A hesperetin derivative plays a role in immunoregulatory effect on human macrophages

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Abstract: The immune system is an important physiological defense system. Its balance and stability are closely related to the body’s health. Once the immune system loses its dynamic balance, the immune response will be blocked, which will lead to the occurrence of various diseases. Hesperetin is a kind of natural flavonoids extracted from citrus fruits of Rutaceae and it has many pharmacological activities. However, its water solubility and liposolubility are poor, and it is easy to be quickly metabolized in vivo, so it is difficult to maintain high blood drug concentration. Therefore, its derivative (HES) was found by structural modification. In this study, THP-1 cells were used as experimental model to investigate the immunomodulatory effect of HES in vitro. The results showed that HES participates in immune response by enhancing phagocytosis of macrophages to promote the release of NO, IL-6 and IL-1β, and enhancing immunity by up-regulating the expression of Bcl-2 and Bcl-XL proteins. This study provides a theoretical and practical basis for the development of HES as an immunomodulator in the future.

Key words: Hesperetin derivative; Immunoregulatory; Macrophages; Cytokine.

Introduction

Immunity is a physiological function of nonspecific or specific recognition and exclusion of antigenic substances in order to maintain the stability of the internal environment and keep health of the body against diseases (1). A variety of pathogenic factors act on the body, thereby reducing the body’s physiological function and immune ability, leading to the dynamic imbalance, making life tend to weaken, increasing the probability of disease. Therefore, the effective regulation of immunity is an important link to maintain the body’s health (2). Macrophages are multifunctional effector cells in the body, which play an important role in infection, inflammation and immune regulation (1, 3, 4).

Natural product monomers have been an important source of drugs and drug leading compounds, with complex chemical structure and diverse biological activities (5). Traditional chemical drugs not only have more side effects and resistance, but also cause drug-induced diseases, so it is very important to develop natural products.

Hesperetin is a kind of natural flavonoids, which belongs to Citrus L. of Rutaceae genus and is the main active components in plant fruits (6). Hesperetin is a kind of aglucon obtained by hydrolyzing hesperidin. Its structure contains ketone carbonyl, ether group, methoxy group and many phenolic hydroxyl groups, which makes it have extensive pharmacological action. Hesperetin does not accumulate in any organ, is safe to use, and has no obvious side effects (7, 8). It is reported that hesperetin has anti-inflammatory, anti-oxidation, anti-allergic, anti-tumor, immune system regulation, radiation protection, antibacterial and antiviral biological activities (9, 10). Although it has a wide range of pharmacological effects, its water solubility and liposolubility are poor, and it is easy to be quickly metabolized in the body, so it is difficult to maintain high blood drug concentration and such low bioavailability limits its clinical use (8, 11). Therefore, it is necessary to modify the structure of hesperetin in order to find the hesperetin derivatives with good water solubility, high bioavailability and better activity. A hesperetin derivative (HES; Fig.1) is a derivative of hesperetin obtained by structural modification of hesperetin by many researchers. In this study, the mechanism of anti-inflammatory and immunomodulatory activity of hesperetin derivatives in THP-1 cells was preliminarily discussed, which provided a certain material basis for further study on pharmacological action of hesperidin derivatives.

Materials and Methods

Materials and reagents

HES (presented by Li Jun, Anhui Medical University)
A hesperetin derivative has immunoregulatory effect.

**Cell culture and macrophage induction**

Human monocyte cell line belongs to human acute monocytic leukemia cell line THP-1, which was purchased from the cell bank of Shanghai Institute of Cell Biology, Chinese Academy of Sciences. RPMI (Roswell Park Memorial Institute) 1640 culture medium (GIBCO company) containing 10% fetal bovine serum (FCS, GIBCO company) was cultured at 37 °C with 5% CO₂. When the cells proliferated to 2 × 10⁵/mL, 100ng/mL phorbol 12-myristate 13-acetate (PMA) was added and induced for 48 h to adhere to the wall and these cells were macrophages. The cells were rinsed with phosphate buffer saline (PBS) for 2 times with the culture medium was changed and cultured for 3 hours for the following experiment.

**Effect of HES on proliferation of THP-1 cells by MTT assay**

The THP-1 cell suspensions with a density of about 2 × 10⁴/mL were inoculated to the 96-well plate for 200 μL/well. After 12 h of culture, the culture medium was removed, the cells were divided into blank control group, LPS positive control group and experimental group (5, 10, 20, 40 μM) with 6 parallel wells in each group. After 24 h, the supernatant was discarded, 200 μL PBS containing 10% MTT was added per well and incubated for 4 h. Then the liquid in the well was removed, 150 μL DMSO was added to each well and oscillated fully for 10 min. Absorbance (A) was measured at 490 nm and cell proliferation rate was calculated.

Cell proliferation rate% = (A2 - A1) / A1 × 100%

In the formula, A1 is the absorbance of the blank control group, and A2 is the absorbance of the experimental group.

