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—**Original**—

Does Carboxy-hemoglobin Serve as a Stress-induced Inflammatory Marker Reflecting Surgical Insults?

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Abstract

Endogenous carbon monoxide (CO) production has been recently observed to be an index of the inflammatory response, reflecting various insults in critically ill patients. Major surgery is supposed to modulate the production of CO by transcriptional regulation of heme oxygenase (HO). CO is easy to measure as carboxyhemoglobin (CO-Hb) by spectrophotometry; however, whether CO-Hb can be used as an index reflecting surgical insults is unknown. We investigated changes in CO generation during coronary artery bypass graft by measuring CO-Hb concentrations and the expression of HO in circulating blood as well as the expressions of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β). The expression ratios of heme oxygenase-1 (HO-1), TNF-α, and IL-1β significantly increased after surgery, and these values correlated significantly with one another. CO-Hb concentrations significantly increased after surgery; however, many of those values during artificial ventilation with high inspired oxygen fraction were within normal limits. Furthermore, changes in CO-Hb concentrations were small when preoperative values were high. On the whole, CO-Hb concentrations significantly but weakly correlated with the expression ratios of the inflammatory mediators. However, they did not correlate in the patients who showed higher preoperative CO-Hb concentrations. These data indicate that CO-Hb concentrations can, in general, reflect the inflammatory response induced by surgical insult; however, CO-Hb measurement may not be a useful form of clinical monitoring because of the limited degree of changes, the variation of baseline values, and the necessity for the management under fixed conditions.
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Key words: carbon monoxide, carboxyhemoglobin, heme oxygenase, tumor necrosis factor-α, interleukin-1β, surgical stress, reverse transcription polymerase chain reaction

Introduction

Major surgery is associated with the development of a systemic inflammatory response. Systemic inflammation can be potentially damaging to major organs and contributes to an increase in postoperative complications[1,2]. Although several anti-inflammatory strategies aimed at attenuating the development of a systemic inflammatory response have been used in recent years[3,4], this phenomenon varies by clinical setting. Changes in the inflammatory response can be detected by measuring plasma concentrations of certain

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inflammatory markers such as complement components, cytokines, and adhesion molecules[5,6]. However, these markers take time to measure and cannot be measured easily in all clinical settings. Thus a simple and accurate index is needed to detect perioperative changes in the inflammatory response.

In recent years, the endogenous generation of carbon monoxide (CO) has been observed to be an index of the inflammatory response after various insults[7–11]. Endogenous CO is mainly synthesized from inducible heme oxygenase-1 (HO-1) and constitutive heme oxygenase-2 (HO-2)[12]. HO-1 is a known stress-inducible heat shock protein 32 that is transcriptionally upregulated by various stressors[13]; in addition, HO-1 is inducible by more diverse stimuli than any other enzyme described to date[12]. Because it is difficult to metabolize CO in vivo, and CO binds with high affinity to hemoglobin[14], it is easy to measure the endogenous production of CO as carboxyhemoglobin (CO-Hb) using spectrophotometry.

Several clinical observations have been published on endogenous CO production in patients. In a study involving 32 surgical intensive care patients, a correlation was found between mean CO-Hb levels as high as 1.9% and severity of illness assessed by the APACHE II score[7]. Another study reported higher CO-Hb levels in 59 intensive care patients compared with 20 control patients[15]. These studies point to the possibility of using CO-Hb levels to determine a patient's severity of illness. On the other hand, another study[16] reported that plasma lactate levels and CO-Hb levels were not correlated in 183 critically ill patients and concluded that CO-Hb levels were not clinically useful as a marker of critical illness. However, previous studies did not measure the gene expression of HO that produces CO and did not compare changes in CO-Hb levels with inflammatory mediators; thus, whether CO-Hb levels are an effective index to measure surgical insults is still unknown.

In this study, we measured CO-Hb levels during elective coronary artery bypass graft surgery (CABG) in patients who underwent cardiopulmonary bypass (CPB) with or without glucocorticoid administration and in patients who did not undergo CPB. Expressions of HO-1 as well as inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) in circulating blood were also measured. We evaluated 1) whether CO-Hb levels are related to HO-1 activity as measured by expression of HO-1 mRNA in circulating leukocytes, 2) whether expression of HO-1 mRNA in circulating leukocytes is elevated in surgical insults, 3) whether reduction in other markers of the inflammatory response decrease expression of HO-1 mRNA.

