



MicroRNA-125a Expression in Isolated Lymphocytes and Decreased Regulated on Activation, Normal T-cell Expressed and Secreted Production during Cardiac Surgery with Cardiopulmonary Bypass

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Background: Cardiopulmonary bypass (CPB) induces postoperative immunosuppression, including decreased T-cells and lower plasma regulated on activation, normal T-cell expressed and secreted (RANTES) concentrations. MicroRNA-125a negatively regulates RANTES expression in activated T-cells. The aims were to investigate microRNA-125a expression in T-cells and RANTES production following CPB. **Materials and Methods:** Twenty-eight patients undergoing elective cardiac surgery were included in this study. Arterial blood was sampled at six sequential points (before anesthesia induction, before CPB, at 2, 4, 6, and 24 h after beginning CPB) for plasma RANTES concentrations by enzyme-linked immunosorbent assay. T-lymphocytes were isolated from whole blood at four points (before anesthesia, before CPB, at 2 and 4 h after beginning CPB) for intracellular microRNA-125a expression by quantitative real-time reverse transcription polymerase chain reaction in 14 patients. Perioperative laboratory data and variables were also recorded. **Results:** The plasma RANTES concentrations decreased significantly at 2–24 h after beginning CPB, with concurrent reduction of postoperative lymphocyte counts, as compared with the preanesthesia level ($P < 0.001$). Intra-T-cell microRNA-125a expression was activated at 2 and 4 h, however, without significance ($P = 0.078$ and 0.124 , respectively). The plasma RANTES levels at 4 h were not correlated with CPB time ($P = 0.671$), anesthesia time ($P = 0.305$), postoperative extubation time ($P = 0.508$), and Intensive Care Unit (ICU) stay ($P = 0.756$). Three patients expired with pneumonia- or mediastinitis-related septic shock in the ICU. **Conclusion:** Plasma RANTES concentrations were depressed till 24 h following CPB, with reduced lymphocytes after cardiac surgery. MicroRNA-125a expression in T-lymphocytes was not correlated with perioperative variables and its role in downregulation of RANTES production needs to be determined.

Key words: RANTES, microRNA, T-lymphocyte, cardiac surgery, cardiopulmonary bypass

INTRODUCTION

Cardiopulmonary bypass (CPB) induces population shifts of the leukocyte subsets, changes their degrees of activation,^{1,2} and contributes to peripheral immune suppression.³ These alterations start immediately after the onset of CPB and

lymphocytopenia lasts in the early postoperative days, mainly from the decrease of helper/inducer T-cells.⁴

Regulated on activation, normal T-cell expressed and secreted (RANTES), known as chemokine ligand 5 (CCL5),⁵ is

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How to cite this article: Lin TC, Huang GS, Lin SL, Lin YC, Ke HY, Tsai YT, *et al.* MicroRNA-125a expression in isolated lymphocytes and decreased regulated on activation, normal T-cell expressed and secreted production during cardiac surgery with cardiopulmonary bypass. J Med Sci 2016;36:234-9.

Received: May 14, 2015; Revised: September 08, 2016;
Accepted: September 28, 2016

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produced by a variety of cell types including T-cells and platelets. It plays a role in regulating T helper cell cytokine production and leukocyte trafficking, migration, and activation, involving in the pathophysiology of acute and chronic inflammatory processes.⁶ In a cohort of male patients undergoing coronary angiography, low baseline plasma RANTES levels are an independent predictor of 2-year cardiac mortality.⁷ Besides, CPB of more than 2 h, longer surgical times, and reoperation were associated with lower RANTES levels in pediatric cardiac surgery.⁸

Recently, circulating plasma microRNAs have been identified as novel biomarkers for screening many inflammatory or cardiac diseases.⁹ MicroRNAs, noncoding RNAs, regulate gene expression by inhibiting mRNA translation or inducing its degradation¹⁰ to regulate over-reactive inflammatory response.¹¹ Expression of microRNA-125a was induced in T-cells and further introduction of microRNA-125a into T-cells from lupus patients alleviated the elevated RANTES expression.¹² we therefore hypothesized that overexpression of microRNA-125a in T-lymphocytes following CPB leads to a significant reduction in RANTES production during cardiac surgery. The aims of this study were to investigate the microRNA-125a expression in circulating T-lymphocytes and plasma RANTES concentrations during cardiac surgery.

