Hypothermia Inhibits Expression of CD11b (MAC-1) and CD162 (PSGL-1) on Monocytes during Extracorporeal Circulation

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Abstract. *Aim: The aim of the present study was to investigate the effect of different hypothermic temperatures on the expression of cellular adhesion molecules on leukocytes. Materials and Methods: Circulation of blood from six volunteers was performed in an extracorporeal circulation model at 36°C, 28°C and 18°C for 30 minutes. Expression of CD11b, CD54 and CD162 on monocytes was measured using flow cytometry. Results: Expression of CD11b significantly decreased at 18°C and at 28°C compared to 36°C. A significant reduction of CD162 expression was found at 18°C compared to 28°C and 36°C and at 28°C compared to 36°C. No association was found between temperature and expression of CD54. Conclusion: Expression of CD11b and CD162 on monocytes has a temperature-dependent regulation, with decreased expression during hypothermia, which may result in an inhibition of leukocyte–endothelial and leukocyte–platelet interaction. This beneficial effect may influence the extracorporeal circulation-related inflammatory response and tissue damage.*

Therapeutic hypothermia has been used clinically for many years to preserve the heart during surgery (*e.g.* coronary artery bypass) and to preserve organs before transplantation

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(1). Applied temperatures are differentiated into 'mild' (34-36°C), 'moderate' (28-33°C), 'deep' (17-27°C) and 'profound' hypothermia (4-16°C) (2). During some cardiac surgical procedures, such as those on the thoracic aorta or during the repair of congenital cardiac defects extracorporeal circulation (ECC) may be performed in combination with deep hypothermia (<20°C) especially during deep hypothermic circulatory arrest (DHCA) (3). Additionally, several studies demonstrated that mild therapeutic hypothermia improves neurological outcome and survival of patients successfully resuscitated after cardiac arrest, without important side-effects (4-6). Hypothermia therapy should be considered, on the basis of current evidence, as the standard-of-care in patients after cardiac arrest irrespective of the initial rhythm, as recommended by the guidelines (6). The mechanisms underlying the beneficial properties of hypothermia are multifactorial (6). On the other hand, there is concern that hypothermia may have adverse effects on cardiac function, coagulation, the immune system, and acid-base status (5).

The contact of blood with artificial biomaterials of the ECC circuit (*e.g.* during heart surgery, hemodialysis, plasmapheresis) induces a humoral and cellular inflammatory response. This response includes a complex activation of the coagulation system, complement activation, release of endotoxins, leukocyte activation along with the expression of adhesion molecules, the release of inflammatory mediators and platelet activation (7-10). These ECC-associated alterations can lead to life-threatening complications such as hemorrhage, thromboembolism, and inflammatory organ dysfunction, and can ultimately lead to multiple organ failure (7, 9, 11).

A large body of experimental evidence currently suggests that activated leukocytes are key mediators of the inflammatory reactions elicited by extracorporeal therapies

through their ability to release tissue-damaging compounds after they have adhered to endothelial cells (12). The interaction between leukocytes and endothelium represents the initial event of tissue injury related to leukocytes. This process involves the interaction between adhesion molecules on the surface of leukocytes and endothelial cells, namely, selectins, integrins, and immunoglobulins (13). After tight adhesion, leukocytes transmigrate through the vessel wall. Additionally, both platelets and leukocytes can modulate each other's function. Leukocytes enhance platelet-mediated aggregation *via* interaction of their P-selectin ligand with P-selectin, and binding of leukocytes to platelets promotes leukocyte activation (10,14). Moreover, monocytes play an important role in regulating the thrombotic and fibrinolytic systems and cellular adhesion molecules trigger cellular interactions at the interface of thrombosis and fibrinolysis (15).

Studies concerning hypothermic conditions have mainly focused on platelet activation, platelet–leukocyte interaction or inflammatory markers, but very few data are available concerning the expression of cellular adhesion molecules on leukocytes during hypothermic ECC. As far as we know, no data are available regarding the effect of different temperatures on the surface expression of cellular adhesion molecules on monocytes. The purpose of the present study was to investigate the expression of cellular adhesion molecules on monocytes at different temperatures at normothermia and hypothermia using an *in vitro* ECC model.