Subjects and Methods

The protocol of this study was approved by the review board of Nippon Medical School, and written informed consent was obtained from all subjects before the study. Patients undergoing elective isolated CABG in Nippon Medical School were included in the study. Patients undergoing re-operation, with preoperative inflammatory disease including infectious disease, perioperative use of intra-aortic balloon pumping or hemodialysis, malignant neoplastic disease, chronic or acute pulmonary dysfunction, smoking history for the past one month, or the preoperative use of steroids were excluded. No patients received anti-inflammatory drugs such as aprotinin and ulinastatin perioperatively, and all operations were performed by a single surgeon.

Preliminary Study

To evaluate the degree of change in CO-Hb concentration during CABG, arterial blood was sampled from the patients who underwent CABG during the period from August 2002 to June 2003. Although these patients fulfilled the criteria, neither the operation method nor the anesthesia methods were controlled. Arterial blood samples for serial determination of CO-Hb concentrations were taken at three timepoints: after induction of anesthesia but before sternotomy (preoperative measurement), after all bypass graftings and anticoagulation was reversed (postbypass measurement), and just before leaving the operating room (postoperative measurement). CO-Hb concentrations were measured immediately by spectrophotometer (ABL625 blood gas analyzer, Radiometer, Copenhagen, Denmark) along with a routine blood count and blood gas analysis. The patients were divided into two groups: patients who showed CO-Hb concentrations 1% or more, and patients who showed less than 1%, and the degrees of change in CO-Hb concentration were compared.

Main Study

Following the end of the preliminary study, 45 patients participated in the main study. Patients were divided into 3 groups: (1) the on-pump group consisted of patients who underwent CPB but who did not receive glucocorticoid therapy; (2) the steroid group consisted of patients who received glucocorticoid therapy during CPB; and (3) the off-pump group consisted of patients who did not undergo CPB. Whether CPB was used for CABG was determined by each attending cardiac surgeon. Whether methylprednisolone was administered during CABG was randomly assigned. To measure gene expressions of inflammatory mediators such as HO-1, HO-2, TNF-α, and IL-1β, 4 mL of arterial blood was collected at two time points: the preoperative and postoperative periods. The sample was immediately mixed with a nucleic acid extraction reagent (ISOGEN-LS, Nippon Gene Co.,Tokyo, Japan) and stored at −70℃ until measurements were made. Arterial blood samples for determination of CO-Hb concentrations were taken at the same two timepoints. The relationships among changes in expression ratios of inflammatory mediators and the change in CO-Hb concentration were evaluated. The patients were divided into two groups: patients who showed CO-Hb concentrations 1% or more, and patients who showed less than 1%, and the degrees of correlations among CO-Hb concentration and expression ratios of inflammatory mediators were compared.

Operative and Anesthetic Procedure

Anesthesia was induced with 0.1 to 0.2 mg/kg midazolam and 2 to 4 μg/kg fentanyl and maintained with fentanyl and sevoflurane with 50% oxygen in air. Muscle relaxation was obtained with vecuronium. Other than during CPB, red cells were transfused to achieve a hemoglobin concentration greater than 10 g/dL. Surgery in all patients was performed thorough 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 ascending aorta were used for CPB with a membrane oxygenator (HPO-2 OH-C; Senkou Ikakougyou, Tokyo, Japan). Two hundred IU/kg of heparin were administered to achieve an activated coagulation time>450 seconds. CPB prime consisted of lactate Ringer's solution and 5% glucose solution at a ratio of 4 : 1. Five mL/kg of mannitol, 1 mEq/kg of sodium bicarbonate, and 40 mL of 25% human

Table 1 Sequences of primers and probes

Heme oxygenase type 1
  Forward primer: 5'-GGCCAGCAACAAAGTGCAA-3'
  Reverse primer: 5'-ACTGTCGCCACCAGAAAGCT-3'
  TaqMan probe: 5'-CTCCCAGGCTCCGCTTCTCCG-3'
Heme oxygenase type 2
  Forward primer: 5'-CAGCCTTTGCCCCTTTGTACT-3'
  Reverse primer: 5'-CAAAGAAATACTCCATGTCCTTGGT-3'
  TaqMan probe: 5'-CCCCATGGAGCTGCACCGGA-3'
Tumor necrosis factor-α
  Forward primer: 5'-ATGTTGTAGCAAACCCTCAAGCT-3'
  Reverse primer: 5'-GATGAGGTACAGGCCCTCTGAT-3'
  TaqMan probe: 5'-CTCCAGTGGCTGAACCGCCGG-3'
Interleukin-1β
  Forward primer: 5'-AGGCTTATGTGCACGATGCA-3'
  Reverse primer: 5'-TGGACCAGACATCACCAAGCT-3'
  TaqMan probe: 5'-TACGATCACTGAACTGCACGCTCCG-3'

