Immunomodulatory Effects of Non-Leukocyte-Depleted and Leukocyte-Depleted Autologous Blood

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Key Words

Preoperative autologous blood donation · Autologous transfusion · Transfusion-related immunomodulation · Leukocyte depletion

Summary

Background: Based on several in vitro experiments, it has been suggested that autologous blood transfusion might lead to immunomodulation and that this effect might be prevented by leukocyte depletion. Clinical studies with surrogate endpoints showed conflicting results, whereas studies documenting clinically relevant outcomes were either too small or methodologically suboptimal to answer this question. **Patients and Methods:** Participants of a randomized multicenter trial were studied. Patients were scheduled for total hip arthroplasty and preoperative autologous blood donation (PABD) of 2 or more autologous whole blood units. Immunological parameters were investigated prior to first donation, prior to surgery and on days 1, 5, and 10 after surgery. **Results:** Leukocyte and lymphocyte subsets changed slightly after donation and considerably after surgery. Monocyte function was slightly suppressed following donation and substantially impaired after surgery. Transfusion slightly counteracted this effect. Leukocyte depletion did not result in any significant changes of the investigated immunological parameters. **Conclusion:** Following PABD immunological changes in patients occur that can be attributed mainly to the donation process and surgery. Transfusion has a minor effect. Leukocyte depletion does not appear to contribute substantially to these changes.

Schlüsselwörter

Präoperative Eigenblutspende · Autologe Transfusion · Transfusionsassoziierte Immunmodulation · Leukozytendepletion

Zusammenfassung

Hintergrund: Auf der Grundlage einer Reihe von In-vitro-Experimenten wird vermutet, dass die autologe Transfusion immunmodulierende Effekte haben könnte und dass diese Effekte durch die Leukozytendepletion vermieden werden können. Klinische Studien mit Surrogat-Endpunkten zeigten widersprüchliche Ergebnisse, während Studien mit klinisch relevanten Endpunkten entweder zu klein waren oder ein suboptimales Design hatten, um diese Frage eindeutig zu beantworten. **Patienten und Methoden:** Die Teilnehmer einer randomisierten, multi-zentrischen Studie wurden untersucht. Bei den Patienten waren ein Hüftgelenkersatz und eine präoperative Eigenblutspende (PEBS) von 2 oder mehr Vollblutkonserven geplant. Immunologische Parameter wurden unmittelbar vor der 1. Spende, am Tag vor der geplanten Operation und an den postoperativen Tagen 1, 5 und 10 untersucht. **Ergebnisse:** Leukozyten- und Lymphozyten-Subpopulationen zeigten geringe Veränderungen nach der Spende und deutliche Veränderungen nach der Operation. Die Monozytenfunktion war nach der Spende leicht und nach der Operation erheblich eingeschränkt. Autologe Transfusionen kompensierten diesen Effekt geringfügig. Leukozytendepletion resultierte nicht in signifikanten Veränderungen der untersuchten immunologischen Parameter. **Schlussfolgerung:** Nach PEBS kommt es zu immunologischen Veränderungen, die hauptsächlich durch die Spenden und den operativen Eingriff bedingt sind. Die autologe Transfusion zeigt nur diskrete Effekte, während die Leukozytendepletion keinen bedeutenden Einfluss auf diese Veränderungen zu haben scheint.

