Impact on red blood cell immunity patterns in postoperative phase following total hip arthroplasty

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Abstract

Objective: In this study, we aimed to measure changes in red blood cell (RBC) immunity and cytokine levels after performing total hip replacement surgery.

Material and methods: Twenty patients receiving total hip arthroplasty were investigated by measuring presurgical and postoperative RBC natural tumor erythrocyte rosette rate (NTERR), RBC C3b receptor rosette rate (RC3bRR), RBC membrane CD35, CD58 and CD59 expression and cytokine levels (including tumor necrosis factor α (TNF-α), interleukin 2 (IL-2), interferon γ (IFN-γ), interleukin 10 (IL-10) and prostaglandin E2 (PGE2)). Blood samples were collected on the day before surgery and on the first day after hip arthroplasty.

Results: Postoperative nTeRR and RC3bRR were significantly lower than presurgical levels (p < 0.05). The RBC membrane CD35, CD58 and CD59 expressions were significantly decreased in the postoperative phase compared to pre-operative levels. Importantly, RBC promoting lymphocyte proliferation rates were significantly reduced after surgery. In addition, postoperative TNF-α, IL-2 and IFN-γ levels in RBC and lymphocyte culture fluid were lower than those pre-operation, whereas IL-10 and PGE2 were significantly increased compared to presurgical levels (p < 0.05).

Conclusions: The modification of RBC immune function may be involved in the occurrence and development of the infection following hip arthroplasty, and this suggests a novel strategy to prevent such infection.

Key words: red blood cell (RBC), immune function, total hip arthroplasty.

Introduction

Infection is a serious complication of total hip arthroplasty. The rate of infection is 0.3-2.2%, despite attempts to prevent it [1, 2]. The immune system plays a critical role in preventing infection, and red blood cells (RBCs) are an important part of the immune response [3-5]. However, it is not known whether total hip joint replacement affects the RBC immune function.

Nelson [6] found that RBCs displayed immune adhesion against pneumococcus and that RBCs could enhance macrophage phagocytosis of bacteria. A number of studies [7-16] have shown that the adhesion of RBC was accomplished by glycoproteins CR1 (or CD35) located in the membrane. RBCs could form rosettes with invasive pathogens, including tumor cells and bacteria, and reduced pathogens in target organs; pathogens were eliminated after RBC adhesion due to a change in surface charge. RBCs present pathogens to phagocytes and promote their removal by macrophages as they adhere to them and pass through the liver and spleen; then RBCs reenter the circulation. After RBCs adhere to pathogens, peroxidases are concentrated at CR1 on the cell membrane and directly destroy adhesive antigens; in this way RBCs act as agents of cell-mediated immunity. After adhesion RBCs also produce cytokines or special marker molecules and thereby regulate the immune response [12, 17, 18].

Co-culture of RBCs and peripheral blood lymphocytes was shown to promote amplification of lymphocytes [19-22], maintain lymphocyte activity and reduce apoptosis of lymphocytes. CD58 and CD59 on the RBC membrane are the natural ligands of lymphocyte receptor CD2 when the cells come into contact. CD58 interacts with CD2 on the lymphocyte membrane to induce production of cytokines by T lymphocytes, and indirectly promotes the proliferation and differentiation of B lymphocytes [23-25]. RBCs may induce B lymphocytes to enter mitosis from the resting stage, and promote their proliferation and differentiation to produce antibodies. The expression of CD35, CD58 and
CD59 on the RBC surface could be used as indicators of the immune function. RBCs interacting with other immune cells may form a complex network of immune regulation and so act as indispensable components of the immune system.

In this study, we hypothesized that RBCs’ natural tumor RBC rosette (NTERR), I-type complement receptor rosette rate (RC3bRR) may decrease after a total hip replacement compared with pre-surgery. The expression of CD35, CD58 and CD59 on the RBC membrane and the promotion of lymphocyte proliferation rates by RBCs might also decline after surgery. Tumor necrosis factor α (TNF-α), interleukin 2 (IL-2), interferon γ (IFN-γ), interleukin 10 (IL-10) and prostaglandin E₂ (PGE₂) concentrations in RBC and lymphocyte culture fluid could also change post-operation. Such modification of RBC immune function may be involved in the occurrence and development of infection after total hip arthroplasty.

Material and methods

Patients

Twenty patients undergoing total hip joint replacement were selected from those presenting with femoral neck fractures at the Second People’s Hospital of Anhui Province between October 2010 and October 2011, including 9 males and 11 females, aged 62 to 75 years old (average 67.7 yrs). No patients had serious cardiovascular disease, diabetes, or other non-associated injuries. No abnormalities were found during routine preoperative examination. No steroids or other drugs affecting the immune system were used during the surgery and one day after surgery.