albumin were added to the priming solution. CPB was conducted with nonpulsatile flow at 2.2 L/min/m^2 with normothermia. In the steroid group, 20 mg/kg of methylprednisolone was administered just before the start of CPB. 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.

Real-time Reverse Transcription - Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from 4 mL of arterial blood using the chaotrophic Trizol method followed by Isogen-chloroform extraction and isopropanol precipitation[17]. One microgram of each total RNA extract was reverse transcribed at 37℃ for 1 h, in a 20-μL reaction mixture containing mouse Moloney leukemia virus reverse transcriptase and hexanucleotide random primers (Takara Bio, Ohtsu, Japan). PCR primers and TaqMan fluorogenic probes were designed using the Primer Express software program (Applied Biosystems, Foster City, CA, USA). Primer and probe sequences are shown in **Table 1**. One microliter of cDNA was used for quantitative PCR in a 50-μL volume including the TaqMan Universal Master Mix (Applied Biosystems; 25 μL); 900 nM of forward and reverse primers; 200 nM of TaqMan probe; and deionized water. PCR conditions were as follows: 50℃ for 2 min and 95℃ for 10 min followed by 40 cycles of amplification for 15 seconds at 95℃ and 1 min at 60℃. The TaqMan probe labeled with 6-FAM was cleaved during amplification, generating a fluorescent signal. Unknown samples and calibration curve samples were run in triplicate. A similar system using a separate glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe and primer set (TaqMan GAPDH control reagent kit; Applied Biosystems) was designed and run for GAPDH along with every unknown sample to correct for total nucleic acid content. The assay used an instrument capable of measuring fluorescence in real time (ABI PRISM 5700 Sequence Detector; Applied Biosystems). Results of the real time PCR data were represented as the threshold cycle (CT) values, where CT was a unitless value defined as the fractional cycle number at which the sample fluorescence signal passes a fixed threshold above baseline. Relative amounts of all mRNAs were calculated by the comparative CT method (Applied Biosystems)[18] using the equation $2^{-\Delta\Delta C_T}$. ΔCT was the difference in the CT values derived from the unknown sample and the GAPDH control, while ΔΔCT represented the difference between the paired samples, as calculated by the formula ΔΔCT = ΔCT of sample before surgery − ΔCT of sample after surgery.

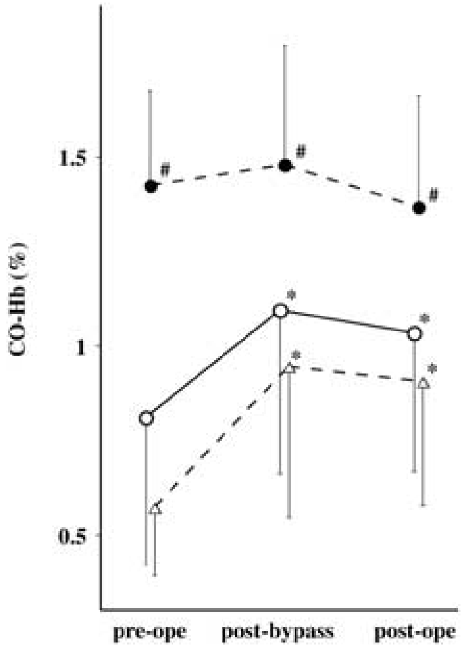

Fig. 1 Changes in the mean values of carboxy-hemoglobin (CO-Hb) concentrations in 118 patients. Open circles = mean − SD of all patients; open triangles = mean − SD of patients who showed CO-Hb concentrations < 1.0% at the preoperative measurement (n = 85); closed circles = mean + SD of patients who showed CO-Hb concentrations ≥ 1.0% at the preoperative period measurement (n = 33). $^{*}P < 0.05$ vs the preoperative measurement; $^{\#}P < 0.05$ vs the patients who showed CO-Hb concentrations < 1.0%.

