

Comparison of m-RNA Expression for Inflammatory Mediators in Leukocytes between On-pump and Off-pump Coronary Artery Bypass Grafting

Naoko Okubo, MD, Nobuo Hatori, MD, PhD, Masami Ochi, MD, and Shigeo Tanaka, MD

Objective: Coronary artery bypass grafting (CABG) with cardiopulmonary bypass (CPB) is associated with a systemic inflammatory response. This is mainly attributed to cytokine release caused by CPB and global myocardial ischemia.

Coronary artery bypass grafting without cardiopulmonary bypass (off-pump CABG, OPCAB) is now accepted as a less invasive technique than conventional CABG. This study was designed to compare the inflammatory response at the m-RNA level of proinflammatory cytokines and adhesion molecules before and after operation in patients undergoing CABG with and without CPB.

Methods: Twenty patients who underwent isolated CABG with CPB (on-pump group, n=10) or without CPB (off-pump group, n=10) were studied. By utilizing a semiquantitative reverse transcription polymerase chain reaction (RT-PCR) technique, gene expression of cytokines, adhesion molecules, and vasoactive substances in leukocytes of peripheral blood were evaluated before and six hours after surgery.

Results: Postoperative expression of m-RNA for interleukin (IL)-1, -8, and -10, tumor necrosis factor (TNF)- α , heme oxygenase (HO)-1, platelet endothelial cellular adhesion molecule (PECAM) and Mac-1 increased significantly in the on-pump group but not in the off-pump group ($p < 0.05$).

Conclusions: In view of the m-RNA level of proinflammatory cytokines and adhesion molecules, it can be concluded that OPCAB is a less invasive technique than on-pump CABG. Direct contact of circulating blood with the synthetic surfaces of the CPB system may be the main cause of the systemic inflammation. (*Ann Thorac Cardiovasc Surg* 2003; 9: 43–9)

Key words: OPCAB, on-pump CABG, cytokines, adhesion molecules, m-RNA

Introduction

Cardiopulmonary bypass (CPB) in cardiac surgery has been recognized as one of the major causes of the systemic inflammatory response associated with multiple organ dysfunction resulting in various postoperative complications. Several factors have been assumed to cause

From Department of Surgery II, Nippon Medical School, Tokyo, Japan

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Address reprint requests to Naoko Okubo, MD: Department of Surgery II, Nippon Medical School, 1-1-5, Sendagi, Bunkyo-ku, Tokyo 113-8603, Japan.

the systemic inflammatory response, including contact activation with the synthetic surfaces of the CPB circuit, reinfusion of shed blood cells, hemodilution, protamine-heparin complexes, and endotoxins released from the temporarily ischemic intestine.¹⁻⁴⁾

To avoid the adverse effects of CPB, coronary artery bypass grafting (CABG) without CPB (off-pump CABG, OPCAB) was developed and is now a widely accepted procedure. This technique is associated with a lower risk of postoperative morbidity when compared with the conventional CABG with CPB (on-pump CABG).^{1,4)} Moreover, recent studies comparing OPCAB with on-pump CABG have shown that OPCAB is associated with significantly lower perioperative serum level of cytokines

than on-pump CABG.^{5,6)}

Since the expression of cytokine m-RNA occurs before cytokine release, measuring cytokine m-RNA expression is a more sensitive and earlier indicator for the detection of systemic inflammatory responses than measuring the serum cytokine level. Kotani et al. reported that CPB induces the expression of genes for proinflammatory cytokines and enhances the production of inflammatory cytokines in alveolar macrophages.⁷⁾ To our knowledge, however, no direct comparison has been made of the m-RNA level of proinflammatory cytokines in circulating blood between OPCAB and on-pump CABG.

This study was designed to compare the amount of surgical stress for the patient who is undergoing OPCAB with that of on-pump CABG, using a semiquantitative reverse transcription polymerase chain reaction (RT-PCR) technique to detect the m-RNA expression of proinflammatory cytokines and leukocyte adhesion molecules in the leukocytes of circulating blood and to estimate the role of CPB in surgical stress experienced by patients undergoing CABG.