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Material and methods

Preparation for lymphocyte suspension:

1) Aspirated, anticoagulant-treated fresh blood (1 ml) was centrifuged and the supernatant removed, RBCs were washed with saline and centrifuged three times, 2000 r/min, 3 ~ 5 min, and suspended at 1.25 x 10⁸/ml.

2) Zymosan reagent was fully dispersed by pipetting in a 37°C water bath to l x 10⁸/ml.

3) 50 μl of zymosan were added to 50 μl of serum in a test tube, then 50 μl of RBC suspension was added and mixed, followed by 50 μl of 0.25% glutaraldehyde, and the cells were smeared by Giemsa staining. Tumor cells were indicated by red color, RBCs by blue. RBC rosettes were observed under a high magnification optical microscope. Three or more RBCs adhered to a tumor cell was identified as a garland. One hundred tumor cells were counted, and the rosette rate was calculated.

RBC C3b detection

1) Aspirated, anticoagulant-treated fresh blood (1 ml) was centrifuged and the supernatant removed, RBCs were washed with saline and centrifuged three times, 2000 r/min, 3 ~ 5 min, and suspended at 1 x 10⁸/ml.

2) Zymosan reagent was fully dispersed by pipetting in a 37°C water bath to l x 10⁸/ml.

3) 50 μl of zymosan were added to 50 μl of serum in a test tube, then 50 μl of RBC suspension was added and mixed well, placed in a 37°C water bath for 30 min, removed and 25 μl of 0.25% glutaraldehyde was added and mixed gently, smeared, and blown dry for Giemsa staining. Under high magnification, RBCs were purple color and yeast was blue; both were counted. Two or more yeast adhered to an RBC was identified as a garland. Two hundred RBCs were counted, and the percentage of positive cells in garlands were counted.

RBC promotion of lymphocyte proliferation

1. Preparation for lymphocyte suspension: 1 ml of sterile anticoagulant-treated blood was diluted with 1 ml of

2. RBC C3b detection

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Hanks fluid, carefully superimposed on an equal volume of lymphocyte separation medium, and centrifuged 20 min with 2000 r/min. Buffy coats were aspirated and transferred to a centrifuge tube, then washed with D-Hanks solution three times (1000 r/min, 10 min). 0.1 ml of lymphocyte suspension from before the last centrifugation was stained with trypan blue and the cells were counted; if more than 95% cells were viable, the concentration was adjusted to 4 × 10^7/ml with 1640 medium.

2. Preparation for RBC suspension: 1 ml of sterile anticoagulant-treated blood was centrifuged at 2000 r/min for 20 min, RBCs were aspirated, washed three times with D-Hanks, then adjusted to 1.25 × 10^7/ml with 1640 medium.

3. Proliferative responses of lymphocytes (MTI method): 96-well culture plates were set up for five groups: 1) experimental group 1 was RBCs + lymphocytes + lipopolysaccharide (LPS); 2) experimental group 2 was RBCs + lymphocytes + phytohemagglutinin (PHA); 3) control group 1 was lymphocytes + LPS; 4) control group 2 was lymphocytes + PHA; 5) blank group was plain 1640 medium. In the experimental group and control group, 100 μl of lymphocytes was seeded in a culture plate. 50 μl each of mitogen and RBC suspension were added and brought to a final volume of 200 μl per well, with 1640 medium. All samples were set up in triplicate. Plates were cultured at 5% CO2, 37°C for 48 hrs. MTT was added at 20 μl/well, and plates cultured for 4 more hours. Supernatant was carefully removed for further testing, and 150 μl of dimethyl sulfoxide (DMSO) was added to each well. Plates were shaken for 30 s on an oscillator, until all crystals were dissolved, then absorbance (A) values were measured with a microplate reader at 540 nm. Promotion of B lymphocyte proliferation by RBCs = (experimental group 1 A – blank group A) / (control group 1 A – blank group A); Promotion of T cell proliferation by RBCs = (experimental group 2 A – blank group A) / (control group 2 A – blank group A).

Cytokine detection

TNF-α, IL-2, IFN-γ, IL-10 and PGE_2 kits (Shanghai Ying-xin Laboratory Equipment Co) were used in accordance with the manufacturer’s instructions to test supernatants removed from cell culture in the experimental groups.