MATERIALS AND METHODS

Participants

The study was approved by the Ethics Committee (TSGHIRB-1-101-05-115), and each written informed consent was obtained from 28 patients undergoing elective cardiac surgery. Patients with cancers, autoimmune diseases, steroid therapy, preoperative respiratory, or hepatic failure were excluded from the study.

Perioperative management

In the operation room, the patients were premedicated with fentanyl and midazolam for arterial catheterization. General anesthesia was induced with fentanyl 1.5–3 µg/kg, propofol 0.5–1.5 mg/kg, and cisatracurium 0.1–0.2 mg/kg and maintained with sevoflurane or isoflurane after tracheal intubation. A pulmonary artery catheter was placed through the right internal jugular vein, and transesophageal echocardiography was used to monitor real-time cardiac performance throughout the whole procedure. Routine median sternotomy and standard CPB (Sarns 8000, Terumo, Ann Arbor, MI, USA) with an extracorporeal membrane oxygenator (Capiiox® SX18, Terumo, Ann Arbor, MI, USA) were carried out in sequence to maintain the body temperature at 28°C–30°C during surgery. The perfusionist adjusted sevoflurane or isoflurane

concentration on the vaporizer to keep mean arterial blood pressure between 50 and 80 mmHg during bypass. Following standard rewarming and deairing, the pump was weaned in the end of the procedure. Routine inotropic support, including dopamine or dobutamine infusion, was added for acceptable cardiac output, if necessary. All patients were transferred to the cardiovascular surgical Intensive Care Unit (ICU) with endotracheal intubation after surgery.

Blood samples and data collection

Each 20 ml arterial blood was sampled from the arterial line at six sequential time points (before induction, just before CPB, and at 2, 4, 6, and 24 h after beginning CPB) into ethylenediaminetetraacetic acid (EDTA)-containing tubes. After immediate centrifugation of blood samples at the speed 1500 rpm for 10 min at room temperature, the plasma samples were divided into avendoff tubes and frozen at –70°C for later plasma RANTES concentration testing.

After obtaining preliminary plasma RANTES concentrations, four time points (before induction, before CPB, at 2 and 4 h after beginning CPB) were selected to isolate T-lymphocytes from whole blood for analyzing intracellular microRNA-125a expression by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). Arterial blood gas analysis was routinely examined perioperatively and immediately upon the ICU admission by GEM Premier 3000 (Instrumentation Laboratory, Lexington, MA, USA). Perioperative variables, including general anesthesia, operation, and bypass times, as well as preoperative and postoperative laboratory data were also recorded.

T-cells isolation

The isolation of human T-cells from whole blood was performed as recently described.¹³ The specific cells were fractioned based on CD3 (T-cells) expression using pluriBead cell separation kit (pluriSelect GmbH, Leipzig, Germany) according to manufacturer's instructions. Briefly, 8 ml EDTA blood sample was respectively incubated with antihuman CD3 antibody (clone: MEM-92, catalog No. 19-00300-10)-coated beads (pluriBead®) with bead sizes 30 µm in diameter (S-size) at room temperature for 10 min on a pluriPlixR roller. Next, the cell–bead mixture was sieved by a 27 µm pluriStrainer which was put onto a separate sterile 50 ml centrifuge tube using a connector ring. Then, the cells (attached to the beads) were washed on the sieve with wash buffer (phosphate-buffered saline, without Ca²⁺ and Mg²⁺, pH 7.4 and with 2 mM EDTA and 0.5% bovine serum albumin) to remove erythrocytes and unbound cells. After washing, the Luer-Lock of the adapter was closed to stop the flow through the mesh, and 1 ml Trizol was added directly on top of the pluriStrainer to lyse the cells.

