Real-time PCR Applications in Haemostasis and Transfusion Medicine

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Chapter 1

Introduction

THE HISTORICAL BACKGROUND OF THE POLYMERASE CHAIN REACTION

The Polymerase Chain Reaction (PCR) was pioneered by Kary B. Mullis, born in 1944 in Lenoir, North Carolina, USA [1]. Charged with making oligonucleotides for DNA sequence analysis, Mullis worked as a scientist for the Cetus Corporation of Emeryville, California. In 1983, whilst driving along a mountain road into northern California's redwood country, Mullis was thinking about a means of determining the nucleotide present at a specific position of the beta-globin gene for diagnosis of sickle cell anaemia [2]. His thoughts were based on the techniques of dideoxy sequencing, a method first described by Sanger and Coulson in 1975 [2,3]. He then had a sudden intuition showing him a way of amplifying a DNA sequence in a single test tube [1,2]. Though we know today that the PCR uses multiple cycles at different temperature conditions to generate double-stranded DNA from a single-stranded DNA template (see 'Basic principles of PCR', page 6), Mullis was not certain during the early phase of his research that the reaction would not cycle itself. Assuming any chemical equilibrium to have some finite value, he believed that a portion of any nominally double-stranded DNA would be single-stranded, meaning that single-stranded DNA templates would be permanently present during the amplification process [1,4]. Thus, the first Polymerase Chain Reaction, labelled 'PCR01' (see figure 1, page 6), was an isothermal process that did not lead to the expected DNA band on ethidium bromide stained agarose gel (see 'Post-PCR Detection of Amplification Products', page 9). It took Mullis and his colleagues from Cetus several months to achieve conditions producing convincing results [4]. Now familiar with exponential amplification of DNA sequences, they were able to present a method for the diagnosis of sickle cell anaemia starting from minimum amounts of genomic DNA [5].

As PCR requires denaturation of double-stranded DNA through application of a high temperature to the reaction tube, a problem in the early phase of PCR was that the enzyme used for DNA amplification (the Klenow fragment of Escheria coli DNA polymerase) had to be replenished after each denaturation step [5,6]. This problem was

overcome by replacing the initially used enzyme with a heat stable protein isolated from an organism native in hot springs (Thermus aquaticus [Taq] DNA polymerase) [7,8,9]. Kary B. Mullis was awarded the Nobel Prize in chemistry in 1993 for his discovery of the PCR method [10,11].

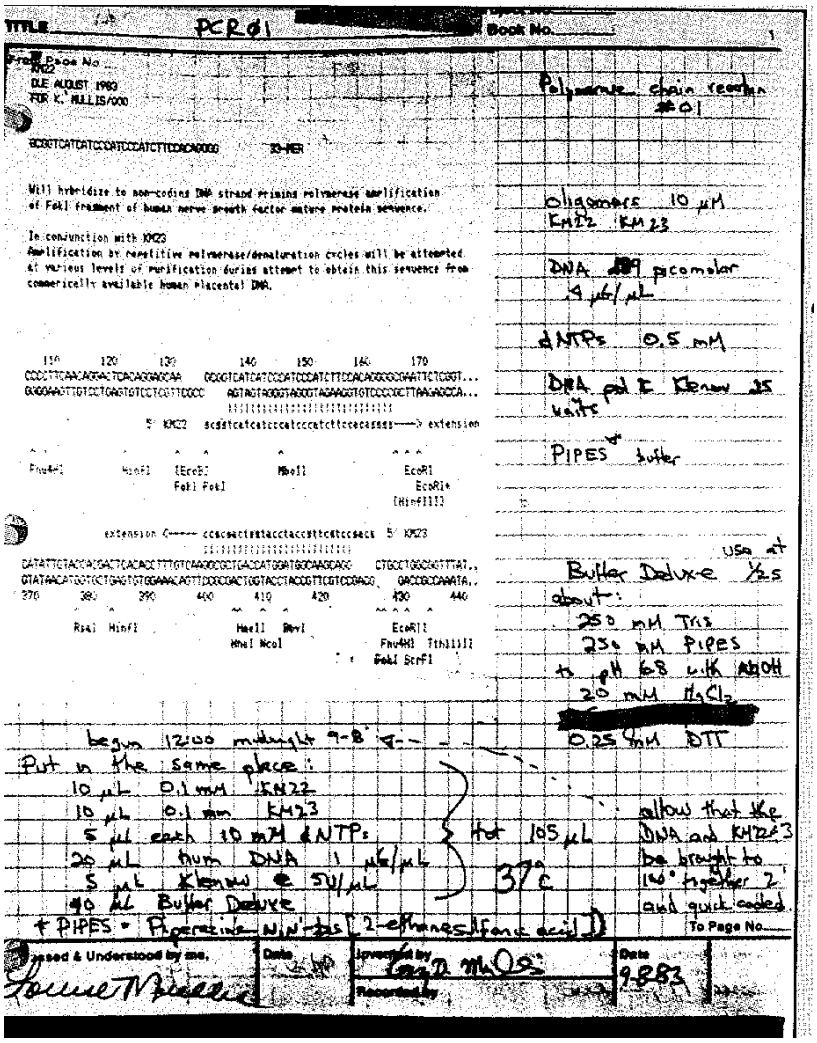

Figure 1.: The first Polymerase Chain Reaction. Page from the notebook of Kary B. Mullis showing the reagents put together for 'PCR01' in a purple-capped tube on September 8th, 1983 [4].

BASIC PRINCIPLES OF PCR

Various reaction components play a role in PCR. The DNA template sequence to be amplified can include purified DNA or RNA sequences converted to cDNA by reverse transcription [12,13]. A large molar excess of small pieces of synthetic single stranded DNA (so-called 'primers') determine the length and sequence of the amplification product [5]. The most frequently used thermostable polymerase is Taq DNA polymerase

[7,8]. Amplification reactions also include PCR buffer, deoxynucleotide triphosphates (dNTPs, an equal mixture of dATP, dTTP, dGTP and dCTP) and magnesium chloride ($MgCl_2$) [7,9,14,15]. The sensitivity and specificity of a PCR reaction depend on the interaction of these reaction components, but the most important aspect is the primer design [16,17]. The ideal primer pair anneals to unique sequences that flank the target and not to other sequences in the sample [18]. A primer needs to be long enough to reduce the probability of the sequence being found at non-target sites. Typical primers are 18 to 24 nucleotides in length [5,6,19]. Despite some general desirable characteristics of primer sequences, the melting temperature (T_m) is another important parameter [20]. T_m is the temperature at which 50% of an oligonucleotide and its complementary sequence are present in a duplex DNA molecule [21]. Ideally, the annealing temperature of a primer is low enough to guarantee efficient annealing but high enough to minimise nonspecific binding [22]. Temperatures for primer annealing are initially set about 5°C below the T_m of the primers. Higher annealing temperatures are then tested to reduce the formation of any formed primer-dimers or nonspecific products [15].

The principle underlying PCR is shown in detail in figure 2 (page 8). In the first stage of the PCR reaction, double-stranded DNA molecules are separated at high temperature into their two component strands. The reaction tubes are then cooled down to a temperature that allows the primers to anneal at specific locations on the opposite strands of the target sequence. While the so-called “forward” (or “sense”) primers anneal to the anti-sense strands of the separated DNA template, the “reverse” (or “anti-sense”) primers anneal to the sense strand downstream from the sequence chosen for the forward primers. Taq DNA polymerases then "extend" the primers by attaching a complementary nucleotide to each nucleotide in the template strand. In this manner, the polymerase creates two identical double-stranded DNA helices from the two separated single strands of the original template molecule. The cycle of denaturation, primer annealing and strand synthesis is then repeated several times. As the products from one round serve as a template for the next amplification cycle, each additional cycle theoretically doubles the amount of accumulated PCR products [2,5,6,19].