RBC membrane CD35, CD58 and CD59 expression by flow cytometry

Anticoagulated whole blood was centrifuged at 1000 r/min for 3 min to enrich for RBCs. We took three 12 × 75 mm test tubes, aspirated RBCs of 1 μl from the bottom of the tube, added 5 μl of antibodies, shook and mixed cells and antibodies well; then incubated at room temperature for 10 min and avoided light. We suspended cells in 500 μl of PBS for flow cytometry, measuring positive percentage and mean fluorescence intensity of CD35, CD58 and CD59 on the RBC surface.

Statistical analysis

Data were expressed as mean ± standard deviation (X ± SD). An independent sample t-test was used to compare

Table 1. Presurgical group was not significantly different compared with healthy adult control group for each index (n = 20)

| Index                                      | Control group | One day before surgery group | t     | p     |
|--------------------------------------------|---------------|------------------------------|-------|-------|
| Type I complement receptor rosette rate    | 23.05 ± 2.34  | 22.37 ± 1.74                | 1.04  | 0.30  |
| Natural tumor cell rosette rate            | 17.33 ± 1.32  | 16.48 ± 1.39                | 1.98  | 0.055 |
| RBC promoting T lymphocyte proliferation rate | 1.69 ± 0.03  | 1.68 ± 0.06                  | 0.67  | 0.51  |
| RBC promoting B lymphocyte proliferation rate | 1.56 ± 0.02  | 1.53 ± 0.09                  | 1.38  | 0.18  |
| TNF-α                                      | 24.97 ± 2.44  | 24.99 ± 2.41                 | −0.02 | 0.98  |
| IL-2                                       | 16.09 ± 0.89  | 15.88 ± 1.11                 | 0.65  | 0.52  |
| IFN-γ                                      | 15.92 ± 0.76  | 15.98 ± 0.99                 | −0.20 | 0.84  |
| IL-10                                      | 16.71 ± 0.96  | 17.13 ± 0.99                 | −1.38 | 0.18  |
| PGE_2                                      | 10.81 ± 0.67  | 11.02 ± 0.83                 | −0.85 | 0.40  |
| CD35 percentage                            | 94.85 ± 1.65  | 94.14 ± 1.21                 | 1.54  | 0.13  |
| CD35 fluorescence intensity                | 1.88 ± 0.51   | 1.76 ± 0.39                  | 0.82  | 0.42  |
| CD58 percentage                            | 91.42 ± 1.85  | 90.75 ± 1.96                 | 1.11  | 0.27  |
| CD58 fluorescence intensity                | 2.98 ± 0.43   | 2.88 ± 0.40                  | 0.77  | 0.45  |
| CD59 percentage                            | 97.89 ± 1.37  | 97.77 ± 1.03                 | 0.30  | 0.77  |
| CD59 fluorescence intensity                | 3.59 ± 0.56   | 3.54 ± 0.55                  | 0.26  | 0.80  |

TNF-α = tumor necrosis factor α; IL-2 = interleukin 2; IFN-γ = interferon γ; IL-10 = interleukin 10; PGE_2 = prostaglandin E_2
each index between the control group and the 1 day pre-surgery group; or the \( t \) test was used. Each indicator in the 1 day postsurgical group and the 1 day presurgical was compared using a paired \( t \) test. We used SPSS11.0 software for statistical analysis, with a significance cutoff at \( p = 0.05 \).

**Results**

**Indices in presurgical group and healthy adult group**

Each index in the one day presurgical group for 20 patients was not significantly different compared with the healthy adult control group \((n = 20)\) as shown in Table 1.

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**Postoperative RBC natural tumor erythrocyte rosette rate (NTERR) and RBC C3b receptor rosette rate (RC3bRR)**

The postoperative natural tumor cell rosette rate was significantly lower than 1 day before surgery \((t = 3.05, p < 0.01)\) as shown in Fig. 1. The postoperative I type complement receptor rosette rate was also significantly lower than one day before surgery \((t = 4.28, p < 0.001)\), as shown in Fig. 1.

**RBC promotion of the lymphocyte proliferation rate**

RBC promotion of the T lymphocyte proliferation rate was significantly lower at one day after surgery than one day before surgery \((t = 4.28, p < 0.001)\) as shown in Fig. 2; RBC...
promotion of the B lymphocyte proliferation rate was also significantly lower at one day after surgery than one day before surgery (t = 4.28, p < 0.001), as also shown in Fig. 2.

