Effect of kidney-reinforcing, blood-activating and stasis-removing recipes on adhesion molecule expression of bone marrow mesenchymal stem cells from chronic aplastic anemia patients

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Abstract

OBJECTIVE: To explore the effect of kidney-reinforcing, blood-activating and stasis-removing recipes on adhesion molecule expression of bone marrow mesenchymal stem cells (MSCs) from patients with chronic aplastic anemia (CAA).

METHODS: We used three Traditional Chinese Medicine recipes, namely a kidney-reinforcing recipe (KRR), blood-activating and stasis-removing recipe (BASRR), and kidney-reinforcing, blood-activating and stasis-removing recipe (KRBASRR), and a normal saline control to prepare herbal medicine serum in Sprague Dawley rats. Thirty CAA patients were enrolled in the experimental group, including 17 kidney-Yang deficient patients and 13 kidney-Yin deficient patients. Ten healthy individuals were included in the control group. MSCs were isolated from bone marrow samples, and the cell density was observed to measure their proliferation ability by microscopy on days 2, 7, and 14 after isolation. In addition, the expression of adhesion molecules of bone marrow MSCs (CD106, CD49d, CD31 and CD44) were detected by flow cytometry after 48 h of treatment with the four different herbal medicine serums.

RESULTS: The proliferation of MSCs from kidney-Yang deficient and kidney-Yin deficient patients was weaker than that of MSCs from the control group. The expression of all adhesion molecules of bone marrow MSCs from CAA patients was obviously lower than that in the control group (P<0.01). The expression of CD49d and CD31 in MSCs from patients with a kidney-Yin deficiency was lower than in those with a kidney-yang deficiency (P<0.05 and P<0.01, respectively). For kidney-Yang deficient patients, CD31 expression in the KRBASRR group was significantly higher than that in the BASRR group (P<0.01), while CD44 in the KRBASRR group was significantly higher than that in both KRR and BASRR groups (P<0.01). For kidney-Yin deficient patients, CD106 and CD49d expression in the KRBASRR group was obviously higher than that in the KRR group (P<0.05), while CD31 and CD44 expression in the KRBASRR group was significantly higher than that in both KRR and BASRR groups (P<0.05 and P<0.01, respectively).

CONCLUSION: The bone marrow microenvironment in CAA patients is abnormal. The effect of KRBASRR may be better than that of KRR and
INTRODUCTION

Immunological factors are important for the pathogenesis of aplastic anemia (AA). Abnormal cytoimmunity destroys hematopoietic stem cells (HSCs) and contributes to suppression of hematopoiesis and pancytopenia. Fatty marrow is the most typical pathogenic feature of AA. It is believed that dysimmunity is the main etiology of acute AA, whereas an abnormal bone marrow microenvironment is the most significant etiology of chronic AA (CAA). Bone marrow mesenchymal stem cells (MSCs), a subset of non-HSCs residing in the bone marrow, can differentiate into various cell types under certain induction conditions, including bone marrow stromal cells, osteoblastic cells, chondroblasts, adipocytes, and muscle cells. It has been shown that MSCs have immunosuppressive effects on regulating the activity of immune cells through various pathways in both in vivo and in vitro studies. Hematopoietic support of MSCs and immunosuppression are found to be significantly reduced in AA patients.

The "11th Five-Year Plan" AA Group Cooperation of the State Administration of traditional Chinese medicine (TCM) in China defines "CAA" as "chronic marrow fatigue". According to Chinese medicine, CAA is a chronic disease and has a close relationship with the spleen, kidney and heart. The kidney stores body essences, supports bones and produces marrow. Kidney deficiency causes a failure to produce marrow and blood is subsequently unable to be produced, resulting in blood stasis. On the other hand, patients always suffer from internal heat due to Yin deficiency or toxic heat entering inwards so that the blood flow becomes frenetic and bleeding occurs. Localized bleeding causes further blood stasis. Blood stasis in the bone marrow results in new blood formation, leading to a poor or disorder bone marrow hematopoietic function. Qi block can result in phlegm accumulation and blood stagnation and vice versa, so both of them interact with each other through formation of Qi block, constituting a chronic disease. As a result, kidney deficiency and blood stasis are the basic pathogenesis of CAA.

Few studies have been carried out to further characterize the mechanism of the damaged inductive hematopoietic microenvironment and bone marrow MSC adhesion. This study investigated the expression of MSC adhesion molecules after treatment of MSCs from CAA patients with kidney-reinforcing, blood-activating and stasis-removing recipes.