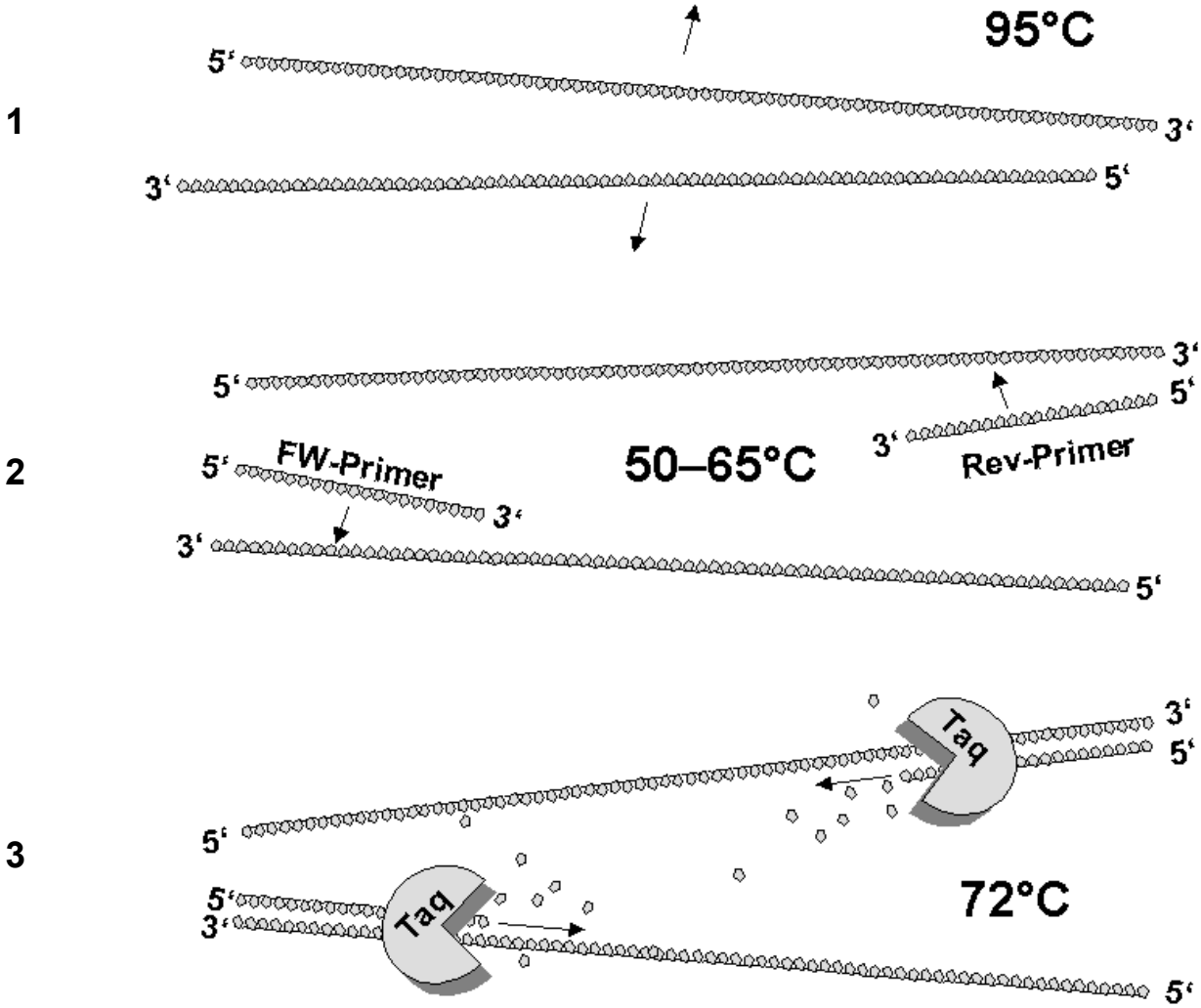

Figure 2. Basic principle of PCR. First, double stranded DNA sequences are separated into single strands by applying high temperatures (usually 95°C) to the reaction tubes. 2. During the subsequent annealing step at an optimised temperature, the forward and reverse primers hybridise to their target sequences located on the opposite strands of the target DNA molecule. 3. Taking the resulting primer:template duplexes as a starting point, Taq DNA polymerases then synthesise a complementary strand to the single-stranded DNA matrices. The process of strand separation, primer annealing and extension is then performed repeatedly, resulting in the production of a large number of identical DNA strands. Since amplified products from the previous cycle serve as a template for the next amplification cycle, PCR is an exponential process and a highly sensitive technique for nucleic acid detection.

KINETICS OF PCR REACTIONS

The amplification of nucleic acids is an exponential process that can be described by the equation

$$P = I * (1+E)^n$$

where P is the copy number of accumulated PCR product, I is the initial copy number of the nucleic acid template of interest, E is the efficiency of the PCR reaction and n is the number of performed amplification cycles [5,23].

The efficiency of a PCR reaction can vary from 0 (no amplification) to 1 (optimal amplification) [24] and depends on various parameters like the characteristics of the designed primer pair, the purity of the nucleic acid template, the performance of the Taq DNA polymerase used for amplification, and the length and the base composition of the PCR product [23,25-27]. Furthermore, the efficiency of the reaction depends on the distribution between the amplification product and the residual amount of free primers and dNTPs and the competition of amplification products for DNA polymerase binding [23,25]. Thus, the reaction trajectory at later stages of amplification diverges from an exponential to a stochastic type, resulting in a plateau phase at the end of the PCR reaction (see figure 3, page 11) [23,25,26].

POST-PCR DETECTION OF AMPLIFICATION PRODUCTS

Gel electrophoresis

Electrophoresis through agarose or polyacrylamide gels is a standard method used to separate and identify PCR products directly after the amplification process or after additionally performed post-PCR modifications such as cleavage by endonucleases for restriction site analysis [19]. Because of their negative charge, nucleic acids become oriented in an electric field [28,29]. While moving to the positive electrode, molecules of double-stranded DNA migrate through gel matrices at rates that are inversely proportional to the $\log_{10}$ of the number of base pairs [30]. Small molecules migrate faster because of lower frictional drag and because they worm their way through the pores of the gel more efficiently than larger molecules [19]. The location of DNA within the gel can be determined directly by staining with low concentrations of a DNA-binding

fluorogenic molecule. The most commonly used fluorescent dyes are ethidium bromide and SYBR® green I. These fluorescent dyes intercalate between single bases of DNA. Because the fluorescent yield of dye:DNA complexes is much greater than that of the unbound dye, small amounts of DNA can be detected by exposing a stained gel to ultraviolet light [19,31]. Through gel electrophoresis, PCR products or DNA fragments resulting from downstream applications of PCR can be assigned after staining according to the corresponding number of base pairs. The application of polyacrylamide gels even permits a distinction between DNA fragments that differ by only one base-pair in length [32,33].

