Pathogen inactivation in cellular blood products by photodynamic treatment

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CHAPTER VIII

SUMMARIZING CONCLUSION

Blood transfusions in developed countries are generally considered to be safe, however recent events illustrated that blood transfusions still need a maximum of vigilance and that considerable efforts are required to maintain optimal safety of blood transfusion practices. The emergence of new virus, such as the WNV in the North American continent or TTV in Asia,[1,2] the potential risk of transmission of variant PrP^{sc} [3,4], the bacterial contamination of blood products responsible for sepsis,[5,6] the reduction but not the elimination of the window-periods despite the increased sensitivity of the tests,[7,8] promote the development of new strategies to increase the safety of the blood products. Chemical and photodynamic pathogen inactivation technologies are the most recent developments for the reduction of pathogen transmission via transfusion of cellular blood products.

This thesis describes the potential of PDT to decontaminate RBCC and CBSC. Besides the inactivation of pathogens, the viability and functions of RBCs and leukocytes, including hematopoietic progenitor stem cells, were evaluated.

FINDING A SUITABLE PATHOGEN PHOTOINACTIVATION METHOD

The original idea to inactivate pathogens in blood products is to add and activate an agent that targets pathogens but leaves the RBCs unharmed. Since RBCs contain no nucleic acids, photosensitizers binding to DNA or RNA were, at least in theory, the most promising photosensitizers to investigate. Photosensitization reaction resulting from the impact of light on the chemical agent leads to disruption of DNA and RNA strands. This results in the incapacity of pathogens to

reproduce and infect new cells. Theoretically, RBCs lacking nucleic acids remain therefore unaffected.

However, all photosensitizers tested so far for pathogen photoinactivation also induce damage to RBCs. Since a photosensitizer needs to bind to a target to induce damage, it is obvious, that the binding of the photosensitizers is not restricted to nucleic acids, but that binding to membranes also occurs. Minimizing photodamage to RBCs can be achieved by using more pathogen-specific photosensitizers or by specific protection of the cells. For this latter, more inside in the molecular mechanisms behind photodynamically induced damage is needed. A possible mechanism of photodamage and the specific protection of RBCs are described in **chapters 2** and **3**. The quest for more pathogen-specific photosensitizers is described in **chapters 4** and **5**.

PROTECTION OF RED BLOOD CELLS

Upon activation of the photosensitizer with visible light, various reactive oxygen species (ROS) are formed. ROS can inactivate a broad range of pathogens. However, as ROS are not selective and can be generated in all locations where sensitizer molecules are present, phototreatment can also damage RBCs.

A better understanding of the molecular mechanisms behind photodynamically-induced damage is needed to improve the selectivity of the photodynamic decontamination technique and to avoid damage to RBCs. Increased permeability of the membrane to cations, as measured by K^+ leakage, is one of the first events that appear after photosensitization of RBCs. This damage results subsequently in delayed RBC hemolysis. In **chapter 2**, we used the photosensitizer DMMB to get more insight behind the PDT-mediated potassium leakage. As it is likely that damage to a membrane protein could be involved in K^+ leakage, the kinetics of photodynamically induced K^+ leakage and inhibition of several transport systems were investigated. Inhibition of band 3 activity by DMMB-mediated PDT showed a comparable light dose dependency as PDT-induced K^+ leakage, whereas glycerol transport activity was inhibited only at higher light doses. Glucose transport was not affected by DMMB-mediated PDT. Dipyridamole (DIP), an inhibitor of anion

transport, protected band 3 against DMMB-induced damage, and prevented the increase in cation permeability of the membrane. These results suggest that there is a direct correlation between DMMB-mediated photodamage to band 3 and K^+ leakage.

Band 3 constitutes the anion transporter of the RBC and, with about a million copies per cell, it is the most abundant protein on the RBC membrane. DIP binds non-covalently, with high affinity to band 3 and has been reported to possess activity as a scavenger of various ROS. An obvious way to minimize the damage to RBCs induced by photodynamic pathogen inactivation would be to use DIP during PDT. In **chapter 3**, we showed that DIP can react with $^1O_2$ which is the main ROS formed by PDT. The presence of DIP during PDT with photosensitizers DMMB and AlPCS4 resulted in a significant protection of the RBCs detected by decreased K^+ leakage and hemolysis. In contrast to the strong protection of RBCs by DIP, hardly any effect on the photoinactivation of the model virus VSV was observed (**chapter 3**). This indicates that DIP has the potential to increase the specificity of the pathogen inactivation of RBC concentrates, thereby increasing the practical applicability of this treatment. However, the results were obtained under experimental conditions illuminating a model virus in diluted RBC suspension. Further research should focus in the effect of DIP on the photoinactivation of other viruses, including HIV, HCV, in a more concentrated RBC suspension. In addition, the effect of DIP on the self-life of phototreated RBC concentrates needs further investigation.