Materials and Methods

Twenty patients undergoing elective isolated CABG were studied: 10 patients with the use of CPB (on-pump group), and the other 10 patients without CPB (off-pump group). Their ages ranged from 50 to 83 years with a mean of 66.9 years. There were 17 males (85%) and 3 females (15%). Patients undergoing redo procedures, with infectious disease, perioperative use of intra-aortic balloon pumping (IABP) or hemodialysis, malignant neoplastic disease, or the preoperative use of steroids were excluded from this study. Written informed consent was obtained before the operation from all patients and the study protocol was approved by the medical ethics committee of our institute.

No patient received corticosteroids, aprotinin or ulinastatin before, during, or after the operation. All operations were performed by one surgeon only.

Operative procedure

Anesthesia was induced with midazolam, propofol and fentanyl and maintained with midazolam, fentanyl, and isoflurane. Muscle relaxation was obtained with pancuronium or vecuronium. Surgery in all patients was performed through a median sternotomy. In the on-pump group, a single two-stage venous drainage cannula in the right atrium and a standard arterial cannula in the ascend-

ing aorta were employed for CPB with a membrane oxygenator (HPO-2OH-C; Senkou Ikakougyou, Tokyo, Japan). Two-hundred international units (IU) per kilogram of heparin were administered to achieve an activated coagulation time of more than 300 seconds. The contents of CPB were lactate Ringer solution and 5% glucose solution at a ratio of 4:1. Five milliliters per kilogram of Mannitol, 1 mEq/kg of sodium bicarbonate and 40 ml of 25% human albumin were added to the priming solution. CPB was conducted with nonpulsatile flow at 2.2 l/min/m² with normothermia. After the aorta was cross-clamped, cold crystalloid cardioplegia was infused into the aortic root. Antegrade and retrograde cardioplegia were intermittently infused during CPB. Terminal blood cardioplegia was used before the aorta was unclamped.

In the off-pump group, after median sternotomy, revascularization was performed on the beating and normothermic heart. Anticoagulation was achieved with heparin at 150 IU/kg after harvesting all grafts. To obtain local stabilization, a mechanical stabilizer (Immobilizer™ system; Genzyme Corp., Cambridge, MA, U.S.A.) was used. Dilthiazem or inderal was used for heart rate control. If the patient's condition did not change during the temporary coronary artery occlusion by tightening the silicon tapes, an anastomosis was constructed. We did not use an intracoronary shunt except for the proximal right coronary anastomosis.

Blood sampling

Blood samples were taken at two points from all patients after induction of anesthesia but before sternotomy (preoperative) and six hours after the first sampling (postoperative). Samples were overlaid on Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) and centrifuged at 400×g for 20 min for isolation of leukocytes, including lymphocytes.

m-RNA was extracted with the acid guanidinium phenol chloroform (AGPC) method to quantitate the amount of m-RNA present in the leukocytes. The main constraint in obtaining quantitative data is inherent in the amplification process. Single-stranded c-DNA synthesis was also performed using random hexamers (TaKaRa, Tokyo, Japan) as primers in the presence of RNase inhibitor. Moloney murine leukemia virus (M-MLV) reverse transcriptase was used as reverse transcriptase.

RT-PCR

All genes in each patient were investigated with the same PCR, at a volume of 50 µl for each gene. A master mix

Table 1. Demographics and surgical characteristics of patients

Group	Off-pump (n=10)	On-pump (n=10)	Statistics
Gender (male/female)	9/1	8/2	NS
Age (years)	72.4±5.9	61.4±10.4	p=0.01
Operation time (min)	260±30.6	273.9±43.7	NS
Basic diseases			
UA	0	2	NS
AP	7	5	NS
OMI	0	1	NS
AMI	3	2	NS
Risk factors			
Smoking	6	6	NS
Hypertension	9	9	NS
Hyperlipidemia	4	9	NS
Diabetes mellitus	3	7	NS
CPB time (min)	–	99.9±2.4	–
Ao clamp time (min)	–	127.8±23.7	–
No. of bypass	2.5±1.1	4.2±1.0	NS