Hybridisation techniques

Although the length of a DNA fragment is an important parameter in the assessment of the specificity of an amplification process, no information about the base composition of a PCR product can be given by gel electrophoresis alone. Thus, it is often desirable to perform an additional oligonucleotide probe hybridisation step to obtain specific product discrimination without the need for DNA sequencing or restriction site analysis. A method to detect DNA fragments by specific hybridisation after gel electrophoresis was described by Southern as early as 1975 [34]. Nowadays, a large number of modifications of this method are used to assess DNA fragments usually resulting from PCR reactions. For example, the presence of particular mutations or polymorphisms can be determined by differential hybridisation with sequence-specific oligonucleotide probes [35,36]. Furthermore, compared to fluorescent dye stained gels, optimised hybridisation assays allow more sensitive detection of PCR products [37]. This aspect is particularly important for test systems established for the detection of infectious agents [37,38].

IN-PROCESS DETECTION OF PCR PRODUCTS (REAL-TIME PCR)

The combination of PCR and subsequent detection of amplification products has been described for a wide variety of applications [5,18,31,32,35-41]. However, all of these approaches require time-intensive post-PCR handling steps and bear the risk of reaction mixtures being contaminated by previously handled amplification products [42,43,44]. The development of various methods for the detection of PCR products during amplification in a closed-tube format (so-called ‘real-time’ PCR) solved these problems

and provided a means of gaining insight even into the early exponential phase of the amplification process [45].

Basic principles of real-time PCR

Real-time PCR is based on continuous quantification of specific fluorescent reporter emission signals during the amplification process. The signal increases in direct proportion to the amount of PCR product accumulated in the reaction mixture [45,46,47]. By detecting the amount of fluorescence emission at each amplification cycle, it is possible to monitor the entire kinetics of an ongoing PCR reaction (see figure 3).

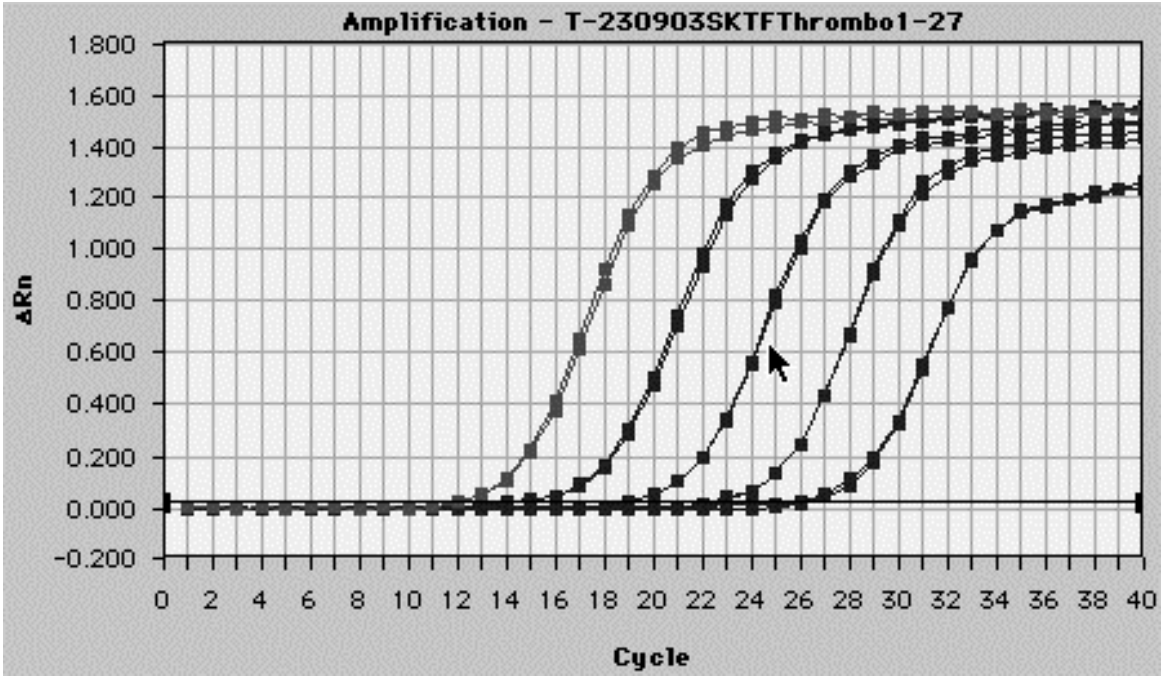

Figure 3. PCR kinetics. Amplification curves of a 5' nuclease assay performed on the ABI Prism® 7700 SDS, showing fluorescence observations from the amplification of a 10-fold dilution series of tissue factor-cDNA after reverse transcription of corresponding mRNA templates. The 40 observations of relative change in fluorescence intensity show the typical sigmoid trajectories of the amplification reactions from an exponential phase to a late plateau phase. Early amplification cannot be viewed, as the specific fluorescence signals from the used reporter dye cannot be distinguished from the background signal. The point at which amplification-derived fluorescence generation becomes significantly detectable is called the threshold cycle (Ct). While higher concentrations of starting templates result in a lower Ct value, lower concentrations produce higher Ct values. Under ideal conditions, a $\log_{10}$ difference in the starting concentration of the template DNA results in a 3.32 cycle difference in the Ct value.

Platforms available for real-time PCR

Nowadays, various real-time PCR systems using different chemistries for amplification-dependent fluorescence generation are commercially available. Adapted thermal cyclers enable irradiation of the reaction mixtures and detection of emitted fluorescence signals by either a computer-controlled CCD camera or photodetection diodes [45,48,49]. Due to the parallel development of different real-time PCR platforms, the absorption and emission characteristics of the used fluorophores depend on the limitations given by the corresponding irradiation and detection system. At present the most commonly used platforms are the ABI Prism® 7000 series sequence detection system (SDS, Perkin Elmer Corporation/Applied Biosystems, USA) and the LightCycler™ instrument (Roche Molecular Biochemicals, Germany) [46,50,51].

Chemistries used in real-time PCR

Real-time PCR has been made possible by the labelling of internal hybridisation probes, primers or the amplification product itself with fluorogenic molecules. The different methods can be classified into sequence-specific or non-specific methods.

DNA-binding fluorophores

The basis of the non-specific detection methods is the use of DNA-binding fluorogenic molecules. Higuchi et al. (1992) pioneered the possibility to monitor the accumulation of PCR products during the amplification process by including ethidium bromide directly into the reaction mixture. Ongoing amplification produced increasing amounts of double-stranded DNA, resulting in an increase in fluorescence after each cycle of the PCR reaction [45]. However, non-specific real-time reactions performed nowadays usually contain the free intercalating dye SYBR® Green I [47,48]. The advantages of SYBR® Green I assays is that they are inexpensive and easy to use. The disadvantage is that SYBR® Green I will bind to any double-stranded DNA in the reaction, including primer-dimers and non-specific reaction products [48,52].

Fluorogenic hybridisation probes

Holland et al. (1991) first described the possibility of incorporating sequence-specific product discrimination by probe hybridisation directly into the PCR process. Their

method was based on a γ-^{32}P-labelled probe designed to hybridise within the target sequence chosen for amplification. The probes also contained a 3’ phosphate moiety, which blocked their extension by the used polymerase. Annealing of the probe to one of the PCR product strands generated a suitable substrate for the 5’-3’ exonuclease activity of Taq DNA polymerase [53]. Thus, annealed probes were degraded during amplification into smaller fragments that could be differentiated from intact probes by radiography performed after thin-layer chromatography [54]. Numerous modifications of this initially described 5’-exonuclease assay led to the development of various fluorogenic hybridisation probes used for specific product detection during the amplification process of the PCR.

Internal fluorogenic oligonucleotide probes provide an additional hybridisation step that improves the specificity of the test system and allows specific product discrimination. Different fluorophores with emission profiles peaking at different wavelengths can be used to perform multiplex real-time PCR assays that allow parallel detection of multiple amplification products in the same reaction mixture [55,56]. For details see below and figure 4 on page 15.