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Finally, the lysed cells containing Trizol solution was collected and stored at -80°C until use.

RNA isolation and quantitative real-time reverse transcription polymerase chain reaction

The expression levels of hsa-microRNA-125a-5p were quantified using TaqMan® MicroRNA Assay. In briefly, Total RNA was isolated using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Then, cDNAs were reverse transcribed from 50 ng RNA (for hsa-microRNA-125a) using the TaqMan microRNA Reverse Transcription Kit with specific looped RT primers, respectively. Finally, 3 μL cDNA samples were used for quantitative PCR performed in an ABI 7500 real-time PCR machine (Applied Biosystems Inc., Lincoln Centre Drive Foster City, CA, USA). The levels of hsa-microRNA-125a-5p were determined by TaqMan® MicroRNA Assay with hsa-microRNA-125a-5p (AB Assay ID: 002198) specific primers and probes, respectively. U6 small nuclear RNA (AB Assay ID: 001973) served as endogenous control in the experiment. The relative expression levels were analyzed using the comparative threshold cycle (Ct) method according to the manufacturer's instructions.

Enzyme-linked immunosorbent assay

The protein concentrations of RANTES in the plasma were measured using the commercially available DuoSet ELISA development kits (R and D Systems Inc., McKinley Place N.E., Minneapolis, USA) according to the manufacturer's protocol (RANTES catalog No. DY278). The absorbance of the color at 450 nm was recorded using a TECAN Sunrise ELISA Reader (Tecan Group Ltd., Männedorf, Switzerland).

Statistical analysis

The patients' demographic data, perioperative variables, postoperative blood cell counts, plasma RANTES concentrations, and microRNA expressions were presented as mean $\pm$ standard deviation and analyzed through SPSS software version 17 (SPSS, Chicago, IL, USA). The RANTES concentrations were analyzed by paired *t*-test, and their correlation with CPB time and ICU stay was further analyzed by Pearson's correlation analysis. $P < 0.05$ was considered statistically significant.

RESULTS

The demographic data of 28 patients were summarized in Table 1, with mean CPB time 124.4 ± 52.3 min. The postoperative extubation time was 35.7 ± 26.0 h after arriving ICU ($n = 25$), except three patients with severe

Table 1: Demographic data and perioperative variables ($n=28$)

	Mean $\pm$ SD	Range
Gender (<i>n</i>)		
Male/female	21/7	
Age (year)	59.4 ± 10.6	33-80
Height (cm)	164.3 ± 7.4	148-179
Weight (kg)	68.4 ± 14.0	43-104
Body mass index (kg/cm^2)	25.2 ± 4.4	17.2-37.3
General anesthesia duration (min)	343.1 ± 71.1	237-495
Operation time (min)	287.6 ± 61.7	184-419
Cardiopulmonary bypass time (min)	124.4 ± 52.3	49-281
Aortic clamp time (min)	71.9 ± 31.2	16-167
Extubation time (h)	35.7 ± 26.0	3.5-140
ICU stay (days)	3.5 ± 1.4	2-9
Hospital stay (days)	11.9 ± 4.0	7-23
Ischemic heart disease (<i>n</i>)	14	
Coronary artery graft	2.8 ± 0.6	1-3
Valvular heart disease (<i>n</i>)	11	
Heart tumor (<i>n</i>)	2	
Atrial septal defect (<i>n</i>)	1	
Hypertension (<i>n</i>)	18	
Diabetes mellitus (<i>n</i>)	8	

The data were presented as mean $\pm$ SD (range). SD=Standard deviation; ICU=Intensive Care Unit

pneumonia- or mediastinitis-related septic shock and expired at postoperative 10, 25, and 31 days in the ICU. The other major cardiopulmonary events included postextubation pneumonia in two and postoperative cardiac tamponade in one, needing re-sternotomy, with prolonged hospital stay as 15, 23, and 21 days, compared with mean 11.9 ± 4.0 hospital days.

