The Effects of Fresh Frozen Plasma on Neutrophil-Endothelial Interactions

Boris Nohé, MD, Ralph Thomas Kiefer, MD, Annette Ploppa, MD, Helene A. Haeberle, MD, Torsten H. Schroeder, MD, and Hans-Juergen Dieterich, MD

Department of Anaesthesiology and Critical Care, University Hospital Tuebingen, Tuebingen, Germany

Leukocyte adhesion to endothelial cells contributes to microcirculatory disturbances during severe shock syndromes. Whereas certain plasma expanders inhibit leukocyte adhesion, contaminants of plasma protein solutions upregulate endothelial cell adhesion molecules in certain cases. We performed this study to determine whether fresh frozen plasma (FFP) affects neutrophil-endothelial interactions in cocultures of neutrophils and human umbilical vein endothelial cells (HUVEC) in vitro. HUVEC (n = 9) were incubated with either 20% FFP or 20% serum in medium for 6 h. Expression of E-selectin, intercellular adhesion molecule 1, and vascular cell adhesion molecule-1 was induced by tumor necrosis factor α (0.5 ng/mL for 4 h) and measured by flow cytometry. Neutrophil adhesion was examined in a parallel plate flow chamber in which isolated neutrophils were perfused over pretreated HUVEC under postcapillary flow conditions. Incubation with FFP decreased E-selectin and intercellular adhesion molecule 1 on activated HUVEC by 28% and 22%, respectively (P ≤ 0.01; analysis of covariance). Consequently, neutrophil adhesion decreased by 20%–41% in FFP-treated cocultures (n = 4; P ≤ 0.01; paired Student’s t-test).

We conclude that FFP attenuates the inflammatory response of endothelial cells with regard to neutrophil-endothelial interactions. Because the composition of patients’ plasma is affected not only by transfusion, but more frequently by shock treatment with IV fluids, plasma dilution in critically ill patients could be important.

(Anesth Analg 2003;97:216–21)

In surgical patients, the administration of fresh frozen plasma (FFP) is usually recommended for the treatment of severe bleeding or complex coagulopathy (1). In addition to the transmission of infectious material, FFP contains leukocyte-derived bioactive substances that are involved in sometimes severe anaphylactic or inflammatory reactions (2,3). In shock syndromes and critical illness, the generalized inflammatory activation of the endothelium plays a central role in the pathogenesis of organ dysfunction (4,5). Although FFP is often administered in such patients, its effects on endothelial cell activation have not been determined.

On activated endothelium, the upregulation of adhesion molecules leads to a multistep process of leukocyte-endothelial interactions. After transient rolling interactions through selectins, binding of intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) to their integrin counterreceptors results in firm adhesion of the leukocyte, which finally transmigrates into the subendothelial tissue (4,6). Although this adhesion cascade is an important feature of host defense, it also leads to endothelial dysfunction, capillary leakage, and tissue damage (4,5). Therefore, the potency of different drugs and plasma substitutes to attenuate this process is a subject of continuing research. Whereas artificial colloids impair leukocyte-endothelial interactions (7), plasma products such as human albumin increase endothelial cell adhesion molecule expression both in vitro and in vivo (8,9). The proinflammatory side effects of human albumin are probably due to contamination of the albumin preparation with undefined mediators during manufacture (9,10).

In contrast to purified albumin solutions, FFP contains all plasma components that are present in donor blood. This composition of numerous immunologically active compounds should render FFP much more likely to interfere with inflammation than human albumin. Because leukocyte-mediated tissue damage is an important feature of shock syndromes, in which
FFP is frequently given, we examined the effects of FFP on endothelial cell adhesion molecules and subsequent neutrophil-endothelial interactions. To minimize the effects of confounding variables usually present in vivo, we chose a flow chamber model in which leukocyte adhesion could be studied under well-defined hydrodynamic conditions of postcapillary venules.

**Methods**

After permission from the local ethics committee, four separate batches of FFP (Group AB; FFP 1–4 from batches 3785, 3802, 3867, and 3799) were derived from the local blood bank after blood donation from healthy blood donors. Written consent was obtained before blood donation, as recommended by the ethics committee. FFP was prepared according to the current guidelines for the preparation of blood components (11). In brief, plasma was separated from whole blood by hard-spin centrifugation (4950g; Cryofuge 6000i; Heraeus, Hanau, Germany) during blood donation. Immediately after separation, aliquots of plasma were frozen within 1 h, as recommended by European guidelines, and stored at −70°C until the experiment.