5’ Nuclease probes

5’ nuclease probes (also called hydrolysis or TaqMan® probes) are based directly on the principle of the 5’-exonuclease assay described by Holland et al. in 1991 [54]. These probes contain a fluorescent dye on the 5' base and a quenching dye typically on the 3' base [57]. When irradiated, the excited fluorescent dye transfers energy to the nearby quenching dye molecule by fluorescence (or Förster) resonance energy transfer (FRET) [58,59]. Thus, the close proximity of the reporter dye and the quencher prevents emission of fluorescence specific for the used reporter dye [46,57]. Probe degradation by the 5’-3’ exonuclease activity of Taq DNA polymerase ends this quenching effect and the reporter dye starts to emit fluorescence at an expected wavelength [60,61]. To date, the 5’ exonuclease assay (also called TaqMan® assay) has been the main application performed on the ABI Prism® 7000 series real-time PCR platform [46].

Adjacent hybridisation probes

In a reverse process to the quenching effect described above, a pair of adjacent, fluorogenic hybridisation probes can also be used for homogenous product detection by FRET [62]. An upstream probe is 3'-labelled with a donor fluorophore while a corresponding downstream probe is 5'-labelled with an acceptor fluorophore. After irradiation, a signal from the acceptor can only be generated when the donor comes into close proximity to the acceptor after adjacent probe hybridisation during the annealing phase of the PCR reaction [63]. This system, also known as the 'HybProbes' system, has become the method of choice for the LightCycler™ instrument [51].

Hairpin probes

Another principle of specific homogenous product detection is the use of molecular beacons or Scorpion primers.

Molecular beacons contain a fluorescent and a quenching dye at either end but they are designed to adopt a hairpin structure while free in solution to bring the fluorescent dye and the quencher in close proximity [64]. This results in quenching either by FRET or direct energy transfer by collision mechanisms [65,66]. When the molecular beacon hybridises to the target during the annealing step, the reporter dye is separated from the quencher and the reporter fluoresces [64,67].

Scorpion primers combine a hairpin structure described above with one of the primer sequences. After annealing of the primer section, the hairpin structure of the probe section is only disrupted by specific hybridisation of the probe sequence downstream from the nascent strand [68,69].

Generally, hairpin structures provide a stable alternative to the binding of the probe sequence to a single-stranded DNA target. Thus, hairpin oligoprobes are a sensitive tool for single nucleotide polymorphism (SNPs) analysis [64,70,71].

Light-up probes

Light-up probes consist of a peptide nucleic acid (PNA) to which an asymmetric cyanine dye (usually thiazole orange) is attached. Upon probe hybridisation, thiazole orange binds to the target DNA and becomes strongly fluorescent [72,73]. Using a single reporter dye permits fluorescence to be detected directly instead of the change in the

fluorescence intensity distribution between two fluorophores having to be measured when FRET is performed. Thus, light-up probes combine the advantage of a non-specific intercalating dye with sequence-specific hybridisation [72,73,74].

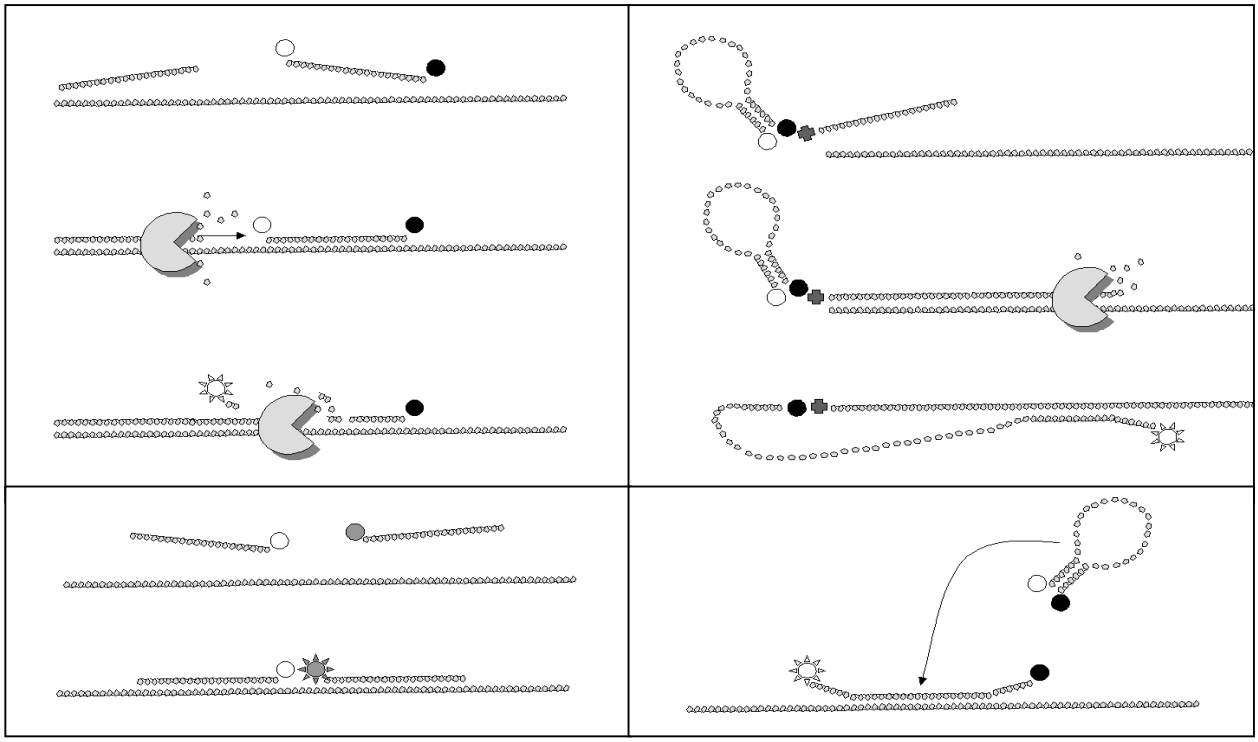

Figure 4. Different chemistries of fluorogenic hybridisation probes. Upper left: 5' nuclease probes. Downstream from one of the amplification primers a dual-labelled hybridisation probe also anneals to one of the single strands of the DNA target molecule present after the denaturation step. During primer extension the 5'-3' exonuclease activity of the Taq DNA polymerase hydrolyses the probe, separating the reporter dye (○) from the quencher (●), giving rise to specific fluorescence emission. **Upper right: Scorpion primers.** After annealing, the primer section is extended by the Taq DNA polymerase. After strand separation, the probe section of the Scorpion oligonucleotide hybridises to a region downstream from the primer sequence during the next annealing step of the PCR reaction. The hairpin structure of the Scorpion primer is blocked from extension (✚) by opposite strand synthesis to ensure that reporter dye (○) and quencher (●) are only separated by specific hybridisation of the probe section to the target sequence. **Lower left: Adjacent hybridisation probes.** Two linear fluorogenic oligoprobes hybridise next to each other to the target sequence during the annealing step of the PCR reaction. The acceptor dye (●) then emits fluorescence due to FRET resulting from the interaction with the donor dye (○) in close proximity. **Lower right: Molecular beacons.** Hybridisation of the probe sequence to the target sequence during the annealing step separates the reporter dye (○) from the quencher (●).