Statistical Analysis

All values are expressed as mean ± SD. Statistical significance of the results for gene expression was

Table 2 Demographics and surgical characteristics of the 45 patients in whom the gene expressions of inflammatory mediators were measured

Group n	On-pump (n = 15)	Steroid (n = 15)	Off-pump (n = 15)	Stasistics
Gender (female/male)	4/11	5/10	5/10	NS
Age (years)	62.6 ± 6.8	63.1 ± 6.4	69.0 ± 8.8	NS
Weight (kg)	58.1 ± 15.9	59.6 ± 11.2	56.4 ± 9.4	NS
Basic diseases				
UA	2	2	1	NS
AP	7	8	9	NS
OMI	3	2	1	NS
AMI	3	3	4	NS
Risk factors				
Smoking history	8	7	7	NS
Hypertension	11	11	12	NS
Hyperlipidemia	8	8	6	NS
Diabetes mellitus	7	8	5	NS
Anesthesia time (min)	354 ± 59	365 ± 71	302 ± 68	NS
CPB time (min)	127 ± 35	132 ± 37	—	NS (On-pump vs Steroid)
Ao clamp time (min)	98 ± 38	108 ± 32	—	NS (On-pump vs Steroid)
No. of bypass	3.9 ± 1.0	3.8 ± 1.2	2.8 ± 1.2	NS
Hemoglobin (g/d*l*)				
Preoperative	11.2 ± 2.3	11.6 ± 2.3	12.1 ± 1.7	NS
Postoperative	10.5 ± 1.0	10.5 ± 1.5	10.7 ± 1.6	NS

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

evaluated using a Kruskal-Wallis analysis of all groups followed by a Mann-Whitney comparison between individual groups. Correlations between two variables were analyzed by the Pearson correlative coefficient. A one-way repeated-measures analysis of variance (ANOVA) followed by Scheffe's test was used to analyze time-dependent changes. Other data were analyzed by one-way ANOVA followed by Scheffe's test for comparisons among groups. A P value ≤ 0.05 was considered statistically significant.

Results

A total of 163 patients participated in the study. All patients were extubated within 24 h of surgery, and none had significant postoperative complications.

Preliminary Study

One hundred eighteen patients (38 female, 80 male) participated in the preliminary study. Their mean (range) age and weight were 69.4 (50 to 87) years and 59.5 (37 to 87) kg, respectively. Thirty-eight patients underwent CABG without CPB (off-pump bypass: OPCAB), and 41 patients were administered with glucocorticoids during CPB. The mean number of bypasses was 3.5. **Fig. 1** shows the changes in the mean values of CO-Hb concentrations. In all patients and in patients who showed CO-Hb concentrations $< 1\%$ at the preoperative measurement, CO-Hb levels were significantly increased at both the postbypass and the postoperative measurements ($P < 0.05$). However, CO-Hb levels were not significantly changed postoperatively in patients who showed CO-Hb concentrations $\geq 1\%$ at the preoperative measurement.

Main Study

Table 2 shows the demographics and surgical characteristics of the 45 patients. There were no significant differences among groups with respect to gender, age, weight, basal coronary disease, risk factors, anesthesia time, CPB time, and pre- or postoperative hemoglobin concentrations. **Fig. 2** shows the expression ratios of HO-1, HO-2, TNF-α,

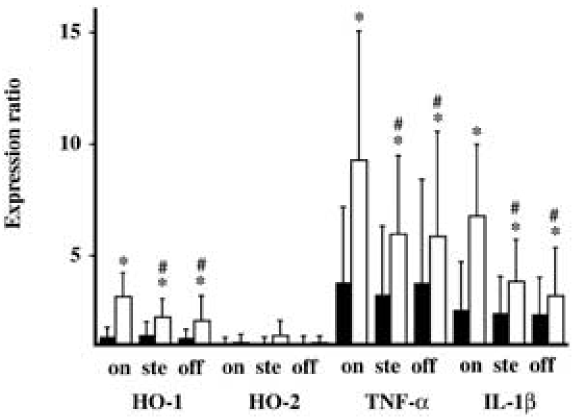

Fig. 2 Expression ratios of hemeoxygenase-1 (HO-1), heme oxygenase-2 (HO-2), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β) in circulating blood in 45 patients. Black bars = preoperative measurement; white bars = postoperative measurement; on = on-pump group; ste = steroid group; off = off-pump group. $^*P<0.05$ vs preoperative measurement; $^\#P<0.05$ vs the on-pump group.

