Prestorage Leukoreduction Prevents Accumulation of Matrix Metalloproteinase 9 in Stored Blood

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Objectives: Transfusion of aged stored blood is associated with many neutrophil proinflammatory effects. The mechanism of these effects remains to be elucidated. The purpose of this study was to determine whether matrix metalloproteinases accumulate in packed red blood cells during storage and are responsible for some of the neutrophil proinflammatory events, and to determine whether prestorage leukoreduction prevents accumulation of matrix metalloproteinases and attenuates proinflammatory effects of stored packed red blood cells.

Design: Laboratory study.

Participants: Healthy human volunteers.

Interventions: Units of blood were drawn from healthy volunteers. Half of each unit was filtered for leukoreduction, removing 99.9% of leukocytes. At biweekly intervals, aliquots were removed from packed red blood cell units, and the plasma fraction was isolated for assays. Plasma was assayed for specific molecules or incubated with isolated neutrophils, with or without a matrix metalloproteinase 9 inhibitor.

Main Outcome Measures: Concentrations of matrix metalloproteinases 2 and 9 and tissue inhibitor of metalloproteinase 1; matrix metalloproteinase 9 activity; and neutrophil apoptosis.

Results: Concentrations of matrix metalloproteinase 9 and tissue inhibitor of metalloproteinase 1 but not matrix metalloproteinase 2 increased over time. This accumulation was abolished by leukoreduction. Matrix metalloproteinase 9 accumulated in an active form. Both leukoreduction and matrix metalloproteinase 9 inhibition reversed stored packed red blood cell–induced, delayed neutrophil apoptosis.

Conclusions: Storage of packed red blood cells for 14 days or more is associated with increases in the concentrations of matrix metalloproteinase 9 and tissue inhibitor of metalloproteinase 1, with the enzyme in excess of its inhibitor. Prestorage leukoreduction prevents this accumulation. Delayed neutrophil apoptosis related to packed red blood cell plasma appears to be due, in part, to matrix metalloproteinase 9. Leukoreduction can help prevent the effects of matrix metalloproteinase 9 on neutrophil apoptosis.

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The immunomodulatory effects of blood transfusion have been recognized for decades. Although renal allograft survival is improved by transfusion, deleterious effects such as postoperative infectious complications and cancer recurrence have been associated with transfusions. In addition, transfusion is a strong independent risk factor for postinjury multiple organ failure. Polymorphonuclear leukocytes (PMNs) have been implicated as primary effector cells in the pathogenesis of postinjury hyperinflammation leading to acute lung injury (ALI) and multiple organ failure. Stored blood delays PMN apoptosis and primes endogenous PMNs for enhanced superoxide production and elastase release. These proinflammatory effects become more pronounced as blood ages to 42 days (product outdate). Furthermore, transfusion-related ALI is exacerbated by older stored blood. The mechanism of this effect remains to be elucidated.

Matrix metalloproteinases (MMPs) represent a group of enzymes involved in the degradation of most components of the extracellular matrix. The MMPs are key participants in tissue remodeling in a variety of pathological situations, including ALI and its most severe form, acute respiratory distress syndrome. Specifically, MMP-2 and MMP-9 appear to play a role in the pathogenesis of human acute respiratory distress syndrome. The biological activities of MMPs are regulated in part by a group of naturally occurring inhibitors known as tissue inhibitors of metalloproteinases (TIMPs). Tissue inhibitor of metalloproteinase 1, the inhibitor of MMP-9, is released along with MMP-9 in most cell types, and it has also been suggested to play a role in human ALI.
The source of MMPs and TIMPs in ALI is not clear, and to our knowledge, the role of MMPs in PMN-mediated inflammation such as transfusion-related ALI has not been investigated. Although the concentration of TIMP-1 has been found to increase during storage in whole blood and platelet-rich plasma, the accumulation of MMPs or TIMP-1 in stored packed red blood cells (PRBCs) has not been described. We hypothesized that levels of MMP-2, MMP-9, and TIMP-1 would increase during 42-day storage of PRBCs and provoke a PMN inflammatory response. We further hypothesized that prestorage leukoreduction (LR) would prevent this accumulation and the associated PMN effect.

BLOOD DRAW FOR PRBC UNIT STORAGE

This study was approved by the Lifespan Human Subject Research Committee, Providence, RI. After giving informed consent, 10 healthy human volunteers each donated 1 U of whole blood that was drawn into citrated 500-mL Triple Blood-Pack Unit bags (Baxter International, Inc, Deerfield, Ill) and processed into components according to the standards of the American Association of Blood Banks, Bethesda, Md. Half of each PRBC fraction was subjected to LR with a gravity-driven leukocyte reduction filter for RBCs (Sepacell R-500 II; Baxter International, Inc). All of the PRBC units were stored at 4°C. At biweekly intervals, a portion of the unit was removed and centrifuged (at 6470 rpm for 7 minutes, then at 10 230 rpm for 10 minutes) to remove the RBCs, leaving a small plasma fraction that was divided into aliquots and stored at −80°C until assayed. This was done every other week until day 42, which is the standard blood product outdate. Prior to use in assays, the plasma aliquots were thawed to room temperature and centrifuged at 4°C at 1000 g for 15 minutes to ensure complete removal of residual platelets.