Fluorogenic primers

In contrast to fluorogenic oligonucleotide probes, a fluorogenic primer system does not further improve the specificity of a real-time PCR reaction, as an additional probe hybridisation step is not performed. Thus, any primer-dimers or non-specific reaction products formed may interfere with expected test results. However, the use of different reporter dyes also permits multiplex reactions and allelic discrimination approaches. Although various strategies for self-reporting primer systems have been described in the literature [75,76,77], only two of these systems, the Sunrise primers (also called Amplifluor™ hairpin primers) and the LUX™ primers, seem to ensure satisfactory results and are therefore commercially available. See also figure 5 (page 17).

Sunrise Primers

Like Scorpion primers, the so-called Sunrise (or Amplifluor™) primers combine a hairpin structure (which provides quenching of a reporter dye) with one of the primer sequences. In contrast to Scorpion primers, the hairpin structure of Sunrise primers is intended to be duplicated by opposing strand synthesis and is therefore incorporated into the PCR product, keeping the quencher and the reporter dye apart [76,78].

LUX™ Primers

The so-called LUX™ (Light Upon Extension) system includes a primer with a single fluorophore attached to its 3' end. The fluorogenic primer also provides a short sequence at its 5' end that is complementary to the 3' end. The resulting hairpin structure ensures quenching of the reporter dye by an adjacent guanine base. However, when the primer is incorporated into the PCR product, the signal of the reporter dye increases up to 10-fold [77,79]. In addition to having the disadvantages of fluorogenic primers described above, this real-time PCR system is restricted to specific sequence characteristics of the target DNA required for successful primer design [80].

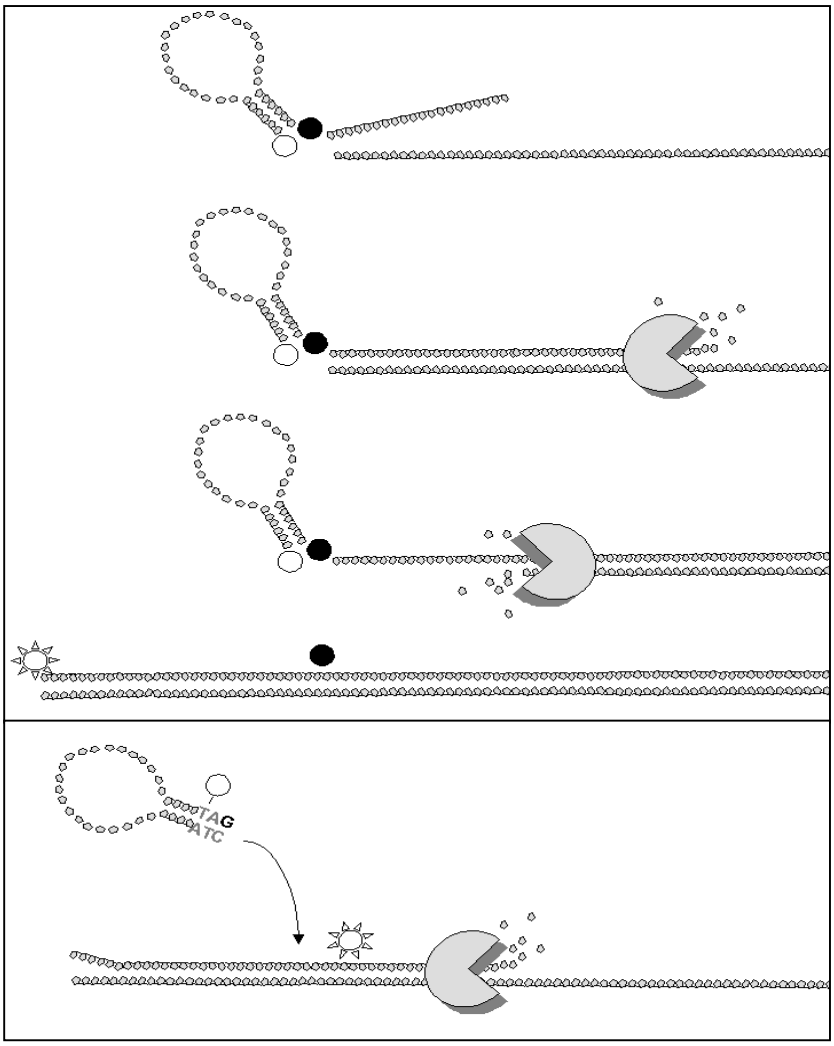

Figure 5. Fluorogenic primer chemistries. Top: Sunrise primers. Strand synthesis takes place after annealing of the primer section of the Sunrise primer to the target sequence. During duplication of the opposite strand, the hairpin structure of the Sunrise primer is incorporated into the PCR product. This separates the reporter dye (○) from the quencher (●), giving rise to specific fluorescence emission. **Bottom: LUX™ Primers.** In the hairpin structure, the reporter dye (○) is sufficiently quenched by a guanine base in close proximity on the same strand. After incorporation of the LUX™ primer into the PCR product this quenching effect decreases, giving rise to increased fluorescence emission.

COMMON APPLICATIONS OF REAL-TIME PCR

The most common applications of real-time PCR are quantitative detection of nucleic acids (mostly mRNA converted to cDNA) in biological samples and discrimination between different sequences of amplification products [56,63,64,69,70,81]. The advantages over conventional PCR assays are that real-time PCR eliminates the need for post-PCR processing and therefore helps to increase throughput, reduces the chances of carryover contamination, allows multiplex reactions and removes potential errors resulting from post-PCR processing [45,46]. Regarding exclusively the quantification of nucleic acids, real-time PCR ensures reliable results from the analysis of single reaction set-ups and offers a broad dynamic range of at least 5 orders of magnitude, eliminating the need for prior sample dilution [46,82,83].

Quantification of nucleic acid templates

As described above, the number of PCR products accumulated after a specific number of amplification cycles in a PCR reaction depends directly on the initial copy number of the nucleic acid template to be amplified. However, a log-linear relationship between these parameters is only given in the exponential phase of the PCR reaction, where the amount of accumulated amplification products does not yet have a negative influence on the efficiency of amplification [23,26]. Prior to the introduction of real-time PCR assays in 1992, quantification of nucleic acids was based exclusively on the quantification of amplification products in post-PCR procedures. The most common and reproducible technique for performing quantitative PCR in a conventional 'endpoint' format is the competitive PCR method that has been used for a variety of applications [84-87]. However, this method is labour-intensive and requires results from multiple reactions for each sample to be analysed [83,88]. Moreover, PCR reactions have to be stopped in the exponential amplification phase to enable reliable quantification of target sequences. This prerequisite limits endpoint quantification assays with regard to their sensitivity and dynamic range [82,83].

Real-time PCR has overcome these limitations, as the entire kinetics of different PCR reactions can be monitored [45]. Even during the exponential phase of amplification, the fluorescence signals rise higher than the unspecific background. This specific point is called crossing point (CP) or threshold cycle (C_t) and represents the parameter used for quantification in real-time PCR assays (see figure 3, page 11).

Absolute quantification requires an absolute calibrator to determine the number of initial nucleic acid targets in the sample reactions [47,83]. A log-linear interpolation between initial copy numbers of the calibration curve and the corresponding C_t values observed is then used for calculation of the initial copy numbers present in the unknowns (see chapter 2, figure 2).

