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Effects of cyclophosphamide on immune system and gut microbiota in mice

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ABSTRACT

Cyclophosphamide (CP) is the most commonly used drug in autoimmune disease, cancer, blood and marrow transplantation. Recent data revealed that therapy efficacy of CP is gut microbiota-dependent. So, it is very important to understand how CP affects intestinal microbiota and immune function. In this study, the effects of CP on mice immuno-activity were firstly evaluated, then, the fecal microbiota from normal and CP-treated mice was compared, and the characteristic bacterial diversity and compositions were identified, using 454 pyrosequencing technology. The results showed that CP reduced the diversity and shifted the fecal microbiota composition. Specifically, CP treatment decreased the proportion of *Bacteroidetes* while increased the proportion of *Firmictutes* in the microbial community. Most importantly, specific microbiota signatures belonging to *Bacteroides acidifaciens*, *Streptococcaceae* and *Alistipes* were also identified, which would provide new insight into the efficacy and side effects in clinical usage of CP. This should be helpful for further demonstration of CP's action mechanism, development of personalized therapy strategies, and prediction of potential side effects related to various treatment regimens of CP.

Introduction

Autoimmune diseases and cancer are currently globe healthy issues (Cooper et al. 2009; Wang et al. 2012a,b). Cyclophosphamide is widely used and listed on the World Health Organization's (WHO) list of essential medicines needed in a health care system with the features of evidence on efficacy and safety, and comparative cost-effectiveness (World Health Organization, 2013). CP is a potent immunosuppressive agent, high-dose CP is increasingly used to treat both autoimmune and alloimmune conditions (Brodsky, 2010; DeZern et al. 2013). It is also the most commonly used drug in blood and marrow transplantation (BMT) (Colvin, 1999), and post-transplantation CP can promote graft-host tolerance after allogeneic hematopoietic stem cell transplantation (Luznik and Fuchs, 2010). Moreover, CP is one of the widely used and most successful anticancer drugs because its therapeutic efficacy is due in part to stimulating immune responses (Emadi et al. 2009; Sistigu et al. 2011). Chemotherapy in combination with antibiotic treatment are commonly used in cancer therapy because of the complications caused by the disruption of mucosal barrier associated with bacteria translocation and infections (van Vliet et al. 2009, 2010). Chemotherapy alone obviously change the fecal microbiota in patients, including the decrease of species richness and absolute bacterial load (Zwielehner et al. 2011). In mice, CP treatment increase the potentially pathogenic bacteria counts and reduce the intestinal tight junctions and adherens junctions (Yang et al. 2013). A recent study indicated that CP's anticancer efficacy is gut microbiota-dependent (Viaud et al. 2013). Germ-free or antibiotics-treated mice severely compromised these antitumor immune responses. This suggests a complex correlation between therapy efficacy of CP and gut microbiota as well as immune system. On the other hand, it was found that the gut barrier was disrupted and the digestive system problems were developed in cancer patients undergoing chemotherapy with CP (Iida et al. 2013), speculating that a link of CP to gut microbiota also exists. So, it is very important to understand how CP affects intestinal microbiota and immune function.

In this study, the effects of CP on mice immunoactivity were firstly evaluated, then, the fecal microbiota from normal (N) and CP-treated (M) mice was compared, and the characteristic bacterial diversity and compositions were identified. This can provide clues for further demonstration of CP's anticancer mechanism and development of personalized cancer therapy strategies. Additionally, it is also helpful for prediction of potential side effects related to various treatments of CP, because dysbiosis of gut microbiota has been linked to various human diseases (Xu et al. 2014).

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Materials and methods

Mice and reagents

Specific pathogen-free male C57BL/6 mice (8 weeks old) were obtained from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). The mice were kept at a temperature of 22 °C and 12-h light/dark cycles environment for at least two weeks before use, and fed on the same batch of standard laboratory diet to minimize the variation of environment factors. The experiments were approved by the Animal Care Welfare Committee of Guangzhou University of Chinese Medicine. Adequate measures were taken to minimize pain of experimental animals.

Cyclophosphamide (CP) was purchased from Shanxi Pude Pharmaceutical CO., Ltd. (Batch No. 04120101, Shanxi, China). CP was dissolved in sterilized physiological saline to an appropriate concentration prior to injection to mice. RPMI 1640 medium and fetal calf serum were purchased from Gibco (Invitrogen Corporation, USA). Trypan blue (T6146), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT, M2128) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 96-well round-bottom microplates (Costar 3799) were purchased from Corning Incorporated (NY, USA). YAC-1 cells were purchased from Sinovac Biotech Co., Ltd. (Shanghai, China). Mouse TNF-α, IFN-γ, IL-1α, IL-2, IL-6, IL-12 Enzyme-Linked Immunosorbent Assay (ELISA) kits were purchased from R&D System (USA). The purity of all chemical reagents was at least analytical grade.

Protocols for CP treatment

Mice were divided into two groups, received intra-peritoneal (ip) injections once a week (day 1, day 8, day 15, day 22) for 28 consecutive days of (1) CP treatment groups (ip CP 150 mg/kg body weight, 8-week old, $n = 4$) (M groups), which were reared in the same cage; (2) normal groups (ip with the same volume of sterile physiological saline, 8-week old, $n = 6$) (N groups), which were reared in the same cage.

