Original Article
Impact of different blood transfusion methods on the expression of perforin and granzyme B in patients with tumors

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Abstract: Objective: The impact of autologous and allogeneic blood transfusion on the postoperative expression of perforin (PFP) and granzyme (Gzm) in CD8+ T and natural killer (NK) cells was examined in patients with tumors. Methods: A total of 87 patients undergoing elective radical tumorectomy were divided into four groups: group I did not undergo blood transfusion; group II underwent allogeneic blood transfusion (ALBT); group III underwent hemo-diluted blood autotransfusion (HBA); and group IV underwent preoperative autologous blood donation (PABD). The CD8+ T and NK cell percentages, as well as PFP and Gzm expression at different time points (before surgery (T0), postoperative 24 h (T1), 3 days (T2), 7 days (T3), and 10 days (T4)) in different groups were compared. Results: The number of NK cells in group II began decreasing at T1 (P<0.05) and reached a minimum level at T2; there was no significant difference in CD8+ T cells among the four groups. The levels of PFP and Gzm decreased at T1 in all groups (P<0.05), remained low in group II and IV (P<0.05), and returned to normal in group IV at T3. The levels of PFP and Gzm in CD8+ T cells returned to normal at T4, but the activity of NK cells remained low (P<0.05). Conclusions: Intraoperative ALBT can significantly inhibit the postoperative number and activity of toxic lymphocytes in patients, and ABD can reduce the occurrence of this damage.

Keywords: Allogeneic blood transfusion, autologous blood transfusion, perforin, granzyme B

Introduction

Most patients with malignant tumors require perioperative blood transfusion (PBT) because of chronic preoperative consumption, tumor complications, or surgical blood loss. PBT can not only maintain normal blood volume and reduce surgical accidents [1], but also impact the prognosis of tumors [2, 3]. The effect of blood transfusion on the postoperative immune function of patients with tumors has been widely examined in clinical studies. In 1981, Gantt [4] first proposed that allogeneic blood transfusion (ALBT) adversely affects the outcome of malignant tumors; subsequently, a large number of experiments has shown that ALBT can cause immune dysfunction in patients with tumors, promote the growth and metastasis of tumor cells, and result in postoperative recurrence of malignant tumors, as well as increase complications such as postoperative infection

[5-7]. ALBT can inhibit the immune function of patients with tumors, but its mechanisms remains unclear. Studies have suggested that residual leukocyte components in the blood lead to changes of immune function [8]; other studies showed that metabolic products present during the preservation process of red blood cells alter immune function [9]. Some scholars have objected to this theory and have suggested that the infusion of red blood cells has no significant relationship with postoperative immune function of patients, and that other confounding factors, such as surgery or tumor pathological features, are involved [10]. Therefore, the impact of ALBT on the immune function of patients with tumors is controversial.

With the increasing use of clinical blood and limited blood supply sources, autologous blood donation (ABD) has increased in the clinic. ABD

can effectively reduce the perioperative ALBT amount and prevent the transmission of hematogenous diseases. The three main sources are preoperative autologous blood donation (PABD), hemodiluted blood autotransfusion (HBA), and salvaged blood autotransfusion (SBA). The clinical application of PABD is limited because of its longer preoperative preparation time, and patients with tumors typically undergo time-restricted surgery. The blood used in SBA may contain tumor cells, which may result in the risk of cancer cell diffusion after transfusion; therefore, whether SBA can be used in patients with tumors is unclear [11]. The currently available HBA techniques are advanced and feasible for patients with clinical tumors.

It is thought that the perioperative immunization of patients with tumors is mainly based on cellular immunity [12], among which CD8+ T cells (with immune memory function) and natural killer cells (NK) (with non-specific immune function) play key roles. Because of their disease features, the impact of blood transfusion on the immune function of patients with tumors is controversial. Existing hematoprotective methods can effectively reduce the amount of perioperative blood transfused, but studies of the impact of this method on the immune function of patients with tumors are limited. In this study, the impact of hematoprotection on the postoperative expression of perforin (PFP) and Gzm in the lymphocytes of patients with tumors was evaluated to examine the effects on immune function and provide a basis for rational blood use during the perioperative period.