Cytokine levels and RBC membrane CD35, CD58 and CD59 expression

The concentrations of TNF-α, IL-2 and IFN-γ in RBC and lymphocyte culture fluids were significantly decreased after surgery (p < 0.001). However, the concentrations of IL-10 and PGE₂ increased significantly after surgery (p < 0.001) as shown in Fig. 3. Expressions of CD35, CD58 and CD59 were significantly lower after surgery than before (p < 0.001) as shown in Fig. 4 and Fig. 6. The fluorescence intensities of CD35, CD58 and CD59 after surgery were also significantly lower than before surgery (p < 0.05) as shown in Fig. 5.

**Fig. 5.** The fluorescence intensities of CD35, CD58 and CD59 of RBC membrane after surgery were significantly lower than before surgery (p < 0.05)

**Fig. 6.** The CD35, CD58, CD59 flow cytometry of RBC membrane after surgery and before surgery (p < 0.001)
Discussion

RBCs account for about 50% of whole blood volume derived from bone marrow pluripotent stem cells. RBCs are the main cellular component of blood. In the past, people thought that RBCs had a simple structure and a single function, serving to transport O_2 and CO_2. Siegel et al. [26] presented the concept of the RBC immune system in 1981. Since then, innate immune molecules related to RBCs have been constantly being discovered [27]. Innate immune molecules on RBCs include CD35, CD58, CD59, CD44, CD55, CR3, NKEF, ECKR, PIE and the SOD enzyme. These immune molecules have a variety of complex innate immune functions and regulate other immune responses. RBCs function to recognize, adhere, remove antigens and clear IC. In addition, RBCs participate in many immune responses and in immune regulation using a complete self-regulatory system. RBC immunity is an important part of the body’s immune system. RBC immunology has received a great deal of attention from researchers recently.

More and more studies have confirmed that changes in the innate immune functions of RBCs are closely related to disease occurrence and development [3, 4]. For instance, a natural RBC immune deficiency was reported to play an important role in the pathogenesis of the autoimmune disease SLE [28]; RBC immune function declined in cancer patients [29]; and abnormal redox reactions in RBCs affected the integrity and function of T lymphocytes, promoting atherosclerosis in carotid arteries [30]. Golenkina et al. [31] found that RBCs interfered in the synthesis of leukotriene by polymorphonuclear leukocytes exposed to Salmonella, and presumed that it played a role by regulating intracellular and extracellular NO exchange and metabolism. Jack et al. [32] proved that RBCs were the core cells of nitrosation in acute inflammation and could trigger uncontrolled blood vessel dilation. They proposed that RBCs directly mediate hypotension through a NO dependent mechanism, and used this theory to explain abnormal blood flow response in sepsis. Beck et al. found that RBCs could adhere to HIV-1 and might be involved in the infection of target cells by the virus [13]. However, to date it has not been reported that major surgery can affect RBC immune function.

In this study, we observed that the RBC rosette rate was significantly lower one day after surgery in patients who had total hip replacement than one day before surgery, or than in healthy controls. RBC promotion of T and B lymphocyte proliferation rates also were significantly decreased ($p < 0.05$). Immune-enhancing cytokines such as TNF-α, IL-2 and IFN-γ in the culture medium after RBCs were co-cultured with lymphocytes decreased, whereas the immunosuppressive factors IL-10 and PGE, increased, which further certified that the immune promoting function of RBCs was reduced. The expression of CD35, CD58 and CD59 on the RBC membrane decreased after surgery compared with before surgery ($p < 0.05$). This could account for the decreases we observed in RBC immune rosette formation and in RBC promotion of lymphocyte proliferation.

Due to leg buckling, dislocation, traction, retractor oppression, prosthesis oppression, etc. for a long time, total hip replacement can cause entire or partial lower limb ischemia, especially severe ischemia in the muscles around the joint. Surgery can also cause an increase in circulating immune complexes and activation of complement and phagocytes, which can lead to massive release of oxygen free radicals and damage to RBCs [33, 34]. RBC membrane peroxidation increased after total hip replacement. These injuries changed the enzyme activity so that the RBC membrane immune receptor was inactivated. This could explain why we observed in this study that the expression of RBC membrane proteins CD35, CD58 and CD59 decreased after surgery compared to levels prior to surgery and in healthy controls. We observed that total hip replacement caused a decrease in RBC immune function, which might affect the occurrence and development of infection after surgery. This study provides a new way to address the problem of infection after total hip replacement.

Our study still had some limitations: the sample size was not large enough and we did not measure dynamic changes in RBC immunity. Further research needs to be conducted in order to improve RBC immune function in infected patients after total hip replacement by therapeutic intervention.