NS, not significant; UA, unstable angina; AP, angina pectoris; OMI, old myocardial infarction; AMI, acute myocardial infarction; CPB, cardiopulmonary bypass; Ao, aorta

consisting of dNTP (1.25 mmol/l), Ampli Taq (TaKaRa, Tokyo, Japan), and PCR buffer was prepared, and divided into two parts for addition of c-DNA. For all genes, the relationship between the number of PCR cycles and the amount of PCR products was investigated before the study. For β -actin (as an internal control), heme oxygenase(HO)-1, platelet endothelial cellular adhesion molecule (PECAM), and l-selectin (SEL), 27 cycles were selected; for HO-2, and Mac-1, 30 cycles; for interleukin (IL)-8, and IL-10, 32 cycles; and for IL-1, tumor necrosis factor (TNF)- α , IL-6, inducible nitric oxide synthase (iNOS), and endothelin (ET)-1, 35 cycles were used. The PCR products were separated by electrophoresis on agarose gel with ethidium bromide (Et.Br.) dyeing and bands were semiquantified with an image analyzer on a UV illuminator. The ratio between the optical density of β -actin and the test gene was calculated to evaluate relative changes in the test gene.

A complete blood cell count and a manual differential were determined at each point.

Statistical analysis

Statistical analysis was performed using SPSS version 11.0J soft package (SPSS Inc., Chicago, IL, U.S.A.). Unpaired Student's t test was used to evaluate differences between the data of two groups, and paired Student's t test was applied to analyse for paired data. Correlations between two variables were analysed by the Pearson cor-

relative coefficient test. Comparisons of proportion were analysed by the chi-square test. A p value less than 0.05 was considered significant. All data are presented as mean±standard deviation (SD).

Results

Patient characteristics

Although the patients were younger (p=0.01) in the on-pump group, there was no significant difference between the two groups with respect to gender, basal coronary disease, risk factors and operation time (Table 1). All patients were extubated within 24 hours of operation. And none had significant postoperative complications.

Leukocyte counts and differentials

The leukocyte counts and their differentials are shown in Table 2. The postoperative leukocyte counts were significantly greater than the preoperative values in both groups (p<0.05). However, no significant difference between the groups was found for any of the values.

Expression of m-RNA

There was no significant difference between the groups in the m-RNA expression before operation (Table 3).

When comparing postoperative expression between the two groups, significant increases were found in m-RNA of the on-pump group for IL-1, IL-8, IL-10, TNF- α , HO-

Table 2. Leukocyte counts and differential of each group

	Preoperation	Postoperation	Statistics (1)
Off-pump group			
WBC	5,300±3,548	11,288±3,878	p<0.0001
Lymphocytes	1,189±273	808±333	NS
Granulocytes	3,801±3,542	9,778±3,809	p<0.0001
Monocytes	310±93	486±228	NS
On-pump group			
WBC	5,620±1,680	12,829±4,165	p=0.007
Lymphocytes	1,432±624	692±476	p=0.026
Granulocytes	3,868±1,093	11,620±3,892	p=0.0001
Monocytes	319±103	516±267	NS
Statistics (2)	NS	NS	-

Data are expressed as counts/ μ l (mean \pm SD).

NS: not significant

Statistics (1) are expressed as comparisons within the group.

Statistics (2) are expressed as comparisons between the groups in each parameter.

1, PECAM and Mac-1 ($p<0.05$). In the on-pump group, the expression was significantly elevated postoperatively ($p<0.05$). In the off-pump group, however, no significant difference in expression for each of these factors was found before or after operation.

Correlation between m-RNA expression and age

No statistically significant correlation was seen between patient age and m-RNA expression level (Table 4), respectively of HO-1 ($p=0.162$ in off-pump group, $p=0.74$ in on-pump), IL-1 ($p=0.90, 0.93$), IL-8 ($p=0.79, 0.77$), IL-10 ($p=0.75, 0.58$), TNF- α ($p=0.14, 0.64$), PECAM ($p=0.22, 0.93$) and Mac-1 ($p=0.72, 0.91$).