Materials and Methods

Inclusion of the healthy volunteers was performed after informed consent was obtained and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the local Ethics Committee (193/2002V).

Blood sampling. Blood from non-medicated healthy male volunteers (n=6) was collected by venipuncture with a 21-gauge needle from an antecubital vein. The first 5 ml of blood were discharged before additional blood samples were drawn for analysis in the extracorporeal circulation (ECC) model. All blood samples were anti-coagulated with 3 U/ml heparin.

Modified Chandler loop. Experiments were performed using an *in vitro* model (modified Chandler loop) as described previously (3, 9). For each experiment, six PVC tubings without additional coating (Jostra, Hechingen, Germany) were filled with 20 ml of blood from the volunteers. Afterwards, the tubings were closed into circuits (tubing length: 50 cm, diameter 3/8×3/32 inch) with a piece of silicone tubing. The tubing loops were rotated vertically (30 rpm) during incubation in a water bath. There were three sets of experiments that were performed at 18˚C, 28˚C and 36˚C. The respective temperatures were established by keeping the water bath at the desired temperature. For each of the three experiments, all test tubings were filled with blood from a single donor and were run obtained parallel. Samples for analysis were obtained after 30 minutes of circulation.

Sub-sample preparation for flow cytometry. The following incubation steps were performed immediately after 30 minutes of circulation with blood of each of the previously described six Chandler loop samples using a previously described method (16). Expression of adhesion molecules on monocytes was measured by flow cytometry (EPICS XL-MCL; Coulter Electronics, Krefeld, Germany). Leukocytes were detected by using fluorescein-isothiocyanate (FITC)-conjugated anti-CD45 (leukocyte common antigen) (Becton and Dickinson Biosciences, Heidelberg, Germany). The following phycoerythrine (PE)-conjugated monoclonal antibodies were used for cell detection in fluorescence-activated cell sorting (FACS): Anti-CD11b (alpha subunit of the beta2-integrin MAC-1), anti-CD54 (intercellular adhesion molecule 1, ICAM-1) (all from Becton and Dickinson Biosciences) and CD162 (P-selectin glycoprotein ligand 1, PSGL-1) (Immunotech; Coulter, Krefeld, Germany). Whole blood (100 μl) was incubated with saturating concentrations of FITC-conjugated anti-CD45 and PE-conjugated monoclonal antibodies for 20 minutes at room temperature. Erythrocytes were lysed and leukocytes were fixed with a commercially available solution (FACS Lysin Solution; Becton and Dickinson Biosciences). Samples were then incubated for 10 minutes in the dark. Thereafter, samples were centrifuged at 200 ×*g* for 10 minutes, the pellet washed with phosphate-buffered saline (Gibco, Karlsruhe, Germany), and re-centrifuged. The pellet was then resuspended in phosphate-buffered saline and applied to the flow cytometer equipped with a 488-nm argon laser. Results are expressed as the mean fluorescence intensity (MFI) of CD11b, CD54 and CD162 on monocytes.

Statistical methods. Statistical calculations were performed using SPSS 19.0 statistical software (SPSS Inc., Chicago, IL, USA) or InStat 3.00 (GraphPad Software Inc., San Diego, CA, USA). Deviations from a Gaussian distribution were tested by the Kolmogorov-Smirnov test. For normally distributed data comparisons were made using a multifactorial analysis of variance (ANOVA). The Kruskal-Wallis test was performed for non-normally distributed data. For *post-hoc* comparisons between conditions Tukey-Kramer or Dunn's test were used accordingly. Data are presented as the mean±standard deviation (SD). Two-tailed *p*-values of less than 0.05 were regarded as significant.

Results

After 30 minutes of blood circulation in an ECC model with different temperatures, a significant association between temperature and expression of CD11b and CD162 on monocytes was demonstrated.