Materials and methods

Subjects

The study was approved by the Hospital Ethics Committee (Gongli Hospital, Lunshenzi 2015-glll-383), and all the patients and their families signed the informed consent. Inclusion criteria: a total of 87 patients undergoing elective radical tumor operation (51 males and 36 females) were selected, including 24 cases of liver cancer, 7 cases of cholangiocarcinoma, and 56 cases of gastrointestinal cancer. The patients aged 48 to 75 years old and weighed 40 to 78 kg, with ASA grade I-II and hemoglobin ≥110 g/L or hematocrit ≥33%, respectively. Exclusion criteria: any patient with a history of blood-borne diseases and transfusions, severe heart, lung, liver, kidney or endocrine diseases, recent serious infection, radiotherapy, chemotherapy or immunosuppressive agents would be excluded.

Anesthesia

Each patient was fasted for 12 hours and restricted drinking water for 6 hours before surgery while didn't apply any preoperative medication. Conventional anesthesia induction: midazolam 0.05 mg/kg, fentanyl 4~5 μg/kg, propofol 1~2 mg/kg, and rocuronium bromide 0.6 mg/kg, together with rapid induction intubation. Intraoperative anesthesia: propofol 6~8 mg•kg-1•h-1, remifentanil 0.1~0.25 μg•kg-1•h-1, and cisatracuronium 2~3 μg•kg-1•min-1 for maintaining anesthesia and muscle relaxation.

Grouping

The patients were divided into four groups according to their blood transfusion and hematoprotective conditions. Group I: control group: the patients' intraoperative blood loss was within 300-400 ml, Hb >70 g/L, with stable circulation and normal oxygenatiokn; no intraoperative blood transfusion or any blood protection measure was performed; intraoperative blood transfusion because of less intraoperative bleeding and no hematoprotection was performed. Group II: ALBT group, patients were transfused appropriate allogeneic blood according to the blood transfusion issued by WHO and their intraoperative bleeding amount and physical conditions (such as intraoperative Hb <70 g/L or HCT <0.25). Group III: HBA group, each patient had blood drawn from the internal jugular vein (rate of 10-20 mL/min) after anesthesia before surgery and were simultaneously infused with the same amount of colloid (6% HES, 130/0.4, Fresenius-Kabi) through the peripheral vein. The reference formula for the bloodletting amount was as follows: bloodletting amount = EBV × 2 × (Hct$_{actual}$~Hct$_{target}$)/ (Hct$_{actual}$ + Hct$_{target}$). EBV was the expected blood volume *in vivo* (male: weight (kg) × 70 ml/kg; female: weight (kg) × 60 ml/kg; the Hct$_{actual}$ and Hct$_{target}$ were the expected hematocrit before and after dilution, and the ideal Hct$_{target}$ was set to 28-30%. Group IV: PABD group, from the patients, 400 mL of autologous blood was obtained in the Department of Hematology, 5

Table 1. Grouping information

Group	N	Information
I (Control)	25	The patients were not performed blood transfusion nor blood protectiondue to less intraoperative blood loss.
II (ABT)	20	The patients were transfused appropriate allogeneic blood depending on the amount of intraoperative bleeding and the patients' physical conditions.
III (ANH)	24	The patients were performed blood dilution via the internal jugular vein before anesthesic surgery, with the bleeding rate as 10~20 ml/min together with quick inputting the same amount of colloidal fluidthrough the peripheral vein.
IV (PABD)	16	The patients were collected 400 ml of autologous blood five days before surgery, together with simultaneous subcutaneous injection of erythropoietin 50 Ul/kg; the preserved blood was re-transfused back to the patient depending on intraoperative bleeding or before the end of the surgery.

Control: Blank control group; ABT: allogeneic blood transfusion group; ANH: acute hemodilution group; PABD: preserved autologous blood transfusion group.

days before surgery, and the patients were subcutaneously administered 50 U/kg erythropoietin. Blood samples were preserved in one acid citrate dextrose blood storage bag and re-infused back into the patient depending on the intraoperative bleeding conditions or before the end of surgery. From each patient, 5 mL venous blood was samples at designated time points (before surgery (T0), postoperative 24 h (T1), 3 days (T2), 7 days (T3), and 10 days (T4)) for detection. The grouping information is shown in **Table 1**.