The postoperative white blood cell and neutrophil counts increased significantly [Table 2], as compared with the preoperative data ($P < 0.001$), whereas lymphocyte decreased from preoperative $1,600 \pm 600$ to postoperative $800 \pm 400/\text{mm}^3$ ($P < 0.001$) and platelet counts reduced from 226.8 ± 71.2 to $150.0 \pm 37.8/\text{mm}^3$ ($P < 0.001$).

As demonstrated in Figure 1, the plasma RANTES levels decreased significantly at 2, 4, 6, and 24 h after the start of CPB, with 5.6 ± 2.8 (range 0.8–11.8), 4.5 ± 2.8 (range 0.7–11.3), 5.6 ± 2.9 (range 1.0–11.6), and 5.5 ± 2.6 (range 0.5–10.9) ng/mL, respectively, as compared with 8.8 ± 2.8 (range 2.4–14.1) ng/mL before induction ($P < 0.001$ in all). Among patients with postoperative mild pneumonia ($n = 2$) and septic shock ($n = 3$), the RANTES levels at 4 h were 3.04, 5.27, 1.75, 2.03, and 11.32 ng/mL, respectively (mean 4.7 ng/mL). Using Pearson's correlation analysis, the plasma RANTES concentrations

Table 2: Pre- and post-operative laboratory data

	Preoperative	Postoperative	P
White blood cell count (/mm ³)	7400±2300	11,300±4100	<0.001
Neutrophil count (/mm ³)	5000±2100	9900±3800	<0.001
Lymphocyte count (/mm ³)	1600±600	800±400	<0.001
N ratio (%)	65.7±8.7	86.2±5.7	<0.001
L ratio (%)	22.7±8.8	7.9±3.3	<0.001
Hemoglobin (g/dL)	13.0±2.1	10.2±1.6	<0.001
Platelet (/mm ³)	226.8±71.2	150.0±37.8	<0.001
BUN (mg/dL)	21.5±14.9	18.4±10.6	0.136
Creatinine (mg/dL)	2.1±3.0	1.6±1.9	0.206
AST (U/L)	30.4±22.9	41.8±16.9	0.002
ALT (U/L)	29.4±23.7	21.3±9.0	0.036
Troponin-I (ng/mL)	-	3.9±2.8	
B-type natriuretic peptide (pg/mL)	-	211.3±247.0	
Cardiac index (L/min/m ²)	-	3.3±1.1	

The data were presented as mean±SD. SD=Standard deviation;
AST=Aspartate aminotransferase; ALT=Alanine aminotransferase;
BUN=Blood urea nitrogen

at 4 h after beginning CPB were not correlated with general anesthesia time ($P = 0.305$) and CPB time ($P = 0.671$) and displayed no differences between patients with less or more than 120-min CPB time (4.6 ± 2.6 vs. 4.5 ± 3.3 ng/mL, $P = 0.459$). They were not associated with extubation time ($P = 0.508$), ICU stay ($P = 0.756$), and hospital days ($P = 0.838$).

The microRNA-125a expression in isolated T-lymphocytes from 14 patients was analyzed using qRT-PCR [Figure 1]. As compared with baseline before induction as 1.0, the intracellular microRNA-125a expression was 1.1 ± 0.8 (range 0.2–3.1) folds before CPB ($P = 0.707$) and elevated up to 3.9 ± 5.7 (range 0.4–18.9) and 3.6 ± 5.8 (range 0.2–17.7) folds at 2 and 4 h after beginning CPB, however, without significance ($P = 0.078$ and 0.124 , respectively).