METHODS

Diagnostic criteria for CAA

Diagnostic Criteria in Modern Medicine (Referring to the 3rd Edition of Diagnostic and Curative Effect Criteria). 1) Clinical Manifestations: the disease occurs relatively slowly and symptoms of anemia, infections and bleeding are relatively mild. 2) Hemogram: the reduction rate of hemoglobin is relatively slow; reticulocytes, neutrophils and platelets are in decrease, but not to the level of acute AA. 3) Bone Marrow Conditions: two to three categories of myeloid production are in decrease and there is at least hyperplastic disorder in one area, such as relatively increased lymphocytes with obviously decreased megakaryocytes, and increased non-hematopoietic cells such as adipocytes in the bone marrow.

Diagnostic criteria in Traditional Chinese Medicine

Diagnostic Criteria in Chinese Medicine (Referring to the Clinical Pathways of 95 Diseases in 22 Special Departments of Traditional Chinese Medicine formulated by the State Administration of TCM). Main Symptoms: pale facial complexion, palpitations, shortness of breath, headache and lack of strength. Yin deficiency pattern: 1) Clinical Manifestations: febrish sensation in the palms, soles and chest, tidal fever, night sweat, frequent epistaxis, macules in the skin, thirst, yellow urine, redness of the tongue edges and tip, thin or scanty tongue coating of fluids. 2) This pattern is more common in kidney-Yin deficiency of CAA. Yang deficiency pattern: 1) Clinical Manifestations: cold feeling, cold limbs, bright pale complexion, poor appetite, loose stool, enlarged tongue bearing tooth marks, white and slippery tongue coating, deep and weak pulse. 2) This pattern is more common in kidney-Yang deficiency of CAA.

Patients and healthy donor selection

Thirty cases were recruited from the Hematology Department of our hospital from June 2010 to December 2011, including 18 male cases and 12 female cases, in which 17 were kidney-Yang deficient and 13 kidney-Yin deficient. The control group consisted of 10 healthy bone marrow MSC donors including five donors of each gender with a median age of 26 (range, 19-38). All patients and healthy donors provided informed consent.

Animals

Eighty Sprague Dawley (SD) male rats of a clean grade weighing (250±20) g were purchased from the Laboratory Animal Center of Zhejiang Chinese Medical Uni-
versity and cared for in accordance with the policies and guidelines by Ethic Committee for Animal Use. All rats were maintained for one week at room temperature before the experiment, with free access to food and water. By means of randomized block method, the 80 male SD rats were randomly divided into kidney-reinforcing recipe (KRR), blood-activating and stasis-removing recipe (BASRR), kidney-reinforcing, blood-activating and stasis-removing recipe (KRBASRR), and normal saline (control) groups with 20 rats in each group. The rats died in a large number of bloodletting.

**Chinese medicinals**
The Chinese medicinals used in this study were standard Chinese medicinal granules produced by Tianjiang Pharmaceutical Co. Ltd.

Kidney-reinforcing medicinals included Shudihuang (Radix Rehmanniae Praeparata), Heshouwu (Radix Polygoni Multiflori), Tusizi (Semen Cuscutae), Buguzhi (Fructus Psoraleae) and Xianlingpi (Herba Epimedii).

Kidney-reinforcing, blood-activating and stasis-removing medicinals included Danshen (Radix et Rhizoma Salviae Miltiorrhizae), Danggui (Radix Angelicae Sinensis), Chishao (Radix Paeoniae Rubra), Mudanpi (Cortex Moutan) and Honghua (Flos Carthami).

Kidney-reinforcing, blood-activating and stasis-removing medicinals included all of the above.

**Laboratory apparatus and reagents**
Laboratory apparatus included a flow cytometer (Becton-Dickinson, New York, USA), adjustable micropipettes (Eppendorf, Hamburg, Germany), and an inverted fluorescence microscope (Olympus, Shanghai, China). The reagents were fluorescence-labeled mouse antibodies against human CD31-FITC, CD49d-PE, CD44-FITC and CD-106-PE, as well as isotype controls (BioLegend, San Diego, USA), Gibco fetal bovine serum (FBS; Invitrogen, New York, USA), low-glucose Dulbecco’s modified eagle medium (DMEM) containing penicillin and streptomycin, 0.25% pancreatin and 0.02% ethylene diamine tetra acetic acid (EDTA) (Hangzhou Genom Biomedical Co., Ltd, Hangzhou, China), and Human Lymphocyte Separation Medium (Ficoll; Tianjin Haoyang Biological Manufacture Co. Ltd, Tianjin, China).