Extraction of peripheral blood mononuclear cells (PBMCs)

Ficoll method: First, 5 mL of venous blood was placed in one heparin-anticoagulated test tube (heparin 20 g/mL), gently shaken, and diluted with phosphate-buffered saline (PBS) at room temperature. The diluted blood was then slowly added to lymphocyte stratification fluid (10 mL diluted blood + 5 mL separation solution) along the tube wall. After centrifugation at 2000 r·min below 20°C for 20 min (LDZ4-1.2 centrifuge, Beijing Jingli, Beijing, China), one 1-mL tip was used to gently sample the mononuclear cells; the resulting PBMCs were then washed with a 5-fold volume of PBS twice; after centrifugation at 1200 r/min for 15 min, the cell concentration was adjusted to 1×10^6/mL.

Detection of PFP and Gzm

To four test tubes, labeled ①, ②, ③, and ④, the prepared PBMC suspension was added and cell surface molecule staining was performed; after blocking non-specific staining, 20 L of APC-anti-CD3 and FITC-anti-CD8 antibodies (BD Biosciences, Franklin Lakes, NJ, USA) were added to tubes ① and ②, respectively, while FITC-anti-CD56 and FITC-anti-CD16 antibodies (BD Biosciences) were added to tubes ③ and
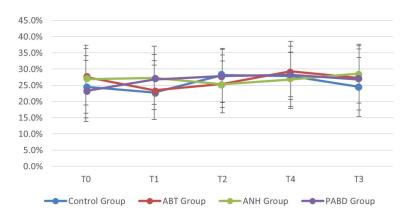
④, followed by incubation for 30 min at 4°C in the dark. The cells were subjected to fluorescent antibody staining of PFP and Gzm (Shanghai Yuayi-Biotech Co., Ltd., Shanghai, China). After washing with 0.5%-FBSA-containing PBS and centrifugation, the cells were mixed with 1 mL of cell fixation solution, shaken, and incubated at room temperature for 20 min. After incubation, 1 mL of prepared membrane drilling solution was added for 15-min incubation in the dark. After washing, PE-anti-perforin antibody (BD Biosciences) was added to tubes ① and ③ and PE-anti-granzyme B antibody (BD Biosciences) was added to tubes ② and ④, followed by 30-min incubation at 4°C and in the dark. After repeated washing and centrifugation twice, PBS solution was used to resuspend the cell precipitate for detection (FACS Calibur flow cytometry, BD Biosciences).
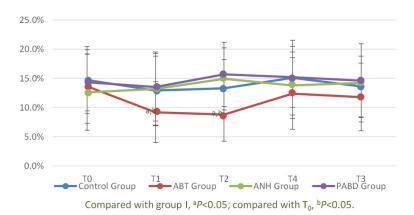
Statistical analysis

SPSS17.0 was used for statistical analysis (SPSS, Inc. Chicago, IL, USA). The normally distributed data expressed as the mean ± standard deviation ($\bar{x} \pm s$). Variance analysis with a repeated measurement design was used for intragroup comparison at different time points. For intergroup comparison, one-way analysis of variance was used. The median M was used to express the data with skewed distribution, while the rank sum test was used for intragroup comparison. The count data were compared using the χ^2 test, with $P<0.05$ considered statistically significant.
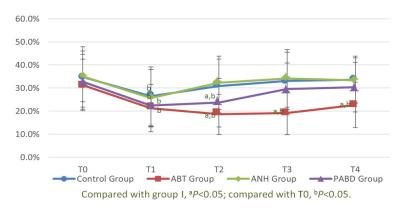
Results

There was no significant difference between the four groups in terms of age, weight, length of operation, preoperative Hct, intraoperative blood transfusion volume, and postoperative Hct, and the results were shown in **Figure 1**.

Figure 1. There is no statistical significance in the percentage of CD8+ T to PBMC of each group before and after surgery (*P*>0.05).

Compared with group I, $^aP<0.05$; compared with T_0, $^bP<0.05$.

Figure 2. Compared Group Control, the percentages of NK cells to PBMC in Group ABT are decreased at T1 and T2; compared with the value at T0, the percentages of NK cells to PBMC in Group ABT are decreased at T1 and T2.

Compared with group I, $^aP<0.05$; compared with T0, $^bP<0.05$.