DETECTION OF MMPs AND TIMP-1 IN RBC UNITS

Quantitative detection of MMP-2, MMP-9, and TIMP-1 levels in each of the PRBC unit plasma samples was accomplished using enzyme-specific sandwich enzyme-linked immunosorbent assay–based Quantikine Immunoassays (R&D Systems, Inc, Minneapolis, Minn). Each kit was specific for its MMP or TIMP, with no detectable cross-reactivity with any of the other MMPs or TIMPs. Purified samples of known concentrations were used to establish a standard curve. Plasma samples with a known dilution factor (1:5 for MMP-2, 1:200 for MMP-9, and 1:50 for TIMP-1) were tested, and their absolute concentrations were extrapolated from the standard curve.

MMP-9 ACTIVITY ASSAY

The MMP-9 activity was assayed using the Fluorokine E Human Active MMP-9 assay kit (R&D Systems, Inc). Enzymes from the plasma samples were captured onto a 96-well plate using a bound specific antibody, and the enzyme activity was measured using a fluorescence-conjugated peptide substrate. The activity was measured using fluorometric detection of substrate cleavage. Known concentrations of purified enzymes were used to create a standard curve, and the absolute concentration of active enzyme was extrapolated from that curve.

PMN ISOLATION AND APOPTOSIS

The PMNs were isolated at 4°C using a previously described protocol. Briefly, whole blood from healthy volunteers was drawn into heparinized Vacutainer tubes (Becton, Dickinson, and Co, Franklin Lakes, NJ) and separated with 3% dextran and Ficoll gradients with 0.2% sodium chloride RBC lysis. Viability was assessed and cell counts were performed using trypan blue. Yield was 98% PMNs, as assessed by giemsa stain. The PMNs were suspended in culture medium in 1 of 3 conditions: (1) without plasma (control); (2) with 20% stored plasma sample; or (3) with 20% plasma plus 25 nmol/L of the MMP-9–specific inhibitor MMP-9 inhibitor I (Calbiochem, La Jolla, Calif). The PMNs were incubated in 5% carbon dioxide at 37°C for 24 hours. Apoptosis was assessed morphologically with acridine orange and ethidium bromide stain and epifluorescence microscopy. Apoptotic cells are expressed as a percentage of the total number of PMNs.

STATISTICS

Statistical analysis of the data was accomplished using analysis of variance with post hoc comparison using the Newman-Keuls method in the SigmaStat 3.0 statistical software program (SPSS, Inc, Chicago, Ill). We particularly noted any data that are significantly different (P<.05) from the initial values.

MMP AND TIMP CONCENTRATIONS

There was an increase in MMP-9 and TIMP-1 concentrations over time. Levels were significantly increased by day 14. The TIMP-1 concentration reached a plateau, but the MMP-9 concentration continued to rise through day 42. The concentration of MMP-2 did not change (Figure 1). Prestorage LR abrogated the accumulation of both MMP-9 (Figure 2) and TIMP-1 (Figure 3).

MMP-9 ACTIVITY

The MMP-9 enzymatic activity increased with the duration of blood storage (Figure 4). Addition of p-aminophenylmercuric acetate, an organomercurial com-
pound that is a universal activator of the metalloproteinase family, only slightly increased the activity in each sample. This indicates that the majority of the MMP-9 present was already in an active form. There was no increase in activity in the stored LR samples, even with p-aminophenylmercuric acetate activation.

**PMN APOPTOSIS**

Addition of 42-day-old stored PRBC plasma to cultured PMNs delayed PMN apoptosis. Prestorage LR attenuated this effect, as did MMP-9 inhibitor I (Figure 5). However, neither condition restored the apoptotic index to the level seen with 1-day-old PRBC plasma.

**COMMENT**

The detrimental clinical effects of transfusing aged stored blood have been well documented, and they include exacerbation of infection in patients with sepsis, increased risk of cancer recurrence, and the development of postinjury multiple organ failure. In an isolated perfused rat lung model, Silliman et al demonstrated that lung injury occurs with perfusion of plasma from PRBCs stored for 42 days but not with fresh plasma. Along with increases in pulmonary artery pressure and pulmonary edema, there were histological changes in PMN infiltration, perivascular cuffing, and hyaline membranes, indicating tissue damage. To date, the mechanism of these effects remains to be elucidated. Many leukocyte-derived bioactive substances, such as lipid mediators, histamine, and cytokines such as interleukins 1 and 8, accumulate over time in the stored blood. However, none have been firmly established as the culprit mediator.