Another way to protect RBC from PDT-induced hemolysis is to choose the optimal RBC suspension media. In this case the protection is indirect: the damaging agent is not inactivated but the effect of damage is suppressed. Studies have shown that solution in which phototreatment was performed could influence the amount of hemolysis induced by PDT.[9,10] Less hemolysis was found when illumination was performed solutions with high osmotic strength as SAG-M. The solution in which the RBCs are stored after PDT has a much more pronounced effect on hemolysis. As shown in **chapter 4**, RBC hemolysis was reduced in SAG-S, SAG-D and AS-3 as compared to the standard RBC additive solution SAG-M. The presence of impermeable solutes, such as sucrose (SAG-S), dextran-100 (SAG-D), and citrate

(AS-3) prevented RBC hemolysis after PDT by counterbalancing the osmotic activity of hemoglobin. RBC integrity was best maintained when illumination was performed in the high osmotic additive solution SAG-M followed by storage in AS-3.

PATHOGEN INACTIVATION WITH TRI-P(4) IN RBC PRODUCT: ADVANTAGES AND DRAWBACKS

In our search to find a photosensitizer that efficiently inactivates pathogens without inducing RBC damage, we focused on a series of meso-substituted positively charged porphyrins. These photosensitizers have been shown to have a strong bactericidal potential.[11,12] From this series photosensitizer Tri-P(4) stood out as the best porphyrin suitable for pathogen inactivation in RBCC (**chapter 4**). Our preliminary studies have shown that PDT of RBC with the amphiphilic photosensitizer Tri-P(4) gives the most satisfactory results combining low hemolysis with efficient virus kill. In contrast, hydrophobic cationic porphyrins such as Trans-P(4), Cis-P(4) and mono-P(4) were supposed, like DMMB, to bind to RBC and were found to induce a rapid potassium efflux. Hydrophilic porphyrin derivatives tetra-P(4), TAP, TNH-P(4) and $AlPcS_4$ (**chapter 3**) may not bind to RBCs but showed a moderate cell lysis.

When illuminated in SAG-M and stored in AS-3, parameters for RBC quality after PDT with Tri-P(4) were satisfactory as shown by in-vitro measurements and in-vivo autologous RBC survival experiments in Rhesus monkeys. To extent the study, we investigated the pathogen-inactivating capacity of PDT with Tri-P(4) for a broader range of pathogens (**chapter 5**). From this study, it became clear that for a >5 log inactivation of various enveloped viruses, and gram positive and gram negative bacteria, a light dose of 360 kJ/m^2 was required. This implies a four times longer illumination time than required for the inactivation of VSV (**chapter 5**). These more rigid illumination conditions clearly increased RBC damage.

Despite these promising results, pathogen inactivation with Tri-P(4) presents many drawbacks. Firstly, the efficacy of PDT with Tri-P(4) to inactivate the non-enveloped CPV is low. Non-enveloped viruses are known to be very resistant to

chemical and photochemical treatments. However, as discussed in **chapter 5**, there is a remarkable difference in sensitivity to PDT within the parvoviridae families. Possibly, by using CPV as a model for human parvovirus B19, the inactivation of parvovirus B19 after PDT with Tri-P(4) is underestimated. Further testing evaluating the inactivation of human parvo B19 and of other non-enveloped viruses such as Hepatitis A virus in RBC using PDT with Tri-P(4) may clarify this issue.

More worrying is that Tri-P(4) is unable to inactivate intracellular viruses (**chapter 5**). In contrast to neutral photosensitizers, cationic dyes do not penetrate the membrane bilayer as shown by the low cytotoxic effects of PDT with Tri-P(4) on WBC (**chapter 6**). Recently, Lambrechts et al. have demonstrated that under hypotonic conditions the influx of the photosensitizer into the yeast Candida albicans was enhanced, thereby potentiating the photosensitizing effect of Tri-P(4).[13] it remains to be elucidated whether there are conditions upon which bacteria, viruses and infected leukocytes, but not the RBCs, become permeable to the photosensitizer, thereby increasing the Tri-P(4) photoinactivation efficiency.