Figure 3. Compared with group Control, the percentages of PFP expression in the NK cells of group ABT are decreased at T2, T3, and T4, and that in group PABD is decreased at T2. Compared with the value at T0, the percentages of PFP expression in the NK cells in all the groups are decreased at T1, which continuously decrease at T2, T3, and T4 in group ABT and decrease at T2 in group PABD.
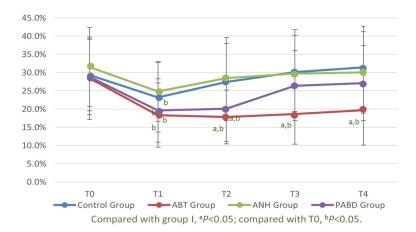
Compared with those at T0, the ratio of NK to PBMC at T1 and T2 in Group II was significantly reduced (P<0.05); the expression percentages of the granzyme B and the contents of perforin in the NK cells of Group II at T1~4 were significantly reduced (P<0.05), and significantly reduced in Group IV at T1 and T2 (P<0.05), among which the decrease was the most significant in Group I and III at T1 (P<0.05). There was no statistical significance in the ratio of CD8+ T to PBMC. The expression percentages of the granzyme B and the contents of perforin in the CD8+ T cells of Group II at T1~3 were significantly reduced (P<0.05), and significantly reduced in Group IV at T1 and T2 (P<0.05), among which the decrease was the most significant in Group I and III at T1 (P<0.05).
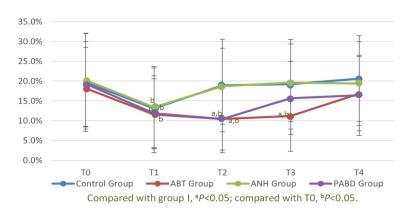
Compared with Group I, the ratio of the NK cells to PBMC in Group II was significantly reduced at T1 and T3 (P<0.05), the expression percentages of the granzyme B and the contents of perforin in the NK cells of Group II at T2 and T3 were significantly reduced (P< 0.05), the expression percentages of the granzyme B and the contents of perforin in the NK cells of Group IV were significantly reduced at T2 (P<0.05). The expression percentages of the granzyme B and the contents of perforin in the CD8+ T cells of Group II and IV at T2 were significantly reduced (P<0.05), and the expression percentages of the granzyme B and the contents of perforin in the CD8+ T cells of Group II at T3 were significantly reduced (P<0.05), the results were shown in **Figures 2-6**.

Discussion

ALBT has immunosuppressive effects on patients with tumors, thus causing adverse

Compared with group I, ᵃP<0.05; compared with T0, ᵇP<0.05.

Figure 4. Compared with group Control, the Gzm B expression percentages in the NK cells of group ABT are decreased at T2, T3, and T4, and that in group PABD is decreased at T2. Compared with the value at T0, the Gzm B expression percentages in all the groups are decreased at T1, which continuously decrease at T2, T3, and T4 in group ABT and decrease at T2 in group PABD.

Compared with group I, ᵃP<0.05; compared with T0, ᵇP<0.05.

Figure 5. Compared with group Control, the PFP expression percentages in the CD8+ T cells of group ABT are decreased at T2 and T3, and that in group PABD is decreased at T2. Compared with the value at T0, the PFP expression percentages in the CD8+ T cells of all the groups are decreased at T1, which continuously decrease at T2 and T3 in group ABT and decrease at T2 in group PABD.

Compared with group I, ᵃP<0.05; compared with T0, ᵇP<0.05.

Figure 6. Compared with group Control, the Gzm B expression percentages in the CD8+ T cells of group ABT are decreased at T2 and T3, and that in

group PABD is decreased at T2. Compared with the value at T0, the Gzm B expression percentages in the CD8+ T cells of all the groups are decreased at T1, which continuously decrease at T2 and T3 in group ABT and decrease at T2 in group PABD.
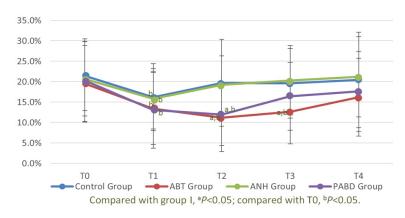
effects on patient prognosis, such as the immune escape of tumors, followed by metastasis and recurrence, postoperative immunocompromised patients, or an increased postoperative infection rate [13]. Such transfusion-induced immune dysfunction is known as transfusion-associated immunomodulation (TRIM) [14]. ALBT can not only reduce the demand for allogeneic blood, but also effectively reduce the occurrence and development of TRIM [15]. However, its mechanisms remain unclear.