Introduction

It has been hypothesized that transfusion of autologous blood might lead to postoperative immunosuppression in a manner similar to the transfusion-related immunomodulation (TRIM) observed after transfusion of allogeneic blood. TRIM is supposed to result in an increased cancer recurrence rate after curative cancer surgery, an increased rate of postoperative infectious complications, and even in an increased postoperative mortality [1, 2]. Whereas in the allogeneic setting this phenomenon has been attributed mainly to transfused leukocytes and immunological effects triggered by the antigenic stimulus exerted by these cells, in autologous transfusion other mediators have been proposed most of which are, however, still related to the transfusion of leukocytes. These mediators comprise soluble HLA class I molecules and/or substances released from disintegrating leukocytes or platelets. A plethora of possible bioactive substances have been investigated [3, 4], e.g. tumor necrosis factor (TNF), IL-1 and IL-6, complement component C3a, histamine, serotonin, myeloperoxidase, eosinophilic cationic protein, eosinophilic protein X, plasminogen activator inhibitor 1, vascular endothelial growth factor, platelet-derived growth factor, prostaglandin E2, thromboglobulin, platelet factor 4, transforming growth factor β 1, and others. Ghio et al. [5] found increased concentrations of soluble HLA-I and Fas-ligand antigens in non-leukocyte-depleted (non-LD) autologous red cell concentrates (RCC), and could demonstrate an inhibition of the antigen-specific cytotoxic T cell response, an inhibition of autologous mixed lymphocyte reactions, as well as an induction of apoptosis of Fas+ cells. This group also found TGF- β 1 in the supernatants of non-LD autologous RCC and were able to inhibit *in vitro* the locomotion and chemotaxis of autologous neutrophils within these supernatants [6]. Apart from these *in vitro* experiments, clinical studies also exist. Avall et al. [7] randomized total hip arthroplasty patients who needed transfusions to receive either autologous whole blood (AWB) or allogeneic buffy coat-depleted RCC. They studied changes of interleukins and components of the complement system following transfusion and found an increase of complement component C3a in both groups, but no change of C5a or C5b-9 complex in either group. Interestingly, IL-6 and IL-8 showed a bigger increase in the allogeneic RCC group, and the authors concluded that cellular immunity had been suppressed in this group. However, since they did not compare their results with patients that had not been transfused, they did not have any data of baseline levels of these cytokines. Thus, their data would also be compatible with a smaller increase of these cytokines in the AWB group which would suggest an activation of the immune system in this group. However, when Avall et al. [7] presented their results, the main scientific interest was in the immunosuppressive effects of allogeneic blood which might explain why the authors did not take into account the alternative explanation for their results. In another clinical trial with a simi-

lar design and studying changes of immunological parameters, i.e. surrogate endpoints, Frietsch et al. [8] randomized total hip arthroplasty patients to receive either (non-LD) AWB or autologous buffy coat-depleted RCC if transfusion was indicated. They measured the phagocytic activity of granulocytes and monocytes and did not find any differences among the groups. In addition, these parameters were also not significantly different from those of patients who had not been transfused. They concluded that autologous transfusion did not have any influence on cellular immunity. At present, to our knowledge only three studies with clinically relevant endpoints addressing the immunomodulatory effects of autologous transfusion have been published. The results of these studies are summarized in table 1. The studies were either too small to detect any significant differences between groups receiving autologous blood products with different leukocyte content [8, 9] or had employed an inferior study design where causal inferences with respect to immunomodulatory effects of autologous leukocytes need to be made with caution [10]. Thus, a randomized controlled trial with sufficient patient numbers to detect meaningful differences in clinically relevant endpoints was initiated [11]. In the subgroup of patients entered into this trial at the trial center Philipps University Marburg we wanted to investigate the impact of leukocyte depletion of AWB on immunological parameters of the patients after taking into account changes of these parameters generated by the donation process, anesthesia, surgery, and transfusion as such.

Patients and Methods

The study was approved by the local ethics committee. All patients were scheduled to donate two units of AWB, 500 ml each, 7 days apart, prior to hip replacement surgery. The first donation usually took place 3–5 weeks prior to surgery in order to allow restoration of the patient's hemoglobin level during a period of at least 2 weeks following the last donation. All patients were prescribed and encouraged to take oral iron 150 mg daily up to the day of surgery.

Laboratory Investigations

All laboratory investigations were performed from blood samples taken on five occasions: i) immediately before the first donation, ii) on the day prior to surgery, and iii–v) on postoperative days 1, 5, and 10. Leukocyte and lymphocyte subsets were determined. In addition, TNF secretion of monocytes after stimulation with lipopolysaccharide (LPS), and IL-2, IL-4, IL-10, and IFN- γ secretion of lymphocytes after stimulation with either concanavalin A (ConA) or phytohemagglutinin (PHA) were studied. Leukocyte and lymphocyte subsets were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, Heidelberg, Germany). CD3+ CD4+ cells (T helper cells), CD3+ CD8+ cells (T cytotoxic cells), CD3– CD19+ cells (B cells), and CD3– CD16/56+ cells (NK cells) were identified. The stimulation of monocytes and lymphocytes was performed in a whole blood cell culture system [12] at 37 °C in 24-well flat-bottomed microtiter plates for suspension cultures (Greiner, Frickenhausen, Germany) in a humidified atmosphere containing 5% CO₂. Monocytes were incubated for 24 h after addition of LPS (from *Salmonella minnesota*; Sigma, Taufkirchen, Germany) at a final concentration of 0.1 μ g/ml. Lymphocytes

Table 1. Summary of studies with clinically relevant endpoints

Study	Frietsch et al. [8]	Tasaki et al. [9]	Karger et al. [10]
Design	Randomized	Randomized	Before/after
Groups	Non-LD AWB vs. autologous RCC*	Non-LD AWB vs. LD AWB	Non-LD AWB vs. autologous RCC*
Patients	25/24	21/13	120/52
Infections	0/0	0/0	10 (8.3%) / 4 (7.7%)†
Remarks		Only published as abstract	No significant differences also for other complications such as thromboembolic events or transfusion reactions

*Buffy-coat depleted.