Furthermore, a broader range of pathogen to inactivate in RBCC including fungi, parasites, and antibiotic resistant bacterial strains remain to be investigated. Studies with skin infection models have shown that fungi as well fungal spores are sensitive to PDT with Tri-P(4).[14,15] This raises the question whether RBCC environment provides the satisfactory conditions for an efficient fungicidal effect. Moreover, besides bacteria, bacterial endotoxins can also be responsible for fatal post-transfusion reactions.[16] The impact of PDT on endotoxin release from photoinactivated bacteria need also to be investigated.

Another major concern about the suitability Tri-P(4) for photodecontamination of RBCC is the increase of IgG binding to the RBC membrane upon PDT. This is a problem also observed with other photosensitizer a.o DMMB and $AlPcS_4$, and so far only observed with human cells (**chapter 4**). Binding of IgG is normally a sign of senescence of RBC which are subsequently removed from the circulation by macrophages. It can be speculated that PDT-induced IgG binding was the cause of a lower in-vivo recovery and a shorter RBC survival after transfusion. The

mechanism behind the process of PDT-induced IgG binding and whether these coated cells are phagocytized is still unknown. We have found that photodamage of both plasma proteins and RBC membrane is required to induce this IgG binding. This suggests a more complex phenomenon than simply the induction of a senescence process exposing phosphatidyl serine onto the RBC membrane. To circumvent interference with transfusion-related diagnostic tests, it is of great importance to prevent IgG binding to RBCs during pathogen inactivation application. A possibility to do this is the addition of GSH to the RBC suspensions. GSH can prevent IgG binding by preventing the formation of sulfur bridges between cysteine amino acids. GSH has an advantage over other scavengers as it is a natural constituent of the blood and therefore might not have to be removed before transfusion. However, when considering the up-scaling of the photo-treatment from small blood aliquots, as used in our studies, to full RBCC units, questions raise regarding the transfusion safety of photo-treated RBCs. With higher RBC volume, a greater amount of GSH would be required. The toxicity of large amount of GSH transfused intravenously needs to be determined. This is a subject of highest importance because the development of antibodies against RBCs treated with pathogen inactivation techniques is a major project killer. Despite the satisfactory in-vitro results and the in-vivo survival of treated RBCs in animals, Phase III clinical studies with S-303 and Pen 110 systems were halted because of the formation of antibodies against neo-antigens on the treated RBCs. Therefore, the future of these pathogen inactivation methods, including Tri-P(4)-mediated PDT, is uncertain until the immunogenicity is completely understood and resolved.

OTHER PERSPECTIVES FOR PDT WITH PORPHYRIN TRI-P(4)

The amphiphilic character of photosensitizers was reported to promote cell uptake and better intracellular targeting.[17] However, the cytotoxicity studies performed with such photosensitizers were often conducted with tumor cell lines cells while relatively little detailed information exists regarding non-transformed cells, especially cells of the immune system.[18]

Although porphyrin Tri-P(4) is amphiphilic and was reported to bind to DNA[19,20], there seems to be very limited uptake of Tri-P(4) by intact cells. As mentioned above, Tri-P(4) is unable to inactivate intracellular virus, most likely because the porphyrin is not taken up by the cells. We also observed no cytototoxic effect upon PDT with Tri-P(4) of leukocytes present in treated RBCs. This observation had lead to conduct photoinactivation studies in CBSC products (**chapter 6**) and to investigate potential immunomodulating effects of PDT with Tri-P(4) (**chapter 7**).