Fluorochrome-conjugated murine monoclonal antibodies against human CD62E and CD106 (immunoglobulin G1 [IgG1]; fluorescein conjugates; Southern Biotechnologies, Birmingham, AL), CD54 (IgG1; phycoerythrin conjugate; Immunotech, Marseille, France), CD15 (Lewis X; IgM; phycoerythrin conjugate; Southern Biotechnologies), CD11b (MAC-1; IgG1; fluorescein conjugate; Caltag, San Francisco, CA), CD45, and CD62L (leukocyte common antigen and l-selectin; both IgG1; fluorescein conjugates; Becton Dickinson, San Jose, CA) were used. Isotype-matched control antibodies were purchased from the same manufacturers from which the specific antibodies were used. Endothelial cell growth medium (EGM), including all supplements, was obtained from PromoCell (Heidelberg, Germany). Collagenase A was purchased from Boehringer Mannheim (Mannheim, Germany), and fetal calf serum and phosphate-buffered saline (PBS or PBS† with or without Mg²⁺ and Ca²⁺) were purchased from Gibco (Paisley, UK). Hanks’ balanced salt solution, trypsin/EDTA, trypsin inhibitor, and Medium 199 with 25 mM HEPES were obtained from BioWhittaker (Verviers, Belgium). Rat tail Collagen I and collagen-treated 12-well plates were purchased from Falcon Biocoat (Becton Dickinson Labware, Bedford, MA). All other chemicals, including tumor necrosis factor α (TNF-α), were obtained from Sigma (St. Louis, MO).

Human umbilical vein endothelial cells (HUVEC) were harvested from freshly prepared umbilical cords by enzymatic treatment of the umbilical vein with Collagenase A 0.1% (12). The cells were grown at 37°C and 5% CO₂ in EGM containing fetal calf serum 2%, epidermal growth factor 0.1 ng/mL, basic fibroblast growth factor 1.0 ng/mL, gentamicin 50 μg/mL, amphotericin B 0.05 μg/mL, and 20 μL of endothelial cell growth supplement/heparin. This preparation yielded a purity and viability of >90% as confirmed by staining for von Willebrand factor and trypan blue exclusion. After the first passage, the cells were plated at high density on either collagen-treated 12-well plates or rectangular coverslips (No. 1.5 thickness; Kindler, Freiburg, Germany) precoated with Collagen I and used for the experiment 48–72 h later, after they had reached confluence.

Human polymorphonuclear neutrophils (PMN) were isolated from citrated blood by density gradient centrifugation at 1700 rpm on a discontinuous Percoll gradient with 63% and 72% Percoll in PBS− (Percoll, 1.130 g/mL; Amersham Pharmacia Biotech, Uppsala, Sweden). Blood was obtained from healthy volunteers. The bottom layer, containing the PMN, was collected, and contaminating erythrocytes were removed by hypotonic lysis in 10% NH₄Cl on ice. After a final wash with cold PBS−, the PMN pellet was resuspended in cold Medium 199 at 5 × 10⁴/mL until immediately before the flow experiment. As determined by light scatter and cell-surface antigen analysis of CD15, CD45, CD62L, and CD11b, separation yielded functionally intact, nonactivated PMN at >90% purity, with only minimal alterations in the surface molecules that are sensitive markers for activation.

To determine whether FFP might influence endothelial cell adhesion molecule expression, aliquots of the four different FFP batches were thawed and diluted in EGM at a final concentration of 20% FFP in EGM. To prevent clotting, we added 10% acid citrate dextrose to the medium of the treatment group and the controls. For incubation of the control cells, FFP was replaced by 20% human AB serum. Serum was prepared by centrifugation of citrated blood and clotting of the plasma layer after the spin was completed. At the beginning of the experiment, the HUVEC monolayers were washed and incubated with the various media. To study the effects of FFP on adhesion molecule expression with and without a concomitant inflammatory stimulus, TNF-α was used for stimulation in half of the experiments on each culture. Two hours after beginning FFP treatment, half of the cell culture was stimulated with TNF-α at 0.5 ng/mL for an additional 4 h, whereas the remaining half was left unstimulated so that a total incubation time of 6 h was achieved in all experiments.