Expression of CD-11b on monocytes was significantly decreased at 18˚C compared to the expression at 36˚C (mean MFI±SD: 14.5±3.6 *vs.* 24.9±9.7, *p*<0.01). At 28˚C the expression of CD11b was also significantly lower compared to 36˚C (16.2±6.0 *vs.* 24.9±9.7, *p*<0.01). After 30 minutes of blood circulation in the ECC model, the expression of CD162 was significantly lower at 18˚C compared to 28˚C (19.2±4.7 *vs.* 27.3±6.9, *p*<0.01) and 36˚C (19.2±4.7 *vs.* 31.5±5.7, *p*<0.05). A significant reduction of CD162 expression was also found at 28˚C compared to 36˚C (27.3±6.9 *vs.* 31.5±5.7, *p*<0.01). No association was found

between temperature and expression of CD54 on monocytes after 30 minutes of blood circulation. The expression of CD54 on monocytes did not differ at 36°C (5.9±2.1), 28°C (5.0±0.9) or 18°C (5.0±1.0, p≥0.05). Medians with quartiles for MFI are depicted in Figure 1.

Discussion

In this *in vitro* study, we investigated the effect of blood temperature on surface expression of cellular adhesion molecules on monocytes. Our experiments give an insight into the effects of normothermia and hypothermia on leukocyte surface markers. We observed a modification of the expression of CD11b and CD162 on monocytes when blood was run through hypothermic circuits compared with blood run through the normothermic circuit.

It has been known for many years that blood exposure to artificial biomaterials of the ECC circuit induces a complex activation of the coagulation system, complement activation, release of various inflammatory cytokines and leads to leukocyte and platelet activation (8-10, 13). Consequently a platelet–leukocyte interaction is induced (9). Enhanced expression of adhesion molecules on neutrophils is mainly responsible for reperfusion injury and postoperative complications (7, 13). ECC-associated alterations of platelet and leukocyte function can lead to life-threatening complications including hemorrhage, thromboembolism, and inflammatory organ dysfunction, and can ultimately lead to multiple organ failure (7, 9, 11). Soo *et al*. demonstrated that higher artificially stimulated neutrophil response was associated with an unfavourable outcome in patients undergoing open-heart surgery (13). One parameter that is known to have a significant impact on inflammatory cell activation is temperature (8). Hypothermia is known to have anti-inflammatory effects by inhibiting leukocyte response following tissue insults such as ischemic brain or liver injury (8). In most cardiac operations employing cardiopulmonary bypass (CPB), mild, moderate or deep hypothermia is used for organ protection against ischemia (10).

The membrane-bound β2-integrin CD11b/CD18 (MAC-1) is basally expressed on leukocytes and can be mobilized after leukocyte activation from intracellular pools in response to various inflammatory mediators (12). It binds to the intercellular adhesion molecule (ICAM) expressed on the endothelial cells and subsequently can prime neutrophil de-granulation and oxidative burst (17). The interaction between leukocytes and endothelium represents the initial event of tissue injury related to leukocytes. The process of leukocyte transendothelial migration involves a cascade of adhesion events, including the up-regulation of CD11b/CD18 (17). β2-Integrins serve to strengthen leukocyte–endothelial cell adhesive interactions (11, 12). Up-regulation of CD11b has been found to enhance the pro-coagulant activity of
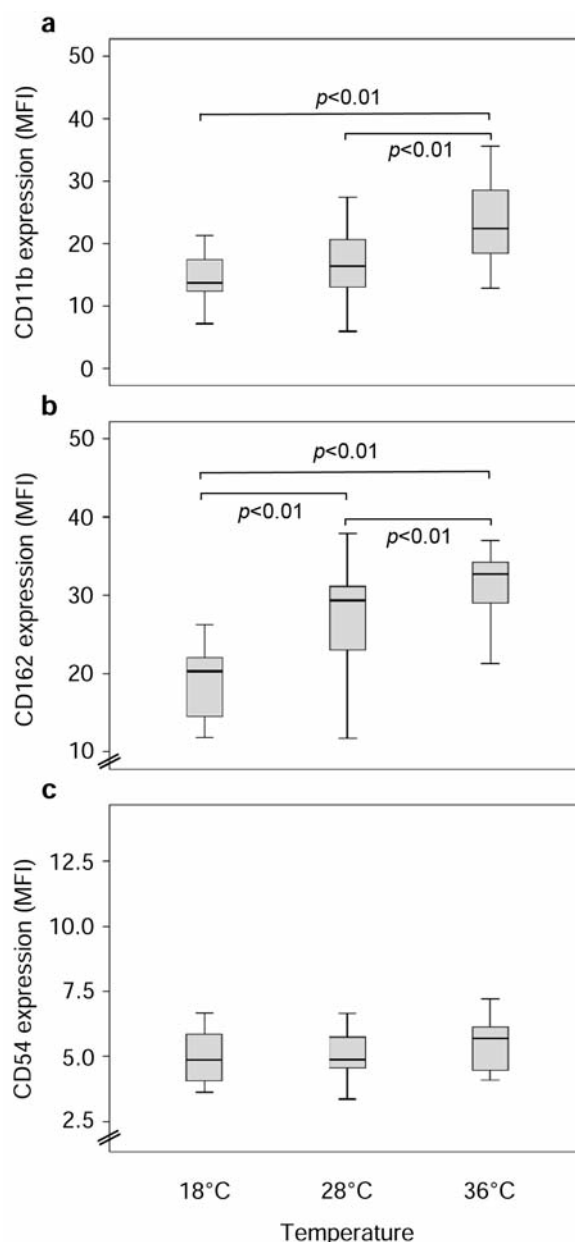