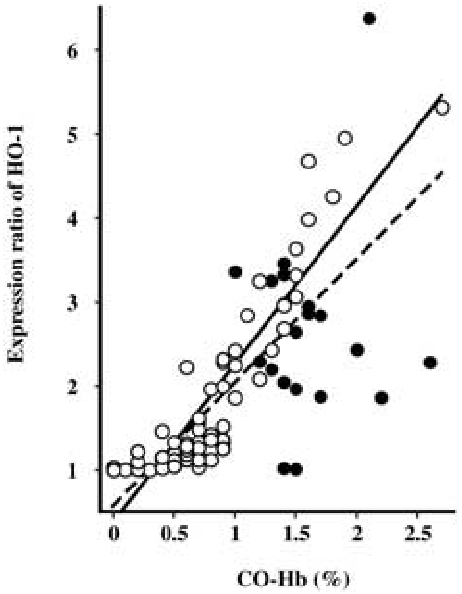

Fig. 3 Relationship between the carboxy-hemoglobin concentrations (CO-Hb) and the expression ratio of heme oxygenase-1 (HO-1). Open circles = patients with CO-Hb concentrations < 1.0% at the preoperative measurement (n = 35); closed circles = patients with CO-Hb concentrations ≥ 1.0% at the preoperative measurement (n = 10). Solid line = regression among patients who showed CO-Hb concentrations < 1.0% at the preoperative measurement; dotted line = regression among all patients.

and IL-1β at the preoperative and postoperative measurements in all three groups. The expression ratio of HO-2 did not show any significant changes in any group. The expression ratios of HO-1, TNF-α, and IL-1β significantly increased after surgery in all three groups ($P<0.05$ for all comparisons) and those in the on-pump group were significantly higher than the other groups ($P<0.05$ for all comparisons) at the postoperative measurement.

Fig. 3 shows the relationship between CO-Hb levels and the expression ratio of HO-1 in 90 measurements from 45 patients. There was a significant but weak correlation between these values ($r^2=0.58$); however, a stronger correlation ($r^2=0.80$) was shown when the data from patients whose CO-Hb levels at the pre-operative measurement were ≥ 1% were excluded. No significant correlation between hemoglobin concentrations and CO-Hb concentrations were seen.

There was a significant correlation ($r^2=0.57$) between the expression ratios of TNF-α and HO-1 (**Fig. 4**). There was a significant correlation ($r^2=0.64$) between the expression ratios of IL-1β and HO-1 (**Fig. 5**). The concentration of CO-Hb showed a significant correlation ($r^2=0.69$) with the expression of TNF-α. A weak correlation ($r^2=0.44$) was shown between these values in patients with CO-Hb values ≥1% at the preoperative measurement (**Fig. 6**). The concentration of CO-Hb also showed a significant correlation ($r^2=0.76$) with the expression of IL-1β. A weak correlation ($r^2=0.42$) was shown between these values in patients with CO-Hb levels ≥1% at the preoperative measurement (**Fig. 7**).

Discussion

Nitric oxide (NO), which is a gaseous monoxide, was identified as an endothelium-derived relaxing factor in the 1980s, and its various physiologic activities have been proved over the years[19]. Although CO, which is a low molecular gaseous monoxide produced by oxygenase like NO and generated with the heme metabolism in vivo, has

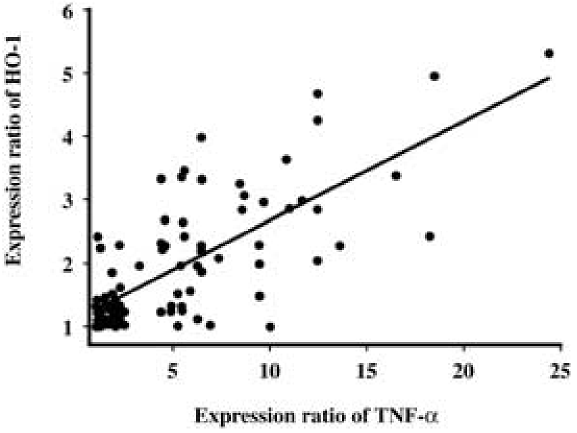

Fig. 4 Relationship between the expression ratios of tumor necrosis factor-α (TNF-α) and hemeoxygenase-1 (HO-1).