Bacterial contamination of CBSC is a major problem. It has been reported that up to 13% of CBSC products have been found positive in bacterial cultures.[21,22] Recently, the FDA guidelines and Netcord FACT standards requested the release of pathogen-free CBSC products for transplantation. All contaminated products have to be discarded. Because of the low number of stem cells, protocols for ex-vivo expansion of CBSC are developed. The presence of contaminants is a critical issue for such cultures which present ideal conditions for bacterial growth.
PDT with Tri-P(4) of CBSC resulted in the inactivation of spiked virus (VSV), the G+ *S. aureus* and, the G- *P. aeruginosa* and *S. agalactiae* (**chapter 6**). However, in comparison with application in RBCs, the treatment required a twofold higher Tri-P(4) concentration. This may be explained by the difference in product composition. CBSC contains plasma proteins which are nearly absent in RBCs. These proteins, in particularly albumin, quench most of the singlet oxygen generated by the action of light on the photosensitizer. Also the presence of high number of macrophages and platelets may influence the bacterial inactivation. G+ bacteria are known to be readily sensitive to PDT when treated in saline or in RBCC containing SAG-M. However when treated in CBSC, the sensitivity of G+ bacteria to PDT decreased and became even lower than this of G- bacteria. Macrophages and platelets, which are abundant in CBSC, can bind to G+ bacteria via the teichoic acid components. The cells may compete with Tri-P(4) for binding to bacteria through the teichoic acid, thereby reducing the efficacy of Tri-P(4) to inactivate G+ bacteria.
PDT with Tri-P(4) had no effect on the viability of hematopoietic progenitor stem cells nor on their ability to differentiate and form colonies in-vitro. Also the ex-vivo expansion and differentiation of CD34-positive cells isolated from photodynamically treated CBSC into megakaryocytes was not affected. Despite

these excellent in-vitro results apparently not affecting lineage-committed cells, transplantation of photo-treated stem cells in NOD/scid mice showed a lower engraftment and hematopoietic recovery as compared to untreated cells. The diminished engraftment could be the result of an impaired homing of the photodynamically treated cells. Many factors have been described to influence migration and engraftment of stem cells, such as cell cycle, cytokines, chemokines, expression of adhesion molecules and cell-cell interaction.[23-29] Further research is needed to reveal whether PDT interacts with one of these critical factors, thereby contributing to the reduced engraftment potential.

As in CBSCs, PDT with Tri-P(4) had no effect on the viability of leukocytes in RBC products (**chapter 7**). However, PDT does affect their functions. Alterations in proliferation, in allo-stimulation, in cell surface antigen expression and cytokine profiles are critical events in Tri-P(4)-phototreated cells. These changes result in an interesting issue, which is steering the immune response to T-cell hyporesponsiveness. For this, a combined effect of dark properties of Tri-P(4) which is binding to antigen-presenting cells (APC) and the effect of photodynamically-generated ROS on T cells was required. Binding of Tri-P(4) resulted in down-regulation of CD80/CD86, up-regulation of HLA-DR and CD14 and in lower release of chemoattractants IL-8 and MIP-1beta. These results may support the formation of alternatively-activated macrophages capable of inducing T-cell hyporesponsiveness. In addition, impairment of the T cell response to allo-antigens upon PDT with Tri-P(4) was strongly dependent on the amount of RBC. The lesser RBCs were present, the more the T cell proliferation was inhibited. The reason of this correlation may be that the increased RBC concentration reduces the amount of light that reached the leukocytes during PDT. Therefore, by adapting the light dose, PDT can modulate the interaction between T-cells and APC. Such modulation can have advantages in establishing appropriate treatment conditions for prevention of transfusion-associated Graft-versus-Host disease and alloimmunization, or for induction of tolerance in a recipient. However, it has been suggested that RBCs have a role as modulators of T-cell growth and survival and thereby may contribute to immunomodulating effects of transfusion.[30,31] Therefore the effect of the presence of RBC during PDT with Tri-P(4) of leukocyte products may not be underestimated. Further research is required to establish if

adjusting the light dose may result in same immunomodulating effects as a variation in concentration of RBC.

In conclusion, due to the particular properties of porphyrin Tri-P(4), many purposes are conceivable for PDT with Tri-P(4) from pathogen inactivation to immunomodulation. The great advantage of this cationic amphiphilic dye is the modulation of its effect by adjusting the environment. PDT with Tri-P(4) can then be applied for various goals.

At first, PDT with Tri-P(4) appeared to be a good method for pathogen inactivation in stem cell containing products, including those derived from à priori contaminated areas, for development of hematopoietic as well as regenerative therapies. Besides cord blood products, PDT with Tri-P(4) may be applied to decontaminate other sources of hematopoietic progenitor cells (e.g. bone marrow or menstrual blood).[32,33]

However, the original purpose to apply photodynamic treatment to enhance safety of RBCs is hampered by the photodynamically-induced damage to RBC. This can only be solved by providing adequate protection to RBC and this led to too many concerns regarding the therapeutic quality of the treated blood products. Therefore, it is unlikely that PDT with Tri-P(4) will be used for pathogen inactivation in RBC. Nevertheless, the studies with RBC revealed another potential of PDT with Tri-P(4) for dividing cell populations and for modulation of allo-immune responses, including tolerance induction.

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