Figure 1. *Effect of hypothermia on expression of MAC-1 (CD11b; a), PSGL-1 (162; b) and ICAM-1 (CD54; c). Data are presented as medians of fluorescence intensity (MFI) with 25th and 75th percentiles (boxes) and 10th and 90th percentiles (whiskers). p-Values ≥0.05 are not indicated.*

leukocytes. CD11b binds to fibrinogen, induces tissue factor gene transcription in monocytes and activates factor X, initiating the coagulation cascade and thrombin generation (15, 16). Activated monocytes possess an alternative plasmin-independent fibrinolytic pathway that uses the integrin MAC-1 (15). Additionally, fibrinogen may enhance leukocyte adhesion and migration by functioning as a

bridging molecule between MAC-1 (and possibly ICAM-1) on the leukocyte and ICAM-1 on the endothelial cell (18). Platelet-leukocyte conjugates are formed, among other mechanisms, _via_ fibrinogen bridging between glycoprotein IIb/IIIa and leukocyte CD11b (19). Nearly all clinical and experimental studies suggest that the expression of integrins on leukocytes is up-regulated during or after CPB (7, 11, 19), whereas some other investigators have reported a decrease (7). Tarnok _et al._ reported that neutrophils isolated from CPB filters had an increased MAC-1 expression, whereas reduced MAC-1 expression was found on neutrophils in the peripheral blood (7). In a study of Paugam _et al._ basal neutrophil CD11b expression rapidly increased during normothermic CPB, and remained elevated until the fourth hour after the onset of CPB (17). Data in the literature show that integrins mediate adhesion of neutrophils and oxidative burst, as a monoclonal antibody to CD18 dramatically inhibited stimulated neutrophil–myocyte adhesion and H_2O_2 production (7). Thus, modulation of CD11b expression (_e.g._ by using hypothermia) may be relevant to reducing the ECC-related systemic inflammatory response and tissue injury. In the present investigation, the expression of CD11b on monocytes was significantly reduced at 18˚C and at 28˚C compared to its expression at 36˚C. Our study is consistent with earlier reports which demonstrated that cooling reduced up-regulation of CD11b on monocytes (8). The use of hypothermia significantly reduced the expression of CD11b during CPB compared with normothermia in cardiac surgical patients (12). In the present study, we demonstrated a relationship between temperature and expression of CD11b on activated monocytes, finding a lower expression at deeper temperatures. Our results, consistent with other data, suggest that hypothermia reduces up-regulation of CD11b expression on leukocytes during ECC. This is associated with a reduced ability of the leukocytes to adhere to endothelium, which is prerequisite for reduced leukocyte-induced tissue damage.