Cellular immunity plays an important role in the perioperative period of patients with tumors. Cytotoxic T (CTL) cells and NK cells are important effector cells of cellular immunity and reflect adaptive immunity and autologous innate immunity. These cells have immune functions in different stages and participate in the occurrence and development of diseases. CTL and NK cells kill target cells mainly by secreting PFP/Gzm [16]. The structure of the glycoprotein PFP is similar to that of complement C, and Gzm is a group of serine proteases stored in the cytoplasmic granules of lymphocytes along with PFP. Three human Gzms have been identified, among which Gzm B is the main type involved in disease immune responses. In precursor cells of CTL and NK cells, PFN is expression is low; however, expression is significantly increased after

activation. When cytotoxic lymphocytes are expose to target cells, such stimulation can increase the cytoplasmic Ca^{2+} level inside the target cells, resulting in the release of PFN into the intracellular gap via exocytosis, which then quickly attaches to the membrane of target cells, embed the bi-phospholipid layers of the cell membrane, and form transmembrane channels so that Gzm B can then enter the cytoplasm and nuclei of target cells through the transmembrane channels. Gzm then cleaves specific nuclear proteins, activates autolytic endonucleases, breaks down the DNA of target cells, and causes apoptosis. After transmembrane channel formation, depolarization of the target cell membrane interferes with intracellular homeostasis, resulting in changes to osmotic pressure and the dissolution of target cells [17, 18]. PFP and Gzm mainly kill and remove virus- or bacteria-infected target cells and tumor cells under pathological conditions, but generally do not kill normal cells.

CD8+ CTLs are the largest *in vivo* CTL subgroup with the largest populations and are the most important effector cells. In this study, we investigated the possible mechanisms of immune dysfunction after blood transfusion by observing the number and cytotoxicity changes of CD8+ T cells and NK cells. The results showed that at T1, the number of NK cells and expression levels of PFP and Gzm B in the four groups were decreased, which may be related to the decrease in immune function induced by surgical trauma and anesthesia stress. Some scholars reported that patients may have transient immunosuppression after surgery and anesthesia, which is clearly observed within 24 h after surgery [19, 20]. The number of NK cells and expression levels of PFP and Gzm B in group ABH decreased to a minimum level at T2 and the number of NK cells recovered at T3, while the expression percentages of PFN and Gzm B and CD8+ T cells recovered to preoperative levels at T4; however, the NK cells did not recover, indicating that ALBT can affect the postoperative level of NK cells, thus affecting the immune function of tumor patients, which can be most clearly observed on the third postoperative day. CD8+ T cell functions recovered, while the NK cells remained inhibited for relatively longer. This may be because CTL cells are involved in adaptive immunity, and thus immune activity will be affected by the antigen presentation of major histocompatibility complex, while NK cells are involved in autologous innate immunity, and thus cytotoxic activity is highly dependent on inflammatory mediators and cytokines (such as interleukin-2 or interferon-γ). After ALBT, cytokines with positive regulatory effects are downregulated, while those with inhibitory regulatory effects are increased, decreasing the immune activity of NK cells [21, 22]. The number of NK cells in group PABD was recovered at T2, but cell viability did not recover until T3, likely because of the 1-week interval between blood collection and transfusion back to the patient. During this period, the blood produced active substances that can inhibit cell immunity, thus affecting the killing effects of toxic cells. Atzil et al. [23] found through animal experiments that blood cells stored for different periods have different impacts on postoperative immune function in rats with tumors; as storage time increased, postoperative immune function decreased. Offner [24] also suggested that changes in the metabolites and biochemical and molecular biology markers in blood cells during storage can lead to decreased immune function and an increased incidence of postoperative complications.

In summary, intraoperative ALBT can significantly inhibit the number and activity of lymphocytes in patients with tumors, which may increase the rate of postoperative TRIM and tumor recurrence. Thus, the transfusion of allogeneic blood should be minimized in patients with tumors. ALBT is effective and beneficial for patients with tumors for not only reducing the demand for allogeneic blood, but also effectively avoiding immune dysfunction in toxic lymphocytes. Although it may produce a series of complications, correct clinical intervention can prevent most of such complications. Therefore, when intraoperative blood transfusion can't be avoided, autologous blood transfusion should be preferred. When selecting ALBT, HBA may be more beneficial than PABD for avoiding damage to immune function by the harmful substances produced during the blood storage process.

Acknowledgements

Disclosure of conflict of interest

None.

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