**Detection of NO release by HES on THP-1 cells by Griess reagent assay**

THP-1 cells were inoculated on 12-well plate with 2 × 10⁵ cells per well, adhered to well for 24 h. The experimental grouping and drug treatment were the same as 2.3. The supernatant of cell culture was collected after 24 h, the supernatant was discarded, 200 μL neutral red kit (Shanghai Beyotime Biotechnology Co., Ltd) was removed, 150 μL DMSO was added to each well for 5 h on 96-well plate, the experimental group (including the control group, LPS positive control group and experimental group) was cultured for 12h, according to the operation method of neutral red kit, 200 μL nutrient solution without NaHCO₃ and 20 μL neutral red dye solution were added to each well, then the supernatant was discarded after 2h culturing. 200 μL neutral red detection pyrolysis solution was added to each well and the absorbance was measured at 540 nm after pyrolysis for 10 min in a rocker at room temperature, then the phagocytic index of neutral red was calculated.

Phagocytosis index of neutral red = A2/A1. In the formula, A1 is the absorbance of the blank control group, and A2 is the absorbance of the experimental group.

**Effect of HES on the secretion of IL-1 β and IL-6 in THP-1 cells by ELISA assay**

THP-1 cells were inoculated into 12-well plate and treated with the same method. After 24 h, the supernatant of the cells was extracted and operated according to the steps of IL-1β and IL-6 enzyme linked immunosorbent assay kit. The absorbency value was detected at the wavelength of 450nm by using an enzyme labeling instrument and the secretion of cytokines IL-1β and IL-6 was calculated. The experiment was repeated three times.

**Analysis of Bcl-2 and Bcl-XL protein expression by Western blot assay**

THP-1 cells were cultured in a 6-well plate for 24 h, then treated with LPS (1 μg/mL) and different concentrations of HES for 18 h. According to the method of literature (12), RIPA cell lystate (20 mM Tris-HCl (pH7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM beta-lycerophosphate, 1 mM Na₃VO₄, 1 μg/mL leupeptin) was used, the total protein was extracted and the content of total protein was determined by Coomassie brilliant blue method. 50 μg total protein samples were collected from each group and separated on 10% polyacrylamide gel electrophoresis. The separated protein was electrotransferred to the nitrocellulose membrane, and the blocking solution was added and acted at room temperature for 2 h. Rabbit anti-mouse anti-Bcl-2 and Bcl-XL monoclonal antibody (1: 1000 diluted by TBS) were added and incubated at 4°C shaking bed overnight, then rinsed with TBST buffer (pH7.6) for 3 times with each time of 5 min. Human horseradish peroxidase labeled anti-rabbit IgG second antibody (1: 5000 diluted by TBS) was added and incubated at 4°C shaking bed overnight, then rinsed with TBST buffer (pH7.6) for 3 times with each time of 5 min. The film was developed and luminesced by electrogenerated chemiluminescence (ECL) and exposed at dark room. The experiment was repeated three times and the film was scanned by Image J software.

**Statistical method**

All the data were processed by GraphpadPrism7.0 statistical software. The measurement data was expressed as mean ± standard deviation (x ± SD) and single factor analysis of variance (ANOVA) was used in the multigroup comparison. Student-Newman-Keuls (SNK) test was used for post-comparison. P < 0.05 represents the statistical results were statistically significant.

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Effects of HES on proliferation of THP-1 cells by MTT assay

MTT assay was used to detect the effect of HES on the proliferation of THP-1 cells. The proliferation rate of THP-1 cells treated with different concentrations of HES for 24 h was shown in Figure 2A. The results showed that low concentration of HES had no obvious effect on the proliferation of THP-1 cells. When the concentration was above 10 μM, HES could significantly promote the proliferation of THP-1 cells, and at 40 μM, the proliferation rate of THP-1 cells reached the maximum and it was dose-dependent. It was concluded that the HES concentration range with increasing cell proliferation rate was selected for follow-up experiments.

Detection of NO release by HES on THP-1 cells by Griess reagent assay

Griess reagent assay was used to detect the level of NO released by LUC on THP-1 cells. The results indicated that both LPS and HES at different concentrations could promote the release of NO, and there was significant difference compared with the blank control group (Fig. 2B) (P < 0.05). Moreover, the release of NO was dose-dependent on the concentration of HES.

Detection of cell phagocytosis by neutral red method

The phagocytic function of THP-1 cells in each group was detected by neutral red method. Compared with the control group, LPS and HES at different concentrations could promote macrophage phagocytosis of neutrophilic red, and HES had significant difference in the concentration range of 5 μM to 40 μM (P < 0.05, Figure 3).

Effect of HES on the secretion of IL-6 and IL-1β in THP-1 cells by ELISA assay

The effect of HES on the secretion of IL-6 and IL-1β in THP-1 cells was detected by ELISA assay. As shown in Figure 4, LPS treated (1μg/mL) cells released a large number of inflammatory factors compared with the blank control group. Meanwhile, the contents of IL-6 and IL-1β were increased in all dose of HES groups (P < 0.05) (Fig.4). The results showed that HES could enhance the secretion of IL-6 and IL-1β in THP-1 cells.