†Difference not significant.

Table 2. Demographic characteristics of study groups

	Not transfused	Transfused	
		LD AWB	Non-LD AWB
Number of patients	18	22	18
Age*, years	58 (10)	58 (10)	62 (8)
Gender† male / female	12 / 6	9 / 13	5 / 13
ASA score† 1 / 2 / 3	4 / 12 / 1	6 / 13 / 3	2 / 14 / 2
Body mass index*	27.1 (2.8)	26.2 (3.7)	26.6 (3.8)
Predonation hemoglobin*, g/dl	14.8 (1.3)	14.6 (1.0)	14.2 (0.7)
Preoperative hemoglobin*, g/dl	13.5 (1.6)	13.0 (1.2)	12.6 (0.8)

*Data presented as mean (standard deviation in parentheses).

†Data presented as number.

were stimulated with either ConA (Sigma) or PHA (Sigma) at a final concentration of 5.0 µg/ml or 2.5 µg/ml, and were also incubated for 24 h. After incubation, the microtiter plates were centrifuged; the culture supernatants were removed and stored frozen at -70 °C until conduction of the cytokine assays. The supernatants were assayed for cytokines with commercial enzyme-linked immunosorbent assay (ELISA) sets (Human OptEIA ELISA sets; BD Biosciences PharMingen, Heidelberg, Germany). Raw data were converted to cytokine concentrations per 10⁶ monocytes (for TNF) or 10⁶ lymphocytes (for IFN-γ, IL-2, IL-4, and IL-10).

Statistical Analysis

The changes of the investigated immunological variables were analyzed over time in a generalized linear regression model for longitudinal data [13]. By employing such a model we were able to take into account the time sequence of the several factors supposed to influence the immunological parameters.

Results

In a group of 40 transfused patients we investigated the immunological parameters at all 5 days of investigation. We also randomly selected a group of 18 patients as controls which had not been transfused during the trial. The final study group consisted of 58 patients. Their baseline demographic characteristics are listed in table 2. In order to demonstrate that the

primary randomization was successful we present the demographic characteristics for patients transfused with non-LD and LD AWB separately. It can be seen that these groups are highly comparable and do not show any substantial difference in the analyzed demographic characteristics, indicating that randomization was successful even in such a small subsample of all randomized patients. However, the control group differed from these two groups in the gender distribution. It is evident, though not surprising, that in the non-transfused patients the proportion of male patients compared to female patients is much higher than in the two transfused groups. This prompted us to enter gender as another factor into our regression model in order to take account of any biases that might have been introduced by the differences in the gender distribution in the different patient groups.

Leukocyte and Lymphocyte Subsets

We always studied the influence of 5 factors on the changes of immunological parameters during preoperative autologous blood donation (PABD) and surgery in our regression model: i) donation, ii) surgery, iii) transfusion, iv) leukocyte depletion, and v) gender. The relative contribution of any of these factors to the changes of leukocyte and lymphocyte subsets is depicted in table 3. The signs in the cells represent the sign

Table 3. Changes of leukocyte and lymphocyte subsets during PABD and surgery

	Dona- tion	Surgery	Trans- fusion	Leukocyte depletion	Gen- der
Leukocytes $\times 10^3/\text{ml}$		++			
Granulocytes, %		++			–
Lymphocytes, %		–			++
Monocytes, %			++		–
T lymphocytes, %	++				(–)
B lymphocytes, %	++	++	–		
NK cells, %	–	–			–
T helper cells, %	++	+	++		+
T cytotoxic cells, %	–		–		

and the statistical significance of the regression coefficient for the respective parameter. A '+' sign indicates an increase of an immunological parameter if a respective factor is present, a '–' sign indicates a decrease. For gender, a '+' indicates an increase in women compared to men. An empty cell represents a regression coefficient with a p value of >0.1 . One sign in parentheses indicates a p value of >0.05 and ≤ 0.1 , one sign indicates a p value of >0.01 and ≤ 0.05 , and two signs indicate a p value of ≤ 0.01 . For instance, the '++' in the cell constituting the crossing of the row 'leukocytes' with the column 'surgery' indicates that surgery led to an increase of the leukocytes and that this increase was highly significant ($p < 0.0001$ in this case). All factors were coded as 1 or 0, indicating whether they were present or not, except for the factor 'transfusion' which was coded as 0, 1 or 2, representing the number of transfused AWB units. A useful practical consequence of the coding was that the p value usually also indicated the magnitude of the change caused by a factor such as that the smaller the p value for a factor was the greater was the change caused by this factor. It can be inferred from table 3 that the most prominent changes were caused by surgery, followed by donation, transfusion, and gender. Leukocyte depletion did not result in any significant changes of leukocyte or lymphocyte subsets.