Thymus and spleen indices examinations and preparation of splenocytes

On the twenty-ninth day, mice were weighted and peripheral blood samples were taken and stored at 4 °C for cytokines determination. Subsequently, mice were sacrificed by cervical dislocation, and spleen and thymus were immediately removed under sterile environment and weighted. The thymus or spleen index was calculated by the following formula: thymus or spleen index = thymus or spleen weight (g)/body weight (g). The small intestine contents and colon contents were sterilely collected separately and immediately stored at −80 °C till for further analysis.

Splenocytes were obtained by gentle disruption of the spleen placed in cold phosphate buffered saline (PBS) and filtration through a 200-mesh sieve mesh to obtain single cell suspension as previously described on the day of sacrifice (Yuan et al., 2006). After treatment with erythrocyte lysis buffer, the cells were resuspended at a final density of 3×10^6 cells/ml in RPMI-1640 medium supplemented with 10% fetal calf serum, 100 U/ml streptomycin and 100 U/ml penicillin.

Cytotoxicity assays of natural killer cell activity of splenocytes

Splenocytes were prepared as described above. The splenic natural killer (NK) cells were effector cells and YAC-1 cells were used as the target cells. The assay was carried out according to the previously described (Wang et al., 2012a,b). The NK activity of effector cells was calculated as cytotoxicity by the following formula: cytotoxicity (%) = $(A + B - C)/A \times 100\%$, where A is the absorbance of the well of target cells, B the absorbance of the well of effector cells, C the absorbance of the experimental well.

Mitogenic and co-mitogenic assay of splenocytes

The co-mitogenic activities were assayed by using a slight modification of a method described previously (Wang et al., 2011a,b). Splenocytes were prepared as described above. The cell suspension (1 ml) was placed in each well of a 24-well flat-bottomed microplate with or without either 75 μl ConA (7.5 μg/ml). It was then cultured for 72 h at 37 °C in a humidified 5% CO_2 atmosphere and then further incubated for 4 h with 50 μl of 5 mg/ml 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT, Sigma, USA) per well. DMSO (150 μl) was added to the culture to dissolve the colored material, and the absorbance at 570 nm was measured with an ELISA reader (Bio-Rad Model 6800, USA).

Determinations of TNF-α, IFN-γ, IL-1α, IL-2, IL-6, IL-12 in serum

The blood samples were centrifuged at $1000 \times g$ and 4 °C for 20 min, while the upper layer contained the serum. Small intestine contents and colon contents were suspended with 1 ml of sterilely PBS, respectively, then centrifuged at $1000 \times g$ and 4 °C for 20 min. The amount of TNF-α, IFN-γ, IL-1α, IL-2, IL-6, IL-12 in the serum were analyzed by the mouse TNF-α, IFN-γ, IL-1α, IL-2, IL-6, IL-12 ELISA kits according to the manufacturer's instruction.

Fecal sample collection and DNA extraction

Fresh fecal samples were collected on the last day in the experiment and immediately frozen in liquid nitrogen before storage at −80 °C for further analysis. Genomic DNA was extracted from fecal samples (N groups, $n = 6$; M groups, $n = 4$) by using the EZNA® Soil DNA kit (Omega Bio-Tek, Inc., GA, USA) according to the manufacturer's instructions.

Pyrosequencing

Pyrosequencing was carried out according to the previously described (Chen et al., 2012; Wu et al., 2013). PCR amplification of the V1–V3 region of bacterial 16S rRNA gene was performed using universal primers (533R 5′-TTACCGCGGCTGCTGGCAC-3′, 27F 5′-AGAGTTTGATCCTGGCTCAG-3′) incorporating the FLX Titanium adapters and a sample barcode sequence. The forward primer (B-27F) was 5′-*TATCCCCTGTGTGCCTTGGCAGT CGACT*AGAGTTTGATCCTGGCTCAG-3′, where the sequence of the B adaptor is shown in italics and underlined. The reverse primer (A-533R) was 5′-*ATCTCATCCCTGCGTGTCTC CGACGACT*NNNNNNNNTTACCGCGGCTGCTGGCAC-3′, where the sequence of the A adaptor is shown in italics and underlined and the Ns represent an eight-base sample specific barcode sequence. Briefly, Each 20 μl PCR reaction included 4 μl of 5 *FastPfu Buffer, 2 μl of 2.5 mM dNTPs, 0.4 μl of Forward Primer (5 mM), 0.4 μl of Reverse Primer (5 mM),0.5 μl of DNA template, 0.4 μl of Fastfu Polymerase, and added ddH_2O to make up the final volume to 20 μl. The cycling parameters were as follows: 95 °C for 2 min; 25 cycles of 95 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s with a final extension at 72 °C for 5 min. Duplicate PCR products were pooled. Then they were visualized on agarose gels (2% in TBE buffer) containing ethidium bromide, and purified using the AXYGEN gel extraction kit (Axygen, USA). Amplicon DNA concentrations were measured using the Quant-iT PicoGreen dsDNA reagent and kit (Invitrogen, Germany) and was quality controlled on an Agilent 2100 bioanalyzer (Agilent, USA). Following quantitation, the amplicon from each reaction mixture were pooled in equimolar ratios