**Medicinal solution**
Standard Chinese medicinal granules were prepared in a water bath to obtain solutions containing 1g/mL crude drug. The solutions were stored at 4°C until use.

**Preparation of serum with Chinese medicinal ingredients**
After living in a barrier system for 4 d, the rats were intragastrically administered the Chinese medicinal solution (5 mL, twice a day). The medicinal dosage was 20 times more than that for a 50 kg adult. According to the routine method of preparation of serum with medicinal ingredients, the rats were denied food for 12 h before blood collection. In the morning of day 4, blood samples were collected under aseptic conditions from the abdominal aorta or heart at 1 h after administration of the drug solution. The collected blood was centrifuged (1500 g, 20 min) to obtain the serum with medicinal ingredients. The collected serum was mixed, inactivated at 56°C for 30 min and then sterilized by passing through a 0.22 µm filter. The serum was then stored at -30°C.

**Isolation and culture of MSCs**
Bone marrow samples (5 mL) were obtained from the posterior superior iliac spine of patients and healthy donors, and diluted with phosphate buffered saline (PBS) at the same volume. The diluted bone marrow samples were layered onto Ficoll at a volume ratio of 1:2 and underwent density gradient centrifugation (2500 r/min, 30 min, upward gradient rate 5 and downward 0) at 4°C. Mononuclear cells were collected and washed twice (1200 r/min, 10 min). Then, the mononuclear cells were counted and suspended in low-glucose DMEM containing 100 U/mL penicillin (except for MSCs from allergic patients), 100 U/mL streptomycin and 10% FBS at a density of 2 × 10^5 cells/cm². Cells were cultured in an incubator at 37°C, 5% CO₂, and saturated humidity. Adherent cells (MSCs) were found scattered on the culture surface after 48 h of culture and resembled fibroblasts with the main body in the clostridial form. Cells reached 90% confluence at an average of 14 days later. MSCs were ready for use at passage 3 and the purity was more than 98%.

**Co-culture of MSCs and serum containing medicinal ingredients**
MSCs were digested with 0.25% pancreatin and 0.02% EDTA, diluted to a density of 5 × 10^5 cells/cm², and placed in 6-well plates (1.8 mL in well). Then, 0.2 mL serum containing medicinal ingredients was added (total volume: 2 mL) for each group. The cells were then cultured at 37°C and 5% CO₂ with saturated humidity for 48 h.

**Flow cytometric analysis**
To prepare cells for flow cytometry, MSCs cultured in the serum containing medicinal ingredients for 48 h were partly digested with trypsin and washed with PBS twice (1000 rpm/min, 5 min). Then, the cell density was adjusted to 1 × 10^6 cells/mL and 100 µL of the cell suspension of each group was incubated with 10 µL each of CD31-FITC and CD49d-PE, or 10 µL each of CD44-FITC and CD106-PE. After mixing, cells were incubated for 20 min at 4°C while protected from light. The cells were washed and then analyzed by flow cytometry. Isotype controls were used accordingly.

**Statistical analysis**
SPSS 15.0 was used for analysis. Paired t-tests were
used to compare the means of the same group before and after treatment. Variance analysis was applied for normality indexes and pairwise comparison was used. For non-normality indexes, nonparametric tests were used. Kruskal-Wallis H tests were applied for multigroup comparison, and Mann-Whitney U tests were used for pairwise comparison.

RESULTS

Patient characteristics
Thirty CAA patients were enrolled in this study, including 17 kidney-Yang deficient patients (10 males and seven females) with a mean age of 27 (range, 9-70 years of age), and 13 kidney-Yin deficient patients (eight males and five females) with a mean age of 23 (range, 9-44 years of age) (Table 1).

Proliferation of MSCs from CAA patients
MSCs were cultured in low-glucose DMEM with 10% FBS and were similar to fibroblasts with the main body in the clostridial form after the first medium change at 48 h. Cells reached 90% confluence at an average of 14 days in the shape of radials or swirls. MSCs were not significantly different in shape between CAA patients and healthy donors. However, at 48 h, 7 days and 14 days, the MSC density of CAA patients was sparser than that of the control group. This observation indicated that the proliferation ability of MSCs from both kidney-Yang deficient and kidney-Yin deficient patients was weaker than that of MSCs from healthy donors (Figures 1-3).