ICAM-1 (CD54) is a member of the immunoglobulin superfamiliy. It is constitutively expressed on the cell surface and is markedly up-regulated by cytokines (12). The binding of MAC-1 (CD11b/CD18) to CD54 results in the adhesion of neutrophils and monocytes to the endothelium (15). Experimental data have demonstrated that CPB results in an increased expression of CD54 on endothelial cells of canine pulmonary capillaries during recovery (7, 20). On monocytes, a decrease in the expression of CD54 was found shortly after the use of CPB in patients undergoing cardiac or thoracic surgery with moderate hypothermia (21). Nevertheless, at present no studies are available, to investigate the effect of temperature on the expression of CD54 on monocytes. In the present investigation, no association was found between different blood temperatures and expression of CD54 on monocytes after 30 minutes of blood circulation in an _in vitro_ ECC model. Our data demonstrate that even though the expression of CD54 was unaffected by hypothermia, the leukocyte–endothelial interaction was reduced by hypothermia due to the reduced expression of its ligand, CD11b on monocytes. Thus, hypothermia would appear to an inhibited leukocyte response during ECC.

The selectin family of adhesion molecules mediates tethering and rolling of leukocytes to the vessel wall by binding to glycoconjugate ligands on apposing cells (22). P-Selectin glycoprotein ligand-1 (CD162) has been demonstrated to generate a specific, high-affinity, biologically relevant ligand for P-selectin (22, 23). The interaction between CD162 and P-selectin mediates the initial tethering of leukocytes to activated endothelium and platelets (22). The generation of platelet–leukocyte aggregates may contribute to the ECC-related systemic inflammatory response (9). In addition, ligation of CD162 by P-selectin may trigger intracellular events in some leukocytes which enable them to respond to mediators expressed at sites of inflammation (22). Davenpeck _et al._ demonstrated that _in vitro_ activation of human neutrophils and moncytes results in a rapid and significant decrease in surface expression of CD162, thereby reducing leukocyte adhesion to P-selectin, but _in vivo_ investigations concerning this finding are still lacking (24). Only few and conflicting data are available regarding the expression of CD162 on leukocytes during CPB. In patients undergoing aortic valve replacement, a significant increase of CD162 on peripheral blood monocytes was observable after CPB. In contrast, in coronary blood, no significant variation of CD162 expression on monocytes was found at reperfusion after cardioplegia compared with measurements before cardioplegia in these patients (25). No studies are available investigating the effect of temperature on CD162 expression on leukocytes. It has been reported by Straub _et al._ that hypothermia induces alpha-granule release, with increased expression of P-selectin on platelets, which mediates platelet–leukocyte binding _via_ interaction with the leukocyte ligand CD162. This finding reveals that hypothermia induces platelet activation (10, 14). However, hypothermia-associated P-selectin up-regulation, and thus platelet activation, was not accompanied by increased leukocyte binding to platelets in their study (14). In the present study, we found that expression of CD162 on monocytes was reduced by hypothermia, with the lowest expression at 18˚C. The fact that leukocytes down-regulate CD162 expression on hypothermia could explain why there was no increase in platelet–leukocyte aggregates in the presence of hypothermia-activated platelets in the study of Straub _et al._ (14). Regarding the data of the present investigation, this finding suggests for the first time that hypothermia reduces CD162 expression on leukocytes, resulting in decreased platelet–leukocyte interaction, which may have a beneficial effect on the ECC-related systemic inflammatory response and organ damage.

This study was conducted in healthy volunteers by using an *in vitro* ECC model and several study limitations have to be noted. Firstly, our results were obtained from *in vitro* experiments. Transference of conclusions to the *in vivo* situation must be done with caution. Secondly, no baseline value of the adhesion molecule expression on monocytes was measured before circulation in the ECC model in the present study. However it was not the aim of the study to demonstrate the activation of leukocytes during ECC, which has been described before; its aim was to investigate the effect of different temperatures on the expression of cellular adhesion molecules on monocytes for the first time. We can assume that in our investigation the baseline leukocyte activation was identical in all three sets (18˚C, 28˚C, 36˚C) of each experiment, since the blood of each volunteer was divided into three portions and these portions were used for each set.

Conclusion

Regulation of expression of CD11b (MAC-1) and CD162 (PSGL-1) on monocytes is temperature-dependent, with a decrease in expression during hypothermia, which may result in an inhibition of leukocyte–endothelial cell interaction and leukocyte–platelet interaction. Thus, hypothermia has an impact on the ECC-associated alteration of leukocyte function. This may be a beneficial aspect of hypothermia that may influence the ECC-related inflammatory response and tissue damage.

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