Subsequently, the monolayers were washed with PBS and labeled with monoclonal antibodies against...
CD62E, CD54, and CD106 for 30 min at room temperature. To stain only living cells, we introduced the antibodies into the plates before trypsinization. After this, the monolayers were washed with HBSS, trypsinized, and immediately analyzed for adhesion molecule expression in the flow cytometer.

For functional studies in the flow chamber, HUVEC were grown on customized coverslips and pretreated with FFP or human serum according to the concentrations described above. Previous analysis of flow cytometric results eliminated systematic variations in adhesion molecule expression between different batches of FFP. Thus, we used FFP only from Batch 3 (No. 3867) in the functional studies. After a 2-h pretreatment, TNF-α (0.5 ng/mL) was added for an additional 4 h. The coverslips were washed and placed into a parallel plate flow chamber that has proven effective in previous studies on cell adhesion (13). Our experimental setup has been described in detail before (14) and consisted of a coverslip that could be inserted into a heatable metal case located on the stage of an inverted microscope (DMIRB; Leica, Bensheim, Germany). By using a transparent cover, the metal case was closed to a certain height, leaving an inner chamber with a defined geometry. From this geometry, the flow rate Q (mL/s) necessary to produce the desired shear stress (τw) could be calculated according to the formula Q = (h²wτw)/6µ, in which h is the chamber height (0.015 cm), w is the width (1.25 cm), and µ is the viscosity of the perfusate (0.007 poise for isolated monocytes in Medium 199). To reproduce the hemodynamic conditions of postcapillary venules (13,15), we performed the experiments at a τw of 2 dynes/cm². Immediately before the perfusion experiment, the neutrophils were resuspended in warm Medium 199 at 10⁶/mL, and the PMN suspension was perfused through the flow chamber via a syringe pump (PHD 2000; Harvard Apparatus, Natick, MA). The entire perfusion time was recorded by a digital camera (CF15 MC; Kappa, Gleichen, Germany) and videotaped. Total PMN adhesion, including rolling and firm interactions with the endothelial monolayer, was determined from 10 fields of view (200× magnification) that were selected by random adjustment of the microscope stage at the end of each 10-min perfusion period. According to the work of Lawrence and Springer (13), neutrophils were considered to be adherent when traveling below a critical velocity of 40 μm/s, as measured from digitized videosequences.

On HUVEC from nine umbilical veins, cell-surface antigen expression was measured from median fluorescence intensities calculated from 5000 single events detected by the flow cytometer for each sample. Because these medians showed a skewed distribution, decadic logarithms were used in the statistical analysis. The log-medians were examined in a multifactorial analysis of variance in which the identity of the cell culture (modeled as a random effect) and the presence or absence of FFP served as nominal influence factors (because the different batches of FFP resulted in similar log-medians and were not statistically different, these data were pooled in the final analysis and labeled FFP positive). PMN adhesion was examined morphometrically from digitized video-recordings of perfusion experiments by using computer-based image recognition, as previously described (14). The effects of FFP treatment on PMN adhesion were examined by a paired Student’s t-test in flow experiments on four HUVEC from four different umbilical cords and PMN from four different volunteers. Unless otherwise stated, data are presented as means ±1.96 SEM, corresponding to the 95% confidence interval. Statistical significance was assumed if P ≤ 0.05. Statistical computation was performed with JMP™ software (SAS institute, Cary, NC).

Results

Only negligible amounts of E-selectin could be detected on unstimulated HUVEC (Fig. 1a). After stimulation with TNF-α, all samples were clearly positive for E-selectin. Incubation with FFP resulted in a 28% reduced expression, which was significantly different (P ≤ 0.01) from that detected on the surface of the control cells (Fig. 1b).

Both control cells and those incubated with FFP expressed basic amounts of ICAM-1 on their surface. In comparison to the control group, FFP significantly decreased ICAM-1 by 35% on unstimulated endothelial cells (P ≤ 0.01; Fig. 2a). In addition, FFP attenuated the upregulation of ICAM-1 after stimulation with TNF-α by 22% when compared with the control group (P ≤ 0.01; Fig. 2b). VCAM-1 was expressed only after stimulation with TNF-α. Treatment with FFP had no effect on the cytokine-induced expression of VCAM-1 on endothelial cells (Fig. 3, a and b).