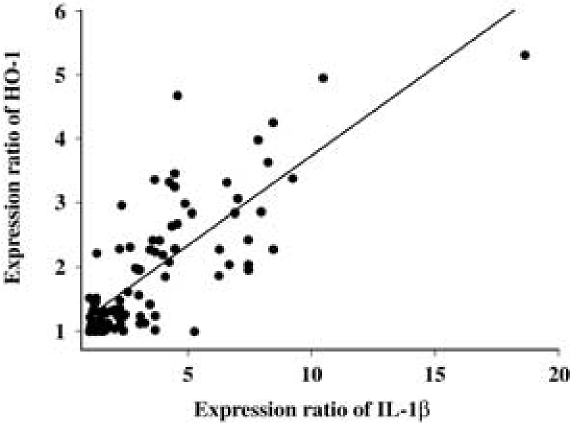

Fig. 5 Relationship between the expression ratios of interleukin-1β (IL-1β) and hemeoxygenase-1 (HO-1).

been known for many years, the physiologic role of CO gained attention in the 1990s after it was reported that CO activates guanylyl cyclase like NO[20]. Endogenous CO is mainly synthesized from HO, and recently, inducible HO-1 has been actively investigated because this enzyme has been shown to have anti-inflammatory, antiapoptotic, antiproliferative, and salutary effects in patients with sepsis[21]. Because CO binds with high affinity to hemoglobin, it is easily measured as CO-Hb by spectrophotometry. Because CO is difficult to metabolize in vivo but is easy to measure, it has become a useful way to evaluate physiologic changes associated with inflammation in vivo.

Locally generated CO is eliminated by hemoglobin in circulating erythrocytes and is gradually released into the alveolar space of the lungs, where molecular oxygen is alternately bound to the heme. Most endogenous-generated CO is thus exhaled into the airway, and the alveolar oxygen tension determines the exchange rate between oxygen and CO[14]. For these reasons, CO-Hb in blood samples collected from patients could be altered by multiple factors such as surgical insults, hemoglobin concentration[10], tissue oxygenation, and pulmonary function[22]. In this study, we chose patients who did not suffer from obvious respiratory or inflammatory diseases, and we fixed the inspired oxygen fraction at 0.5 during the study except for during intubation. We tried to maintain Hb concentrations >10 g/dL so that hemoglobin concentrations at sampling time did not differ among groups and were not correlated with CO-Hb concentrations. In spite of the strict patient selection and the fixed patient management, CO-Hb values varied at the preoperative measurement. We also found that changes in CO-Hb concentrations were small in patients who showed CO-Hb concentrations $\geq 1\%$ at the preoperative measurement. It is presumed that these patients had functional changes in CO dynamics, such as CO production, binding to Hb, and excretion from pulmonary circulation, or that they already had certain inflammatory changes.

We selected patients undergoing CABG to evaluate changes in CO-Hb concentrations because cardiac surgery with CPB has been recognized to provoke a systematic inflammatory response and the anti-inflammatory strategies, such as administration of corticosteroids[18] or not using CPB[23], were expected to change the inflammation level. Several factors have been assumed to cause the systematic inflammatory response, including contact activation with artificial surfaces of the CPB circuit, reinfusion of shed blood cells, hemodilution, protamine-heparin complexes, operative trauma, and endotoxins released from the temporarily ischemic intestine[24]. Off-pump CABG has been reported to be associated with a lower risk of postoperative morbidity and with significantly lower perioperative serum level of inflammatory cytokines than conventional CABG with CPB[23,24]. Pharmacologic intervention such as administration of