Relative quantification is most commonly used in gene expression analysis and is based on the relative expression of a target gene compared to a reference gene [89]. Two methods used for relative quantification purposes have been described: the calibration curve method and the comparative C_t value method [90]. In the calibration curve method the input amounts of both the target and the reference gene transcripts are calculated from individual calibration curves (see chapter 3). This approach is the method of choice

if the efficiencies of the different amplification systems are either unknown or unequal. Use of the comparative C_t value method (also called ddC_t method) is subject to the prerequisite that the amplification efficiencies of the target and reference transcripts are approximately equal and close to 1. If these requirements are fulfilled, relative expression patterns of the target transcript in different samples can be derived direct from the corresponding differences of the C_t values obtained from amplification of target and reference transcripts [90,91]. Basically, all chemistries described for use in real-time PCR applications can be applied for quantitative detection of nucleic acids as described above. Nevertheless, only the use of fluorescence-labelled oligonucleotides offers the possibility to apply multiplex reactions in order to detect both the target and a possible reference template in a single amplification reaction [77,92,93,94]

Sequence-specific discrimination

Discrimination between different target sequences in a multiplex single reaction set-up by internal fluorescence-labelled oligonucleotide probes was first applied by Lee et al. [56]. Although the 5' nuclease principle was used in this initially described assay, all probe or primer chemistries described above can theoretically be adapted to sequence-specific discrimination approaches. To date, applications for sequence-specific discrimination have been used primarily for allelic discrimination of single nucleotide polymorphisms (SNPs) in human genes. Two basic methods are currently performed for single-tube genotyping: multicolour applications and melting curve analysis.

At present, the multicolour method is performed mainly with 5' nuclease probes or molecular beacons [64,70,95]. Generally, a pair of different fluorescence-labelled allele-specific hybridisation probes is added to the reaction mixture. During the annealing phase of the amplification reaction the probes hybridise perfectly only to their complementary target sequences, giving rise to a fluorogenic emission spectrum that is specific for the corresponding genotype. During amplification the reporter signals further increase to a level that enables allelic discrimination by end-point measurements of specific fluorescence intensities (see figure 6, page 20) [56,67,95].

Melting curve analysis is adapted primarily to the LightCycler™ instrument with a pair of adjacent oligoprobes. On completion of amplification, the reaction is cooled to a temperature ensuring hybridisation of both the donor and the acceptor probe. During

subsequent heating of the reaction mixture according to a time-dependent temperature ramp, the emission profile of the acceptor dye is constantly monitored. Software is then used to calculate the first derivative of the measured fluorescence over time, showing clear peaks representing the T_m of the probe-target hybrid. Even if a single base-pair substitution meets the target sequence of one of the two probes, the T_m is shifted to a lower temperature and this very shift can be used for genotyping purposes [96,97,98].

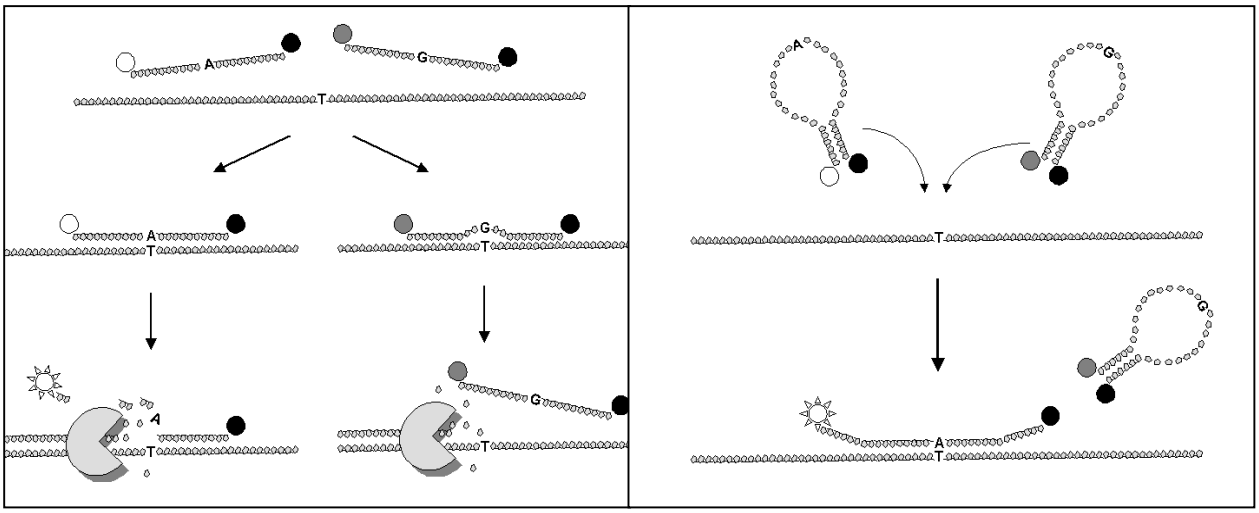

Figure 6. Main principles of allelic discrimination using fluorogenic hybridisation probes. Left: 5' nuclease probes. Two differently labelled allele specific hydrolysis probes compete for corresponding target sequences present in the DNA sample. During the extension of the target strand, only perfectly matched probes are hydrolysed by the 5'-3' exonuclease activity of the Taq DNA polymerase while mismatched probes are only put aside and remain intact. **Right: Molecular beacons.** Two differently labelled molecular beacons compete for corresponding target sequences. Due to competition between the stem and the target sequence, only perfectly matching target sequences lead to disruption of the hairpin structure, giving rise to fluorescence.

PCR APPLICATIONS IN HAEMOSTASIS AND TRANSFUSION MEDICINE

The general introduction of PCR into the medical field broadened the possibilities of both diagnostics and research substantially. In transfusion medicine, PCR has been used for the screening of blood products for viral nucleic acids and the genotyping of transfusion-relevant antigen systems. In the field of haemostasis, PCR has become a powerful tool in the diagnosis of genetically determined risk factors associated with either thrombosis or bleeding disorders. In general, the advent of real-time PCR increased the throughput

in routine diagnostics and improved the research potential in the field of haemostasis and transfusion medicine.

Viral safety of blood products

In 1999, nucleic acid amplification testing (NAT) for the screening of blood donations for genomes of the hepatitis C virus (HCV) was made mandatory by the Paul-Ehrlich-Institute, the German regulatory authority for transfusion medicine [99]. This step was justified by the fact that the long-lasting preseroconversion phase of HCV infection leads to a diagnostic window period of up to 70 days if donations are tested exclusively for antibodies against HCV. This window period is reduced by 41-60 days if blood donations are additionally tested by NAT [100].

In the case of human immunodeficiency virus (HIV) infection, antibodies become undetectable before 22 days on average. The use of NAT for HIV-RNA has the potential to reduce the window period to 10-15 days [100]. As a consequence, the screening of blood donors for HIV-1 group M genomes became mandatory in Germany from May 1, 2004 [101].

With obligatory NAT testing the residual risk of unrevealed HCV or HIV-1 window period donations is now estimated to be less than 1 in 5,000,000 [102].

A variety of procedures is available for the detection of viral RNA in blood donations. Among the different techniques used for NAT, reverse transcriptase polymerase chain reaction (RT-PCR) is the most common used [50,69,81,85,103,104]. Protocols applying isothermal methods like nucleic acid sequence-based amplification (NASBA) and the very similar transcription-mediated amplification (TMA) have also been described [105,106,107].

Regarding the screening of blood donations for viral DNA, the principle underlying PCR is without an applicable alternative [108]. However, obligatory viral DNA screening of blood donations used for the production of single therapeutic units is not yet scheduled in Germany but is hotly debated in the case of the hepatitis B virus (HBV) [109,110].