PMN adhesion to untreated endothelial cells was negligible after 10 min of perfusion under venular shear forces (not shown). On cytokine-activated HUVEC (TNF-α, 0.5 ng/mL, 4 h), a 10- to 20-fold increase in the number of adherent PMN was observed. On cytokine-activated HUVEC, adherence of PMN was reduced significantly by 20%–40% when FFP-treated cultures were compared with the serum-treated controls in each of the four paired flow experiments (P ≤ 0.01; paired Student’s t-test; Fig. 4).

Discussion

Using cell-surface antigen analysis and a dynamic adhesion assay, we demonstrated that pretreatment of cytokine-activated HUVEC with FFP reduced both
adhesion molecule expression and subsequent adhesion of PMN under postcapillary shear forces. Although VCAM-1 was not attenuated by FFP, the 28% and 22% downregulation of E-selectin and ICAM-1 on activated HUVEC resulted in a 20%–40% reduction in neutrophil-endothelial interactions. Both affected molecules participate in the transition from rolling into firm adhesion (4,16). Whereas E-selectin decelerates transiently rolling leukocytes, ICAM-1 contributes to firm adhesion through its binding to \( \beta_2 \)-integrins on the neutrophil at slow rolling velocities (6,16). Therefore, the additive functions of both molecules might explain why FFP affected neutrophil-endothelial interactions to a slightly greater degree than endothelial cell adhesion molecule expression. Despite its well-preserved upregulation, VCAM-1 could not compensate for the decrease in E-selectin and ICAM-1 with regard to PMN adhesion. This is due to the cellular distribution of its ligand CD49d, which is expressed only on mononuclear leukocytes and eosinophils but is absent on neutrophils (17). P-selectin is another important endothelial cell adhesion molecule. However, we did not attempt to analyze its expression, because P-selectin is known to be weak even on primary HUVEC, making flow cytometry questionable.

This becomes particularly true after several hours of stimulation with TNF, which represents the more sustained phase of inflammation in which E-selectin predominates over P-selectin (18).

Despite the high correlation between reduced adhesion molecule expression and subsequently impaired adhesion after treatment with FFP, we were somewhat surprised by the attenuated inflammatory response in FFP-treated cell cultures. Although the effects of FFP on neutrophil-endothelial interactions have not been examined, previous studies on adverse reactions of FFP indicate a proinflammatory rather than an antiinflammatory effect. In vivo, infusion of FFP has been demonstrated to cause fatal myocardial depression (19), anaphylaxis (3), and transfusion-related lung injury (20) both in animal models and in patients. In addition to specific reactions with donor antibodies (20), these adverse reactions seem to be due mostly to increased concentrations of leukocyte-derived substances, e.g., histamine, myeloperoxidase, and cytokines (2,21); anaphylatoxins (22); or complement complexes (19). Pre-storage leukocyte depletion or bedside filtering have been demonstrated to reduce these contaminations (23). Although we did not use additional filtering, we could not observe any upregulation of

---

**Figure 1.** Effects of fresh frozen plasma (FFP) on the endothelial expression of E-selectin. Human umbilical vein endothelial cells (HUVEC) were incubated with 20% FFP as indicated in the text. Controls were left in medium supplemented with 20% serum. Expression of E-selectin (CD62E) was measured on unstimulated HUVEC (a) and cytokine-activated HUVEC (tumor necrosis factor \( \alpha \), 0.5 ng/mL, 4 h) (b). Cell-surface expression was measured as median fluorescence intensities (MFI) by flow cytometry. Results are given as mean values and 95% confidence intervals; \( *P \leq 0.01 \) compared with control.