The authors declare no conflict of interest.

References

1. Gmerek K, Stachurska A, Fabijańska-Mitek J (2013): Expression levels of CD47 on red blood cells during their storage in blood bank. Centr Eur J Immunol 38: 187-189.

2. Merollini KM, Zheng H, Graves N (2013): Most relevant strategies for preventing surgical site infection after total hip arthroplasty: Guideline recommendations and expert opinion. Am J Infect Control 41: 221-226.

3. Garratty G. Advances in red blood cell immunology 1960 to 2009. Transfusion 2010; 50: 526-535.

4. Teklemariam T, Zhao L, Hantash BM (2012): Full-length HLA-G1 and truncated HLA-G3 differentially increase HLA-E surface localization. Hum Immunol 73: 898-905.

5. Bishlawy IM (1999): Red blood cells, hemoglobin and the immune system. Med Hypotheses 53: 345-346.

6. Nelson RA Jr (1953): The immune-adherence phenomenon; an immunologically specific reaction between microorganisms and erythrocytes leading to enhanced phagocytosis. Science 118: 733-737.

7. Hess C, Schifferli JA (2003): Immune adherence revisited: novel players in an old game. News Physiol Sci 18: 104-108.

8. Passantino L, Altamura M, Cianciotta A, et al. (2002): Fish immunology. I. Binding and engulfment of Candida albicans by erythrocytes of rainbow trout (Salmo gairdneri Richardson) Immunopharmacol Immunotoxicol 24: 665-678.
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9. Baum J, Ward RH, Conway DJ (2002): Natural selection on the erythrocyte surface. Mol Biol Evol 19: 223-229.
10. Jiang N, Tan NS, Ho B, Ding JL (2007): Respiratory protein-generated reactive oxygen species as an antimicrobial strategy. Nat Immunol 8: 1114-1122.
11. Liepke C, Baxmann S, Heine C, et al. (2003): Human hemoglobin-derived peptides exhibit antimicrobial activity; a class of host defense peptides. J Chromatogr B Analyt Technol Biomed Life Sci 791: 345-356.
12. Passantino L, Massaro MA, Jirillo F, et al. (2007): Antigenically activated avian erythrocytes release cytokine-like factors: a conserved phylogenetic function discovered in fish. Immunopharmacol Immunotoxicol 29: 141-152.
13. Beck Z, Brown BK, Wieczorek L, et al. (2009): Human erythrocytes selectively bind and enrich infectious HIV-1 virions. PLoS One 4: e8297.
14. Passantino L, Altamura M, Cianciotta A, et al. (2004): Maturation of fish erythrocytes coincides with changes in their morphology, enhanced ability to interact with Candida albicans and release of cytokine-like factors active upon autologous macrophages. Immunopharmacol Immunotoxicol 26: 573-585.
15. Bishlawy IM (1999): Red blood cells, hemoglobin and the immune system. Med Hypotheses 53: 345-346.
16. Passantino L, Altamura M, Cianciotta A, et al. (2004): Maturation of fish erythrocytes coincides with changes in their morphology, enhanced ability to interact with Candida albicans and release of cytokine-like factors active upon autologous macrophages. Immunopharmacol Immunotoxicol 26: 573-585.
17. Rabinowitz R, Pokroy R, Yu Y, Schlesinger M (1998): Activated human T-cells bestow T-cell antigens to non-T-cells by intercellular antigen transfer. Hum Immunol 59: 331-342.
18. Ebert EC (1985): Sheep red blood cells enhance T-lymphocyte proliferation. Clin Immunol Immunopathol 37: 203-212.
19. Arosa FA, Pereira CF, Fonseca AM (2004): Red blood cells as modulators of T cell growth and survival. Curr Pharm Des 10: 191-201.
20. Fonseca AM, Porto G, Uchida K, et al. (2001): Red blood cells inhibit activation-induced cell death and oxidative stress in human peripheral blood T lymphocytes. Blood 97: 3152-3160.
21. Porto B, Fonseca AM, Godinho L, et al. (2001): Human red blood cells have an enhancing effect on the relative expansion of CD8+ T lymphocytes in vitro. Cell Prolif 34: 359-367.
22. Antunes RF, Brandao C, Maia M, et al. (2011): Red blood cells release factors with growth and survival bioactivities for normal and leukemic T cells. Immunol Cell Biol 89: 111-121.
23. Siegel I, Liu TL, Gleicher N (1981): The red cell immune system. Lancet 2: 556-559.