Comparison of MSC adhesion molecule expression between CAA patients and healthy donors
The expression of adhesion molecules, CD106, CD49d, CD31 and CD44, in MSCs from CAA patients, regardless of kidney-Yang or kidney-Yin deficiencies, was obviously lower than that in the control group. The expression of CD31 and CD44 in kidney-Yin deficient patients was lower than that in the kidney-Yang deficient patients (P<0.01 and <0.05, respectively). There was no significant difference in CD106 and CD49d expression between kidney-Yin deficient and kidney-Yang deficient patients (Table 2).

Comparison of adhesion molecule expression in MSCs cultured with different TCM recipes for kidney-Yang deficient CAA patients
For kidney-Yang deficient patients of the KRR group, CD49d, CD31 and CD44 expression was significantly higher than that in the control group (P=0.029, 0.016 and 0.004, respectively). In the BASRR group, CD44 expression was significantly higher than that in the control group (P<0.01). In the KRASRR group, expression of the four MSC adhesion molecules was obviously higher than that in the control group (P=0.000, 0.001, 0.000 and 0.034, respectively). The expression of all four molecules showed no significant difference between KRR and BASRR groups. CD31 expression in the KRASRR group was significantly higher than that in the BASRR group (P<0.01), CD44 expression in the KRASRR group was significantly higher than that in both KRR and BASRR groups (P=0.000 for both groups) (Figure 4).

Comparison of adhesion molecule expression of MSCs cultured with different TCM recipes for kidney-Yin deficient CAA patients
For kidney-Yin deficient patients of the KRR, BASRR and KRASRR groups, CD49d, CD31 and CD44 expression was significantly higher than that in the control group. In KRR and BASRR groups, the expression of these four molecules showed no significant difference (P>0.05). In the KRASRR group, CD106 and CD49d expression was not different from that in the KRR group (P>0.05). CD31 and CD44 expression in the KRASRR group was significantly higher than that in both KRR and BASRR groups (CD31: P=0.015 and P=0.003, respectively; CD44: P=0.011 and

Table 1 General clinical data of CAA patients

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Chronic Aplastic Anemia [%]</th>
<th>Mean Age [Year (range)]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Kidney-Yang Deficiency</td>
<td>17</td>
<td>10 (58.8%)</td>
<td>7 (41.2%)</td>
</tr>
<tr>
<td>Kidney-Yin Deficiency</td>
<td>13</td>
<td>8 (61.5%)</td>
<td>5 (38.5%)</td>
</tr>
</tbody>
</table>

Note: CAA: Chronic aplastic anemia.

Table 2 Comparison of MSC adhesion molecule expression between CAA patients and healthy donors (% ±s)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>CD106</th>
<th>CD49d</th>
<th>CD31</th>
<th>CD44</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Control</td>
<td>10</td>
<td>34.1±3.4</td>
<td>30.4±1.9</td>
<td>7.4±1.5</td>
<td>98.5±1.3</td>
</tr>
<tr>
<td>Kidney-Yang Deficiency</td>
<td>17</td>
<td>2.5±2.4*</td>
<td>2.2±1.7*</td>
<td>3.1±1.4*</td>
<td>80.4±4.4*</td>
</tr>
<tr>
<td>Kidney-Yin Deficiency</td>
<td>13</td>
<td>1.7±1.4*</td>
<td>2.6±2.8*</td>
<td>0.5±0.4a</td>
<td>83.9±4.3*</td>
</tr>
</tbody>
</table>

Notes: MSCs: Bone marrow mesenchymal stem cells; CAA: Chronic aplastic anemia; Compared with the normal control group, *P<0.01; Compared with Kidney-Yang deficiency, **P<0.01; P=0.05.
Ye BD et al. KRBASRR on adhesion molecule expression of bone marrow MSCs from CAA patients

**Effects of different TCM recipes on the expression of MSC adhesion molecules in different TCM patterns of CAA patients**

Expression of MSC adhesion molecules, CD106, CD49d, CD31 and CD44, increased at varying degrees after treatment with KRR, BASRR or KRBASRR. However, only CD31 expression showed a significant difference after treatment for different patterns of CAA patients ($P<0.01$) (Table 3).

**DISCUSSION**

CAA is a disease that causes hematopoietic failure of the bone marrow, and the pathogenesis is related to immune hyperfunction, bone marrow stem cell dysfunction, and hematopoietic inductive microenvironment disorders. Termed as "marrow fatigue" in Chinese medicine, CAA is clinically defined as a kidney-$Yang$ deficiency, kidney-$Yin$ deficiency and deficiencies of both kidney $Yin$ and $Yang$. KRR is a common clinical therapy for CAA, but the actual clinical results for some CAA patients are not desirable and methods that are more effective need to be further explored.