Viral safety aspects are of equal or much greater importance in the industrial production of coagulation factor concentrates, e.g. factor VIII, factor IX, PPSB or FEIBA, that are manufactured from plasma pools containing thousands of single donations. Thus, single

plasma units destined for use in production pools should routinely be tested for hepatitis A virus (HAV), HBV, HCV, HIV-1 and parvovirus B19 genomes [111,112].
The high sensitivity of NAT assays allows the strategy of pool testing [100]. Potentially present viral nucleic acids are either directly isolated from a pool of single donations or, in order to enhance sensitivity, from a corresponding ultracentrifugal pellet [113,114].The isolated nucleic acids then serve as a template in subsequently performed NAT assays [115].
A variety of methods may be used to detect an amplified viral sequence. The screening of blood donations by real-time PCR assays even allows reliable quantification of the viral load in positive samples [81,116,117,118]. This is of special importance if plasma donations used for production pools are to be tested for parvovirus B19 DNA (see Chapter 2). Another advantage of real-time PCR applications is the use of an internal inhibition control to monitor the efficiency of the amplification process [103,108]. This feature designed to avoid false negative test results is made possible by the multiplex configuration of real-time PCR assays.

Diagnosis of genetic determinants

PCR diagnostics simplified the genotyping of several phenotypic variants proving to be of importance in transfusion medicine substantially. For example, the development of corresponding PCR assays allow the genetic determination of the AB0, rhesus or Kell blood groups [119,120,121]. Furthermore, genotyping of human leukocyte antigens (HLA) or human platelet antigens (HPA) by PCR approaches enhanced the scope of currently applied transfusion medicine [122,123,124].
In the field of haemostasis, a variety of specific polymorphisms or mutations have been identified to be genetic risk factors for either venous or arterial thrombosis. The most frequently discussed allelic variants are located within the genes coding for plasmatic coagulation factors or platelet surface antigens [125 -129]. To date, however, routine diagnostics for genetic thrombophilic risk factors is restricted mainly to the factor V Leiden mutation and the prothrombin G20210A polymorphism [125,127]. The most common methods used for determining these variants are the amplification of the target region followed by restriction site analysis (RFLP) or the use of sequence specific primers (SSP) [130 - 132]. In order to enable a higher throughput of patient samples

without the need for time-consuming post-PCR steps, several real-time PCR applications for the detection of these as well as of several other thrombosis-associated polymorphisms/mutations have been developed [95,97,133,134].

Hereditary bleeding disorders are mainly associated with mutations located in the genes of coagulation factors VIII or IX, causing haemophilia of type A or B, respectively [135,136]. A variety of point mutations, deletions or inversions in these genes have already been described [137 - 140]. It was only the implementation of PCR that enabled the individual genetic variant in the genes of either factor VIII or IX in haemophiliac patients to be identified, e.g. by heteroduplex analysis in an acceptable time window [141,142].

AIM OF THE THESIS

The aim of the present thesis was the application of novel real-time PCR approaches into the haemotherapeutic field. The ABI Prism® 7700 sequence detection system platform was used for the development of 5' nuclease assays applied for diagnostics and research in haemotherapy.

- Chapter 2 describes the development of a fully validated 5' nuclease assay for the screening of blood donations for parvovirus B19 DNA.

- An evaluated 5' nuclease assay for the quantification of tissue factor (TF) mRNA in circulating blood monocytes is presented in Chapter 3.

- A study described in Chapter 4 shows the potential of 1-deamino-8-d-arginine vasopressin (DDAVP) to upregulate TF gene expression in blood monocytes in vivo. TF mRNA levels in white blood cells were quantified using the method described in chapter 3 with modifications.

- In order to evaluate a newly established method for global amplification of platelet mRNA sequences, a quantitative 5' nuclease assay for the detection of von Willebrand factor (vWF) and GAPDH mRNA sequences in a multiplex configuration was developed (Chapter 5).

- Chapter 6 describes a study on the prevalence of molecular thrombophilic risk factors in patients with dural arteriovenous fistulas (DAVFs). Several polymorphisms were detected by partially unpublished restriction site analyses or 5' nuclease assays.

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Chapter 2

Development and Validation of a Real-Time PCR Assay for Routine Testing of Blood Donations for Parvovirus B19 DNA*

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SUMMARY

BACKGROUND: Human parvovirus B 19 (B19) is a single-stranded and non-enveloped DNA virus. In the majority of patients infections with B19 are benign and characterised only by malaise and exanthema of the skin. In a small subset of patients B19 infections induce severe complications including aplastic anaemia, chronic affections of the joints and fetal injuries up to abortion during pregnancy. Since B19 is transmitted through contaminated blood donations, testing for B19 DNA is taken into consideration to further increase virus safety of blood products. **MATERIAL AND METHODS:** TaqMan technology, a homogenous and real-time fluorescence PCR approach, has been applied for identification of B19 DNA. Primers were designed that amplify a DNA fragment located within a conserved region of non-structural protein 1 (NS1). A B19 variant showing a 27 base-pair inversion was constructed to be used as internal control. The assay was validated according to the criteria of the European regulatory authorities. **RESULTS:** The assay had a sensitivity limit of 104 genome equivalents per ml. False-negative results due to problems with amplification were ruled out by the use of an internal control DNA. Reliable quantification of B19 DNA was possible by the use of a standard curve. **CONCLUSIONS:** A real-time PCR procedure for B19 virus DNA detection that is based on the TaqMan-technology has been developed and validated. The procedure is applicable for routine testing of single blood donations as well as for analysing large scale plasma pools.

INTRODUCTION

Human parvovirus B19 (B19) is a single-stranded DNA virus of the genus erythrovirus within the family of parvoviridae. The viral genome of 5.4 kb encodes two capsid proteins (VP1 and VP2) and the three non-structural proteins NS1, P11, P7.5 [1,2]. B19 is an ubiquitous virus. By the age of 15, about 50% of individuals have serologic evidence of a past infection [3,4]. In immunologically component individuals the B19 infection may be asymptomatic or cause the common childhood disease erythema infectiosum. The infection is cleared within several weeks by specific antibodies and cytotoxic T-cells [5]. However, even in the presence of neutralising antibodies circulating B19 viruses can be detected up to one year after infection [6]. In patients with absent or dysfunctional humoral immunity, persistent infection can occur, which induces chronic haematological disorders including chronic anaemia, neutropenia, and thrombocytopenia [7,8]. In addition, B19 infections in pregnancy are associated with a high risk for fetal injuries and spontaneous abortion [9].

There are several reports that describe transmission of B19 through blood products [10,11,12]. Recent studies have shown that outside an epidemic period up to 1 in 3,300 donations and within an epidemic period 1 in 167 donations may contain detectable B19 virus DNA [13]. To enhance transfusion safety, testing of blood donors for B19 should be taken into consideration, especially for recipients of blood products that are exceptionally at risk in case of B19 infection. Screening for IgM antibodies is not very reliable in order to identify viremic blood donors, as IgM antibodies do not emerge until 10 days after infection but an intense viraemia develops approximately after 5-8 days [2,14]. Furthermore, IgM antibodies are still detectable several weeks after complete clearance of the B19-virus [15]. Thus, nucleic acid amplification techniques (NAT) are the most suitable method to identify B19 contaminated blood donations.

The possibility of viral load quantification is an additional advantage of NAT screening. This is especially important for screening of plasma donations used for production pools. Donations containing high titers of B19 can be identified and removed from pooling, whereas donations containing low titers of B19 should not be discarded because of their desirable high content of protective antibodies against B19 [16].