DISCUSSION

In this study, CPB induced significant depression of plasma RANTES concentrations till 24 h, with a predominant neutrophil/lymphocyte ratio and decreased lymphocyte and platelet counts after cardiac surgery. MicroRNA-125a expression in isolated lymphocytes was transient activated after beginning CPB but without significance. Further studies are needed to determine its contribution to downregulation of RANTES production following cardiac surgery.

RANTES, a member of the C-C or β chemokine subfamily, is classified as CCL5⁵ and expressed in activated T-lymphocytes, airway epithelial cells, platelets, fibroblasts, and renal epithelial and mesangial cells.⁶ It is a potent chemoattractant for T-cells and

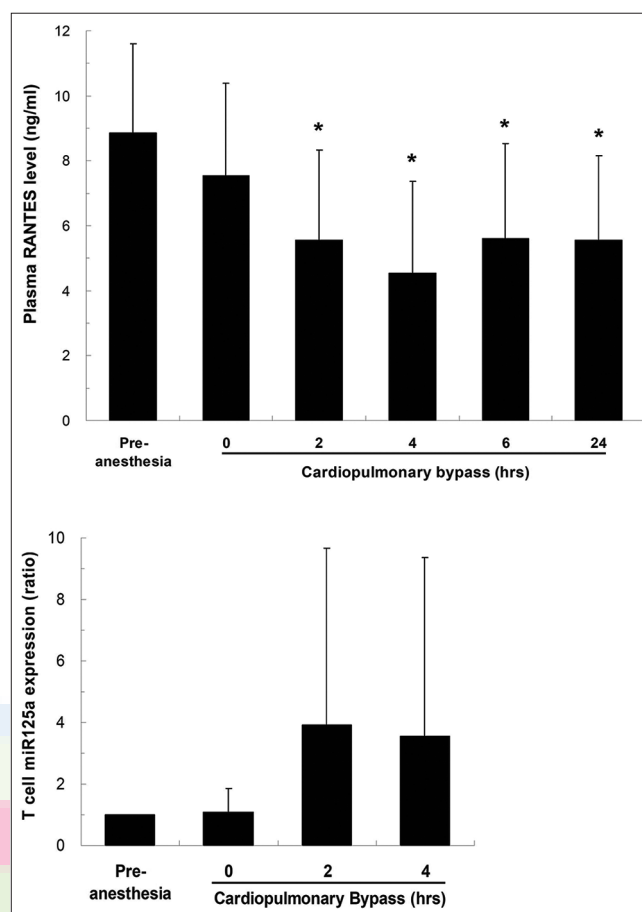


Figure 1: Plasma regulated on activation, normal T-cell expressed and secreted concentrations of 28 patients (upper) decreased significantly at 2, 4, 6, and 24 h after beginning cardiopulmonary bypass as compared with the preanesthesia level (all $P < 0.001$). In isolated T-lymphocytes from 14 patients, microRNA-125a expression (lower) elevated at 2 and 4 h after the start of bypass, however, without significance ($P = 0.078$ and 0.124 , respectively)

monocytes, which has been shown to enhance inflammation,⁵ including higher serum levels in patients with systemic lupus erythematosus (SLE),⁶ gastric cancer,¹⁴ and healthy younger men with metabolic syndrome,¹⁵ which is correlated with the activated platelet-associated markers, including plasma interleukin 6 and platelet-derived microparticles.¹⁵ On the other hand, lower plasma RANTES levels are an independent predictor of 2-year cardiac mortality in a cohort of male patients undergoing coronary angiography⁷ and are associated with CPB time over 2 h, longer surgical times, and reoperation in pediatric cardiac surgery.⁸ In our patients, the RANTES level decreased at 4 h after beginning CPB, which was consistent with the simultaneously reduced lymphocyte counts, but not the activated and consumed platelets after cardiac surgery, which were supposed to degranulate abundant RANTES protein into the blood and cause higher plasma concentrations at 2–4 h after beginning CPB.