Discussion

The introduction of CPB in the 1960's has contributed to the safety of open heart surgery. It is generally agreed, however, that postoperative morbidity in cardiac surgery stems from the damaging effects of the CPB itself.

The sequestration of white blood cells generates oxygen free radicals and protease enzymes, thus causing interstitial edema of the lung with the need for postoperative respiratory support.⁸ Several studies have indicated that the CPB-induced systemic inflammatory response was due to the activation of complement, macrophages, monocytes, neutrophils, and the release of cytokines and vasoactive substances.^{9,10} Consequently, OPCAB is believed to have a significant advantage in that it can be performed without CPB, in which the circulating blood

is in direct contact with artificial surfaces. It has been reported that OPCAB is less invasive than on-pump CABG in that the serum level of cytokines increases much more in on-pump CABG than in OPCAB.^{5,6}

Since the production of serum cytokines follows the expression of their m-RNA, the m-RNA level is believed to be a more sensitive mediator than the serum cytokine concentration.

Therefore we compared the m-RNA level of cytokines in the two groups. It is known that the magnitude of the inflammatory reaction, which correlates with the degree of tissue injury, is compatible with the degree of the cytokine level.^{9,11} Particularly, serum levels of IL-1, IL-6, IL-8, IL-10 and TNF- α have been examined in many clinical studies during and after CPB.¹²⁻¹⁷ In the present study, m-RNA expression of IL-1, IL-8, IL-10 and TNF- α increased only in the on-pump group, results which also support previous observations.^{5,6,18} The expression of IL-6 m-RNA did not increase in our study. A possible explanation for this is that there were no macrophages in the circulating blood except for monocytes. This finding is also supported by a study of Kotani et al., who reported that IL-6 was increased in alveolar macrophages but not in leukocytes.⁷

Blood contact with the synthetic surfaces of the CPB circuit activates the cellular components of inflammation. Leukocytes activated with CPB adhere to the vascular endothelium as a crucial early step in tissue injury.^{14,19,20} The interaction of specific neutrophils and endothelial adhesion molecules plays an important role in the leuko-

Table 3. Expression of each gene before and after operation

Parameter	Preoperation	Intra-group	Postoperation
HO-1	off-pump	0.60±0.16	←NS→ 0.64±0.28 ←
	on-pump	0.54±0.17	←★→ 0.94±0.27 ←
HO-2	off-pump	0.65±0.27	←NS→ 0.77±0.39
	on-pump	0.74±0.34	←NS→ 0.82±0.42
IL-6	off-pump	0.04±0.09	←NS→ 0.07±0.18
	on-pump	0.00±0.00	←NS→ 0.05±0.10
IL-8	off-pump	0.43±0.24	←NS→ 0.27±0.20 ←
	on-pump	0.29±0.11	←★→ 0.53±0.23 ←
IL-10	off-pump	0.21±0.12	←NS→ 0.39±0.26 ←
	on-pump	0.11±0.12	←★→ 1.09±0.41 ←
IL-1	off-pump	0.96±0.43	←NS→ 0.73±0.29 ←
	on-pump	0.77±0.30	←★→ 1.27±0.40 ←
TNF-α	off-pump	0.65±0.29	←NS→ 0.45±0.19 ←
	on-pump	0.53±0.11	←★→ 0.93±0.36 ←
iNOS	off-pump	0.22±0.17	←NS→ 0.28±0.31
	on-pump	0.11±0.11	←NS→ 0.12±0.14
ET-1	off-pump	0.32±0.28	←NS→ 0.31±0.30
	on-pump	0.25±0.30	←NS→ 0.33±0.37
PECAM	off-pump	0.77±0.22	←NS→ 0.51±0.25 ←
	on-pump	0.68±0.28	←★→ 1.09±0.56 ←
l-SEL	off-pump	0.83±0.22	←NS→ 0.97±0.33
	on-pump	0.89±0.36	←NS→ 1.26±0.51
Mac-1	off-pump	0.57±0.51	←NS→ 0.67±0.56 ←
	on-pump	0.55±0.55	←★→ 1.47±0.84 ←

★ shows the statistical significance (p<0.05) between two values indicated with a black arrow.