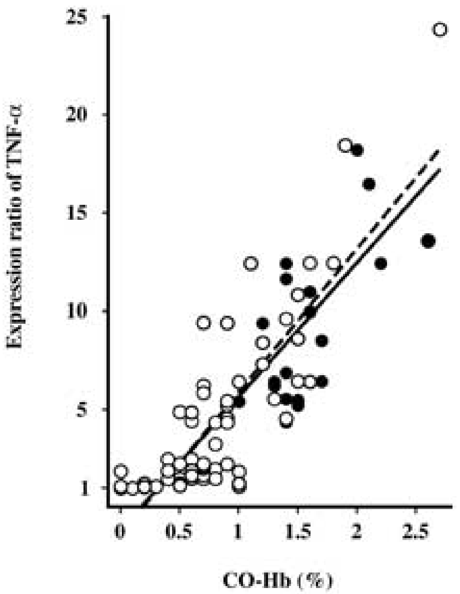

Fig. 6 Relationship between the carboxyhemoglobin concentrations (CO-Hb) and the expression ratio of tumor necrosis factor-α (TNF-α). Open circles = patients with CO-Hb concentrations ≥ 1.0% at the preoperative measurement (n = 35); closed circles = patients with CO-Hb concentrations < 1.0% at the preoperative measurement (n = 10). Solid line = regression among patients who showed CO-Hb concentrations < 1.0% at the preoperative measurement; dotted line = regression among all patients.

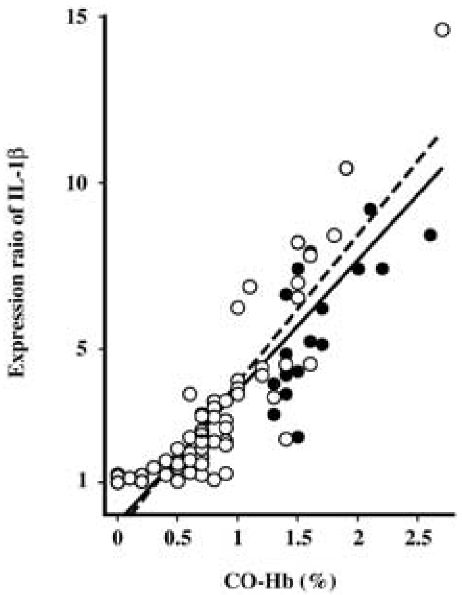

Fig. 7 Relationship between the carboxyhemoglobin concentrations (CO-Hb) and the expression ratio of interleukin-1β (IL-1β). Open circles = patients with CO-Hb concentrations < 1.0% at the preoperative measurement (n = 35); closed circles = patients with CO-Hb concentrations ≥ 1.0% at the preoperative measurement (n = 10). Solid line = regression among patients who showed CO-Hb concentrations < 1.0% at the preoperative measurement; dotted line = regression among all patients.

glucocorticoids to regulate the CPB-induced inflammatory response also has been shown[3] to attenuate the increase in serum inflammatory cytokines and increased anti-inflammatory cytokines. In this study, we measured the mRNA expression of key inflammatory cytokines such as TNF-α and IL-1β because the mRNA level is thought to be a more sensitive and earlier indicator than the concentration of serum cytokines that follows the expression of mRNA. The results of this study clearly showed that the use of CPB during CABG increased the gene expressions of TNF-α and IL-1β as well as HO-1 in circulating blood, and that anti-inflammatory strategies attenuated those increases. In addition, significant correlations of HO-1 expression with inflammatory cytokines expression indicate that expression changes of HO-1 may reflect surgical stress-induced inflammation. Results of a previous study[25] that measured gene expressions by less quantitative method in off-pump CABG and standard CABG support our results.

Whether CO-Hb is a clinical useful index reflecting the inflammatory response induced by surgical insults remains unknown. In this study, the concentrations of CO-Hb varied before surgery and the change rates were lower than those shown in CO poisoning or sepsis[8]. In addition, the correlation between CO-Hb concentrations and the expression of inflammatory mediators was low in patients who showed high concentrations of CO-Hb before surgery. These results indicate that the usefulness of CO-Hb concentrations as a marker of surgical stress might be limited based on the facts that an excessive inflammatory response can influence morbidity in more critical patients than those included in this study, and that CO-Hb concentrations before the surgery may vary further in such patients.

In conclusion, the expression of inflammatory mediators such as TNF-α, IL-1β, and HO-1 in circulating blood increased during CABG, especially when CPB was used. Treatment with glucocorticoid therapy attenuated these increases. CO-Hb concentrations also increased during CABG, however, CO-Hb concentrations did not change and were less well correlated with inflammatory mediators in patients who showed CO-Hb values≥ 1% before surgery, despite the fact that we chose patients with no obvious signs of inflammation or respiratory disease, and that the inhalation oxygen concentration and hemoglobin concentration were managed in a uniform fashion. CO-Hb measurements might not be useful in the clinical monitoring of surgical stress because of the limited degree of changes, the variation of baseline values, and the necessity for management under fixed conditions.

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