RANTES production in cardiac surgery

Many postoperative complications after cardiac surgery are associated with some degree of immune dysregulation.¹⁶ Cardiac surgical patients may be predisposed to critically compromised illness, additional immune depleting effects by CPB, and varying degrees of malnutrition early postoperatively,¹⁶ resulting in pulmonary infection, dysfunction, and multiple organ failure, especially in patients undergoing valve surgery, who require longer CPB runs.¹⁷ Notably, a soaring inflammatory response before cardiac surgery¹⁸ following CPB or sepsis¹⁹ is usually accompanied with immune suppression. Brown *et al.*¹⁸ reported cardiac surgical patients with elevated white blood cell counts ($>10,000/\text{mm}^3$) before surgery had higher 30-day readmission after discharge than those with lower levels. The so-called T-cell exhaustion, a newly recognized immunosuppressive mechanism, may causes profoundly suppressed cytokine production in postmortem patients by sepsis.²⁰ In our patients, leukocytosis with neutrophil predominance was detected upon arrival of ICU, indicating an acute systemic inflammatory response following CPB. Meanwhile, the decreased lymphocyte count and RANTES level demonstrated the bad aspect of immunosuppression following cardiac surgery.

Recently, circulating plasma microRNAs have been novel biomarkers for screening many inflammatory or cardiac diseases.⁹ MicroRNAs are noncoding RNAs and regulate posttranscriptional gene expression by inhibiting mRNA translation or inducing its degradation,¹⁰ to modulate the expression of target genes to an optimum level, rather than participating in on/off decisions in the inflammatory response.²¹ Therefore, a lower microRNA-125a level has been observed in autoimmune lupus patients. Zhao *et al.*¹² also demonstrated microRNA-125a expression was induced in T-cells in a dose- and time-dependent manner and overexpression of microRNA-125a led to a significant reduction in the expression of RANTES mRNA. The introduction of microRNA-125a into T-cells from SLE patients alleviated the elevated RANTES expression. They concluded that microRNA-125a negatively regulates RANTES expression in activated T-cells. So far, the targets of microRNA-125 researches are metastasis and invasion of carcinomas, suppression and promotion of autoimmune diseases, and immunological host defense in response to bacterial or viral infections.²² However, there are no available reports regarding interaction between microRNA-125a expression and CPB-induced systemic inflammatory response or immunosuppression. In this study, microRNA-125a expression in isolated T-lymphocytes elevated after beginning CPB and was accompanied by reduced plasma RANTES concentrations following cardiac surgery. Further, *ex vivo* introduction of microRNA-125a antagonists or mimics into the T-cells is needed to verify the T-lymphocyte-mediated

RANTES activation and production during cardiac surgery in the future studies.

Two limitations should be addressed. First, only plasma RANTES concentrations and clinical parameters were analyzed during postoperative acute phase in this study, needing further *ex vivo* or experimental data to verify the biological changes of immune suppression and the following infectious complications. Second, limited cases may diminish the clinical manifestation and its statistical significance for the correlations. More participants with prolonged duration of follow-up are needed to verify the microRNA-125a expression and RANTES concentration as clinical indicators for potential immune dysfunction after cardiac surgery.

CONCLUSION

We demonstrated the reduced plasma RANTES concentrations for 24 h and elevated microRNA-125a expression till 4 h following CPB. The predominant increase of neutrophil/lymphocyte ratio and decrease of lymphocyte counts upon arriving ICU might predispose to immune suppression in patients receiving cardiac surgery.

Acknowledgment

This study was supported by grants from Taiwan Ministry of Science and Technology (NSC102-2314-B-016-026) and Tri-Service General Hospital (TSGH-C94-60 and TSGH-C96-15-S04).

We gratefully acknowledge the research assistants, Jui-Chi Tsai, PhD and Hsin-Hua Chung, for laboratory analysis.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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