Analysis of Bcl-2 and Bcl-XL protein expression by western blot assay

Western blot was used to demonstrate the effect of
HES on the expression of Bcl-2 and Bcl-XL in THP-1 macrophages. As shown in figure 5, 1 μg/mL LPS stimulation could significantly induce the up-regulation of Bcl-2 and Bcl-XL protein expression in THP-1 macrophages. Moreover, the expression of Bcl-2 and Bcl-XL in THP-1 macrophages was up-regulated in different dose of HES groups (P < 0.05).

Discussion

Macrophages, derived from mononuclear cells, are important immune cells involved in the process of nonspecific defense and specific defense (13), and are one of the most important immune cells in both specific and non-specific immunity. There is no killing effect in non-activated state of the macrophages. After activation, macrophages phagocytose and digest pathogens in a fixed or free form, release a variety of cytokines and NO and other mediators, indirectly activate lymphocytes and other immune cells, and make the body respond to inflammation (14). Macrophages are often used as model cells in immunomodulation evaluation, and have strong phagocytosis. Phagocytosis is one of the pathways for macrophages to play immune regulation effect (15). Macrophages recognize the invading pathogenic microorganisms and other antigenic foreign bodies by their receptors on the surface, and transfer the foreign bodies to the cells for phagocytosis or pinocytosis, forming phagocytic bodies, and clearing them out through their own sterilizing system in the phagocytic body. At the same time, macrophages can also act as presentation cells, presenting phagocytic foreign bodies to T lymphocytes and initiating immune response (16). On the other hand, the release of some cytokines such as IL-1β, IL-6 and active effector molecules such as NO can promote macrophages to recognize antigenic foreign bodies, enhance phagocytosis of macrophages, and enhance the body’s resistance (17). Therefore, detection of macrophage proliferation, phagocytosis, release of NO and the expression of many cytokines can be used as an important index to evaluate the activation of macrophages.

In this study, macrophages induced by human monocyte line THP-1 were used to simulate macrophages involved in immune regulation in vivo. The cell activity was detected by MTT assay (18) and the results showed that HES at different concentrations was not toxic to THP-1 cells and could promote cell growth and proliferation in varying degrees.

Bacterial infection is mainly caused by Gram-negative bacteria. LPS is a component of cell wall of Gram-negative bacteria, has toxic effect on the host and can cause fever, endotoxin shock and so on (19). LPS specifically binds to the TLR-4 receptor on the cell surface and promotes macrophages to secrete IL-1β, IL-6, NO and other cytokines to resist foreign invasion and play immunomodulatory role. NO is an important immunomodulatory factor (20), and all kinds of external stimuli, such as LPS can induce macrophages to release NO. The immunomodulation of NO is very extensive and complex, which is the basis for macrophages to play the role of immune regulation, participate in killing pathogenic microorganisms and tumor cells, and mediating a series of immune response processes. IL-6 and IL-1β are important cytokines secreted by macrophages, both of which are involved in immunity and are consistent in specific biological characteristics (21, 22). IL-1β can promote the differentiation of B cells into plasma cells to produce immunoglobulin and enhance the body’s resistance through humoral immune pathway (23). IL-6 participates in the transduction of P-STAT3 signaling pathway and induces the expression of anti-apoptotic genes Bcl-2 and Bcl-XL. It is the key cytokine that inhibits Th1 type immune response and mediates Th2 type immune response (24, 25).

In this experiment, LPS was selected as positive control, and the regulation of immunomodulatory effect of LPS and HES on macrophages was studied through the immunomodulation related indexes. The results showed that different concentrations of HES had the same effect as LPS, which could stimulate the release of NO from macrophages, enhance the phagocytosis of macrophages, and the phagocytosis increased with the increase of HES concentration. Meanwhile, different concentrations of HES and LPS could promote secretion of IL-1β and IL-6 cytokines by THP-1 cells, and induced the up-regulation of the expression of Bcl-2 and Bcl-XL protein in THP-1 macrophages to different degrees.

In conclusion, the effects of HES on the secretion of IL-6 and IL-1β were investigated and good results were obtained. HES participates in the immune response by enhancing phagocytosis of macrophages and promoting the release of NO, IL-6 and IL-1β. At the same time, by upregulating the expression of Bcl-2 and Bcl-XL proteins, HES can enhance the body immunity. It is proved that HES is a kind of substance with immunomodulatory function and has the potential to enhance nonspecific immunity.

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None.

Conflict of Interest

There is no conflict of interest in this study.

Author’s contribution

All work was done by the author named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Jian-ili Ma; Jian-li Ma and Chun Li collected and analysed the data; Jian-li Ma wrote the text and all authors have read and approved the text prior to publication.

References

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