Stimulation Experiments

The results of the stimulation experiments are depicted in table 4. The table shows that donation led to a slight decrease in monocyte function, whereas cytokine secretion of lymphocytes was increased. This effect was particularly noticeable in the ConA stimulation experiments. In contrast, surgery resulted in a suppression of stimulated cytokine secretion of lymphocytes. In addition, the suppression of monocyte function already generated by donation was greatly aggravated by surgery. Transfusion, apart from counteracting the suppressive effects of donation and surgery on monocyte function, had only questionable immunosuppressive effects. The influence of gender was even less relevant. Again, leukocyte depletion did not result in any significant changes of stimulated cytokine secretion of monocytes or lymphocytes.

Table 4. Changes in stimulated cytokine secretion of monocytes and lymphocytes during PABD and surgery

	Dona- tion	Surgery	Trans- fusion	Leukocyte depletion	Gen- der
TNF	–	–	++		
IL-2 (PHA)	+	(–)			(–)
IL-2 (ConA)	++	–			
IFN- γ (PHA)			(–)		
IFN- γ (ConA)	(+)	–			
IL-4 (PHA)		–			
IL-4 (ConA)	(+)	–			
IL-10 (PHA)			(–)		(–)
IL-10 (ConA)	(+)	–	(–)		–

Discussion

The results of this study demonstrate that during PABD, ensuing surgery and the postoperative hospital stay considerable changes of immunological variables occurred. During the donation phase the number of NK cells decreased which has already been shown in a prior investigation [14]. However, we also found a relative increase in T helper and B cells. In addition, when we investigated a greater patient group and focused on the change observed during the weeks of PABD only, we found significant changes for several other leukocyte and lymphocyte subgroups (unpublished data). On the functional level, PABD led to a suppression of LPS-stimulated TNF secretion by monocytes. This effect may be important because surgery causes an even more pronounced suppression of this parameter of monocyte function. Furthermore, a relative increase in B cells and a decrease in NK cells were also major effects of surgery. On the other hand, surgery led to an absolute rise in granulocytes, probably as a result of a systemic inflammatory reaction. Surgery also led to suppression of the cytokine secretion of mitogen-stimulated lymphocytes. Transfusion of autologous blood provoked only minor changes. We could find a relative increase of T helper cells and a partial restoration of the TNF secretion capacity of monocytes. The observed changes of immunological parameters might also have been slightly modulated dependent on a patient's gender. However, this influence was only very discrete. When all these factors were taken into account, the fact whether transfused AWB was LD or not, did not have any significant influence on the measured immunological parameters.

Taken together, our data suggest that PABD and surgery synergistically suppress innate, i.e. unspecific, immune responses. The influences of these factors on cellular immunity are complex and in part divergent. Autologous transfusion is of minor importance. The clinical relevance of these findings is unclear because the net effect of the observed changes on the propensity to infection cannot currently be estimated. Further studies are needed to address this question. A couple of other issues remain unresolved. We cannot definitely rule out that other

factors that changed concurrently during the donation period might have caused the immunological changes that we attributed to the donation process. However, we are not aware of any of such unknown factors that might have generated the pronounced changes we observed in this study. The same holds true for surgery. The changes observed between the preoperative day of investigation and the days of investigation after surgery might not have been caused by surgery itself but also by other measures such as preoperative medication, anesthesia and so on. However, because all of these factors are directly related to surgery, this distinction is largely academic and of little clinical relevance. Another unresolved issue is the influence of the age of the transfused products on the immunity of the patients [15]. We did not address this question in our analysis because the donation scheme did not result in a great variability of the age of the blood products. Thus, we believe

that our study lacked the power to answer this question. We could also not address the question whether the effects we attributed to transfusion might actually have been caused by the circumstances that led to transfusion. And finally, as already mentioned, it is unclear how the observed immunological changes do translate to clinically relevant outcomes such as the rate of postoperative infections. The already mentioned randomized trial, which this in vitro study is a part of, might possibly provide an answer.

In conclusion, PABD and ensuing surgery result in considerable changes of immunological parameters. The main factors are (in this order of relevance): anesthesia/operation (hip arthroplasty), autologous donation, autologous transfusion (AWB), and gender. Leukocyte depletion does not have any significant influence in this context.

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