MSCs are a significant part of the bone marrow immune microenvironment, and are able to establish immunosuppressive loci, regulate normal hematopoiesis, repair the hematopoietic microenvironment and promote bone marrow hematopoiesis. For AA patients, hematopoietic support of MSCs and immunosuppression are significantly reduced. T-cell suppression mediated by MSCs is deficient in patients with severe AA, and bone marrow MSCs derived from children with severe AA have poor potentials for proliferation and differentiation. However, Xu et al. found that the immunosuppressive ability of MSCs is not decreased in children with AA. Our study found that the proliferation...
ability of MSCs from CAA patients, regardless of kidney-Yang or kidney-Yin deficiencies, is weaker than that of MSCs in the normal control group. Thus, a functional impairment of MSCs exists in CAA patients. CD106 which is also called vascular cell adhesion molecule-1 (VCAM-1) and its receptor are expressed on bone marrow stroma cells but rarely on hematopoietic cells. CD49d is also called very late antigen-4 (VLA-4) that is expressed mainly on CD34+ cells. These adhesion molecules participate in migration, homing of HSCs, and formation of HSC-MSC aggregations and play a key role in localization in the bone marrow microenvironment, and proliferation and differentiation of HSCs through cell-cell adhesion and signal transmission. CD31 is mainly expressed on the surface of vascular cells and blood cells. It is essential for the differentiation, maturation and release of HSCs into the peripheral blood from the bone marrow. CD44 is a receptor of lymphocyte homing. It is mainly involved in cell-cell interactions and cell adhesion for HSC homing. VCAM-1, VLA-4 and CD44 participate in the homing of HSCs and promote interactions between HSCs and MSCs, thereby assisting the homing of HSCs to niches in the bone marrow microenvironment, which are necessary for growth and development of HSCs for hematopoiesis. A previous study used monoclonal antibodies to block VCAM-1, VLA-4 and CD44 on bone marrow cells and found that 66%, 86%, 77% of HSCs could not home, respectively, and interactions with MSCs was weakened, indicating the importance of adhesion molecules in hematopoiesis. The expression of CD106, CD44 and CD31 in AA mice is much lower than that in normal controls. Our study shows that the expression levels of CD106, CD49d, CD31 and CD44 in MSCs from CAA patients is obviously lower than those in MSCs from healthy donors. These results indicate that there is lower expression of bone marrow mesenchymal cell adhesion molecules and an impaired bone marrow microenvironment in CAA patients, thus weak adhesion and interactions between HSCs and MSCs may play a role in the pathogenesis of CAA. Currently, improving the

Figure 4 Comparison of Adhesion Molecule Expression of MSCs cultured with Different TCM Recipes for Kidney-Yang Deficient CAA Patients

TCM: Traditional Chinese Medicine; MSCs: bone marrow mesenchymal stem cells; CAA: chronic aplastic anemia; Compared with the control group, P<0.01, P<0.05; Compared with the KRR group, P<0.01; Compared with the BASRR group, P<0.01.
microenvironments of HSCs is the main strategy for treating AA. However, our study focused on promoting the expression level of MSC adhesion molecules, which is different from targeting dysfunctional lymphocytes. Our previous study showed that treating both the phlegm and blood stasis can affect the apoptosis of nucleated bone marrow cells from AA mice, and the recipe for invigorating the kidney and eliminating phlegm and stasis can obviously inhibit the apoptosis of bone marrow hematopoietic cells and thus improve the hematopoiesis of bone marrow. In this study, we used animal serum containing medicinal ingredients of KRR,
BASRR and KRBASRR to observe the effects on the expression of bone marrow stromal cell adhesion molecules of CAA patients. MSCs from kidney-"Yang deficient or kidney-"Yin deficient patients in the KRBASRR group showed significantly higher expression of MSC adhesion molecules compared with those in KRR and BASRR groups ($P<0.05$ and $P<0.01$, respectively). The results show that treatment of kidney-"Yin deficient patients is more difficult than that of kidney-"Yang deficiency patients.

Our study indicates that the bone marrow MSCs in CAA patients are characterized by an abnormal quantity and a deficient proliferation ability. Furthermore, the expression of bone marrow MSC adhesion molecules is significantly lower than that in healthy individuals. These results validate that the bone marrow microenvironment in the CAA patients is abnormal, and that KRBASRR treatment can exert a better effect, probably by improving the expression levels of MSC adhesion molecules and the bone marrow hematopoietic microenvironment. This study also indicates that the KRBASRR treatment is an efficient therapy for CAA. However, further research on the specific mechanism is required to be carried out.

REFERENCES