Another potential group of mediators of transfusion-related ALI are MMPs. In human ALI, MMP-2 and MMP-9 are involved in basement membrane disruption. Furthermore, MMP-9 has been implicated in the...
pancreatitis-associated ALI. Matrix metalloproteinase 9 is present at low levels in normal healthy lung tissue, but in certain disease states, levels are significantly increased. It is found in serum, bronchoalveolar lavage fluid, and tissue samples in chronic and acute asthma, idiopathic pulmonary fibrosis, and chronic obstructive pulmonary disease. Both MMP-9 and TIMP-1 are involved in airway remodeling, fibrosis, and inflammatory changes, and their ratio may be important. In those processes that involve acute inflammation, the ratio of MMP-9 to TIMP-1 is generally greater than 1; in those favoring remodeling and fibrosis, the ratio of MMP-9 to TIMP-1 is less than 1. Our data indicate that MMP-9 and TIMP-1 levels increase in stored PRBC units over time, with MMP-9 increasing in excess of its inhibitor. This imbalanced protease-antiprotease relationship could be a contributor to tissue damage.

Matrix metalloproteinase 9 has other proinflammatory effects in relation to substrates other than extracellular matrix proteins. For example, it increases the biological activity of precursors of interleukins 8 and 13. Our data demonstrate another potential proinflammatory mechanism of MMPs to augment the cytotoxic potential of PMNs. Aged stored blood delays PMN apoptosis, prolonging the functional lifespan of the PMN so that it may continue to respond to inflammatory mediators. This effect can be attenuated by prestorage LR or by inhibition of MMP-9 in the stored blood.

Comment on the clinical significance of the current findings is purely speculative at this point. While prestorage LR could prevent MMP accumulation and attenuate the proinflammatory potential of stored blood, the cost-benefit ratio of this strategy needs to be critically evaluated. In Rhode Island, the annual costs associated with universal LR amounted to $1.1 million to serve a population of roughly 1 million people. This would extrapolate to well over $300 million yearly in the United States. The primary benefit noted in a meta-analysis of randomized trials was that patients who received LR transfusions had fewer postoperative infections. Mortality was lower but did not reach statistical significance, possibly owing to underpowered studies. A subsequent before-after cohort study found that mortality rates were lower following adoption of national universal LR in Canada. These findings are compelling, but more data are needed before universal LR is adopted in the United States. In the meantime, poststorage or bedside filtration might be used in high-risk patients such as those with high transfusion requirements or without transfusion of older stored units. The comparative efficacy of poststorage LR has not been fully evaluated. Because it removes the leukocytes but not the inflammatory mediators that accumulate during storage, the theoretical benefit is less.

In summary, active MMP-9 accumulates in stored PRBCs and potentially plays a role in stored blood-mediated PMN cytotoxicity. Further investigations are needed to clarify the effects of these MMPs on the endothelium and PMN-endothelial interactions as well as their effects in vivo.

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DISCUSSION

Kenneth Burchard, MD, Lebanon, NH: Have there been studies of the infusion of MMP-9 to see if it produces lung injury directly?

Mr Frake: Are you referring to animal studies?

Dr Burchard: Yes, because you are inferring that the accumulation of that enzyme per se and infusing it would result in a proinflammatory condition that could be detrimental, so the question would be, what’s the half-life of MMP-9 when it’s infused? What’s the total body systemic reaction to that? And if that’s all in place and known, then there is a neatly packaged relationship between accumulation of that enzyme in the blood and the potential ill effects.

Mr Frake: That’s an excellent point. The material that was presented here is all in vitro work and was not done with exogenous MMP-9. An animal model would be very important to study, and that would be an appropriate follow-up to this work.

Patricia Donahoe, MD, Boston, Mass: Very nice paper, Dr Frake. Just one caution. Stem cells have been detected in transfused blood; if you do leukoreduction, will you be removing potentially salutory stem cells? Is there a way to separate and preserve stem cells during leukoreduction?

Mr Frake: That’s a good point. Clearly, there is more to learn about the clinical effects of leukoreduction.

Roger Foster, MD, Shelburne, Vt: I no longer follow this area, but I’ve been interested in it in the past. There have been a number of trials of leukocyte-depleted blood transfusions, a number of which have been negative. Can you bring us up to date on what sort of leukocyte depletion procedures have been demonstrated to have a clinical effect? What’s the current status of that data?

Mr Frake: A multicenter Canadian study found lower mortality following the adoption of a national universal leukoreduction program (JAMA. 2003;289:1941). A recent meta-analysis of randomized trials suggests that patients receiving leukoreduced blood benefit from a decrease in postoperative infections (Can J Anaesth. 2004;51:417). But, there hasn’t been a prospective trial that specifically addresses acute lung injury after leukoreduction.