**Figure 2.** Effects of fresh frozen plasma (FFP) on the endothelial expression of intercellular adhesion molecule 1 (ICAM-1). Human umbilical vein endothelial cells (HUVEC) were incubated with 20% FFP as indicated in the text. Controls were left in medium supplemented with 20% serum. Expression of ICAM-1 (CD54) was measured on unstimulated HUVEC (a) and cytokine-activated HUVEC (tumor necrosis factor \( \alpha \), 0.5 ng/mL, 4 h) (b). Cell-surface expression was measured as median fluorescence intensities (MFI) by flow cytometry. Results are given as mean values and 95% confidence intervals; \( *P \leq 0.01 \) compared with control.
adhesion molecules, which are sensitive markers for endothelial cell activation.

The mechanisms responsible for the downregulation of ICAM-1 and E-selectin remain unknown. It seems nearly impossible to identify a certain component of FFP that could have attenuated adhesion molecule expression due to the large number of proteins with immunological functions in plasma. Our goal was to study neutrophil-endothelial interaction instead of contamination, and, therefore, we did not attempt to test the FFP batches for an arbitrary subset of molecules. Additionally, we did not investigate dose-response relationships, because the activity of plasma components varies considerably among different donors and plasma batches (24), which would have largely confounded these relationships. Nevertheless, some conclusions can be drawn on the basis of our experimental setup. First, the use of a serum control excludes nonspecific effects of serum proteins or acid citrate dextrose on antibody binding. Furthermore, preliminary experiments on medium-treated HUVEC devoid of any serum indicate that the serum itself did not upregulate adhesion molecule expression, thus representing a valid control. Different concentrations of soluble E-selectin and ICAM-1 in serum and in plasma might have represented an attractive explanation for our findings. Soluble forms of adhesion molecules have been shown to inhibit the binding of cell-bound adhesion molecules dose dependently (25). This means that they could have also inhibited antibody binding during flow cytometry. However, removing the plasma by repeated washing steps before the perfusion experiment should have also removed soluble adhesion molecules, thus preventing an effect on PMN adhesion in the functional adhesion assay. Therefore, neither soluble E-selectin nor ICAM-1 can explain both decreased fluorescence intensity and PMN adhesion. Finally, the unchanged fluorescence intensity of VCAM-1-stained HUVEC is a strong indication that unspecific coating of the cell surface with a plasma layer did not occur.

The serum used in the experiment was derived from platelet-poor plasma layers after centrifugation of blood cells. Therefore, the main difference between serum and FFP was the induction of coagulation after cell removal during serum preparation. Whether inhibitory factors in plasma might have been depleted during the coagulation process cannot be answered from our study or from the literature. Although there are no reports on the nature of such compounds, other studies on FFP demonstrated beneficial effects on immunological functions. In one study, autologous plasma reduced apoptosis in lymphocytic leukemic B cells in vitro (26). With regard to multiorgan failure and shock, an early study of Busund et al. (27) is interesting. Two hours after inoculation of Escherichia coli, animals were treated with either albumin or FFP. Despite larger levels of endotoxin and TNF-α in the plasma group, the authors found a beneficial effect of
early plasma versus albumin infusion on vital organ functions in septic piglets (27). Finally, Stratford (28) showed that IV fluids can influence inflammatory reactions by reducing oxidative injury via amino acid constituents such as sulfhydryl, hydroxyl, and carboxyl groups. By examining the antioxidant potential of different infusion fluids, they demonstrated that the antioxidant potential of plasma exceeds that of albumin by 150%.

Intravascular fluid administration can be accompanied by a massive dilution of plasma components. Although plasma expanders attenuate leukocyte adhesion and reperfusion injury in vivo, their effects are usually compared with those of crystalloids or human albumin (8,29,30). Taking the presently poor knowledge on the antiinflammatory effects of plasma into account, much is left to be determined. Our study is the first that examined the effects of plasma on leukocyte-endothelial interactions under normal postcapillary flow. In addition to its antioxidant potential and the identification of antiinflammatory proteins, future studies should determine the effect of plasma under low-flow conditions as they occur during varying degrees of ischemia. Because leukocyte accumulation largely affects tissue perfusion in vivo, our results indicate that plasma may influence the course of septic and nonseptic shock apart from its effects on coagulation.

We thank Alice Mager and Christof Zanke for their help with the cell culture and videomicroscopy. Furthermore, we thank Birgit Neumeister for providing us with plasma batches, Joan Robertson for copyediting the manuscript, and Martin Eichner, Institute for Biometry, for his expert advice on statistics.

References