Data are expressed as the ratio between the optical density of β-actin and the test gene, which was calculated to evaluate relative change in the test gene.

HO, heme oxygenase; IL, interleukin; TNF, tumor necrosis factor; iNOS, inducible nitric oxide synthase; ET, endothelin; PECAM, platelet endothelial cellular adhesion molecule; SEL, selectin

cyte-mediated tissue injury associated with CPB.²¹⁾ The increase in the m-RNA expression of Mac-1 and PECAM in the on-pump group shows that activated neutrophils start the high-affinity binding for infiltration. However, l-SEL did not increase in either group in our study. This may be because the l-SEL molecules, which mediate leukocyte rolling, which is described as a low-affinity binding state, always exist on the surface of neutrophils.

HO-1 increased in the on-pump group. HO-1 is induced by inflammatory conditions, and it represents an endogenous protective mechanism against oxidative stress.^{22,23)}

In contrast, HO-2, which is not inducible, did not increase in either group.

In this study, the patient age differed significantly between the two groups (off-pump 72.4±5.9 years versus on-pump 61.4±10.4 years, p=0.01) (Table 1). However,

this factor may not have influenced the result because there was no statistical correlation between each m-RNA expression level and patient age (Table 4).

Although several factors, such as CPB, cardioplegia, reperfusion in the ischemic heart, and others during CABG are possible causes of the inflammatory reaction, it has been reported that the contact of blood with foreign materials used in the CPB circuit is one of the major factors which cause deleterious inflammatory reactions.^{24,25)}

The inflammatory reaction occurring with CPB principally involves four blood elements: the plasma protein contact activation system, the complement system, neutrophils, and monocytes. Kallikrein produced by the contact system of plasma proteins directly mediates neutrophil activation. Neutrophils are also activated by C5b, which is one of three anaphylatoxins of the complements

Table 4. Correlation between m-RNA and age in two groups

	HO-1	IL-8	IL-10	IL-1	TNF- α	PECAM	Mac-1
Off-pump							
r value	0.48	0.10	0.11	0.05	0.50	-0.42	-0.13
p value	0.162	0.79	0.75	0.90	0.14	0.22	0.72
On-pump							
r value	-0.12	-0.11	0.20	0.03	0.17	-0.03	0.04
p value	0.74	0.77	0.58	0.93	0.64	0.93	0.91

Correlations between m-RNA and age were analysed by Pearson's test. No statistical relation was observed between m-RNA and age in two groups.

activated by both the classic and alternative pathways.²⁶⁾ This activation leads to increased neutrophil interaction with the similarly activated endothelium, resulting in enhanced neutrophil-endothelial cell adhesion and neutrophil sequestration. The adhesion molecule family of selectins mediates the initial rolling phase of neutrophil attachment to the endothelium. Subsequently, interaction between the adhesion molecule families of integrins and immunoglobulins facilitates a firm binding of the neutrophils to the activated endothelium.²⁷⁾ This adhesion molecule activation cascade may agree with the results of the m-RNA expression in our study.

In an in vitro perfusion circuit, monocytes are activated more slowly than contact and complement proteins and neutrophils.²⁸⁾ In the present study, however, the significant increase in m-RNA of IL-8, possibly originating from monocytes, was observed immediately after operation in the on-pump group. This may indicate that the expression of m-RNA is a sensitive indicator for inflammatory response.

In conclusion, we examined gene expression of leukocyte adhesion molecules and proinflammatory cytokines in the human circulating blood after CABG with or without CPB. Gene expression of inflammatory mediators in circulating leukocytes was more evident in patients undergoing on-pump CABG when compared to those with on-pump CABG. In this sense, OPCAB was less invasive than on-pump CABG.

Expanded studies of gene expression analysis may elucidate the precise mechanism of the systemic inflammatory reaction caused by CPB.

A limitation of this study was that the observation was only done twice. Further studies are necessary to investigate the m-RNA expression at multiple time points and to detect the relationship between m-RNA expression and serum cytokine levels.

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