This report details the development and validation of a quantitative PCR-assay based on real-time PCR using ABI Prism SDS7700 (TaqMan) for large-scale screening

of blood products for B19 DNA. TaqMan is a homogeneous fluorescence generation system that detects target DNA as the PCR proceeds in real time. It functions by including an oligonucleotide probe designed to hybridise downstream of one of the primers. These probes are blocked from extension at their 3´-terminus and are labelled with a fluorescent reporter at the 5´-terminus. The probes are also conjugated to another fluorophore, which quenches the fluorescence of the reporter when both labels are in close proximity. During PCR, the probe is cleaved by the 5´-exonuclease activity of DNA polymerase. This cleavage separates the fluorescent reporter dye from the quencher, giving rise to an increase in fluorescence [17,18]. The TaqMan technology measures the emission in each PCR cycle. The cycle at which the fluorescence rises higher than the background signal is called threshold cycle (Ct-value) and is proportional to the concentration of target DNA added to the PCR mixture.

Especially in a blood bank setting inhibition of amplification must be reliably identified to prevent false-negative results. Reporter dyes showing different fluorescence emission characteristics are commercially available. Therefore, the TaqMan approach allows analysis of several amplicons simultaneously. This enables the construction and use of an internal standard that was used to monitor the efficiency of the amplification process.

MATERIALS AND METHODS

Extraction of viral DNA from plasma

Viral DNA was isolated from 140µl plasma by spin column procedure using the viral RNA Mini Kit ® (Qiagen, Hilden, Germany) according to the manufacturer's instructions. This protocol does not separate RNA from DNA and can therefore be used for purification of DNA as well. After adding the lysis buffer, 50 copies of an internal control were added to each sample to evaluate both, extraction and amplification. Purified DNA was eluted in 50µl of water.

B19-TaqMan-PCR-assay

Primers and probes were designed using Primer Express Software ® Version 1.5 (PE Applied Biosystems, Foster City, USA). The primer set amplifies a 78 base pair fragment in a conserved NS1 region of B19. The primers used were 5'-AAGCC-

ATTTTAGGCGGGC-3’ (forward) and 5’-CCACAGGTACTCCAGGCACAG-3’ (reverse). The wild type specific probe was 5’-CCCACCAGGGTAGATCAAAAAATG- CGT-3’, labelled with the fluorescent dye FAM (6-carboxyfluorescein) at the 5’ end and the quencher TAMRA (6-carboxytetramethylrhodamine) at the 3’ end. The internal control probe was 5’-TGCTAAAAAACTAGATGGGACCACCC-3’, labelled with the fluorescent dye VIC at the 5’ end and TAMRA at the 3’ end. Nucleotide homology compared to sequences other than Parvovirus B19 was checked for all oligonucleotides against EMBL, GenBank and DDBJ using the BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST). Primers were purchased from Eurogentec (Seraing, Belgium), probes were obtained from PE Applied Biosystems (Weiterstadt, Germany).

Optimal amplification conditions in a final volume of 25 µl were found to be 1x PCR buffer (20mM Tris-HCl, pH 8.5, 50mM KCl), 7mM $MgCl_2$, 200µM dATP, dCTP and dGTP, 400µM dUTP, 300nM forward primer, 900nM reverse primer, 200nM wildtype- and internal control-probe, 0.5µl Rox reference dye, 1.25 U PlatinumTaq DNA Polymerase ®, 0.5U uracil-N-glycosylase and 10 µl of the sample preparation. PlatinumTaq DNA Polymerase ® and Rox reference dye were purchased from Invitrogen (Groningen, Netherlands). Thermal cycling was performed using the ABI Prism 7700 Sequence Detection System ® (PE Applied Biosystems). The following amplification profile was used: 50°C for 2 min, 95°C for 10 min followed by 45 cycles consisting of denaturation at 94°C for 20s and annealing and extension at 57°C for 1 min.

Internal and external controls

For construction of an internal inhibition control, a synthetic 180 base pair oligonucleotide was used. The sequence of this internal control sequence is identical to the B19-DNA sequence ranging from nucleotide 1526 to nucleotide 1705 [19], thus including the segment being amplified by the B19 TaqMan-PCR with the only exception that the 27 bp spanning hybridisation area where the TaqMan probe anneals was inverted. The synthetic oligonucleotide was amplified by PCR. The primers used for amplification were 5’-CAGGGAAAAGCTTGGTGG-3’ (forward) and 5’-CGCTTACAACAAAAGTAATGTCA-3’ (reverse). The control oligonucleotide and amplification primers were purchased from Eurogentec. After purification using the PCR

Purification Kit ® (Qiagen, Hilden, Germany), the amplification products were ligated in pGEM-T vectors (Promega,Woods Hollow Road, USA) and used as an internal control after purification from transformed TOP10 ® E.Coli culture (Invitrogen, Groningen, Netherlands). The concentration of the internal control was determined by photometric measurement of the purified plasmid-DNA.

A quantification standard was prepared from B19 positive plasma, that was obtained from the National Institute for Biological Standards and Control (NIBSC, Potters Bar, Great Britain). This plasma contains 10^7 genome equivalents per ml (geq/ml) and represents a 1:10 dilution of a B19 DNA positive single donation that was used to prepare the NIBSC B19 DNA working reagent (Code 97/542) containing 1,000 geq/ml. Standard plasma was aliquoted and stored at –80°C until used. For construction of a standard curve, B19 DNA was extracted from standard plasma and serially diluted with water to achieve final concentrations ranging from 3 to 28.000 geq/PCR-reaction corresponding to 1 x 10^2 to 1 x 10^6 geq/ml.

In addition, a run-control containing 250 geq/ml was prepared from standard plasma by diluting it with negative plasma and used as a positive control in every run.

Reference samples

Twelve Parvovirus B19 DNA positive samples were provided from the Institute of Medical Microbiology and Immunology, University of Bonn. All samples were previously tested for B19-DNA using a nested PCR assay [20].

Probit analysis on experimental data

Probit analysis was performed using software (SSPS ®, Base-Line, Munich, Germany).

RESULTS

Development of the B19 TaqMan PCR

A single, correctly sized PCR product was found on an ethidiumbromide stained 2% agarose gel using the described primer combination. Real-time-PCR conditions were optimised in order to define an amplification protocol that combines a maximum of sensitivity with stable reproducibility. Parameters evaluated include the concentrations of primers and probes and the $MgCl_2$ concentration of the PCR reaction mix. For optimisation of primer concentrations, both primers were tested in a primer matrix at concentrations ranging from 50 nM to 900 nM. Optimal results were obtained at concentrations of 300 nM and 900 nM for the forward and reverse primer, respectively. Magnesium chloride concentrations ranging from 2 to 10 mM were studied. The highest fluorescence intensity was obtained at a $MgCl_2$ concentration of 7 mM. Varying the concentrations of the hybridisation probes (50 – 300nM), the highest level of fluorescence generation was seen at concentrations ranging from 200 to 300 nM for both probes. Therefore, a concentration of 200 nM was defined as the working concentration used for both probes.

Amplification of the internal control showed a single band that was identical in size to the 78 bp B19-wildtype-PCR product. In a second series of experiments, the internal control was serially diluted to establish the optimal working concentration, found to be 50 copies/extraction. Based on these results 50 copies of the internal control were added in the extraction-step in all further experiments. A possible influence of the internal control on the sensitivity of the B19-TaqMan-PCR was assessed by B19 DNA amplification (1,000 geq per ml of the plasma sample) in the presence and absence of 50 copies of the internal control. Identical amplification efficacies ruled out an interference of the control reaction with the B19 DNA amplification (data not shown).

Specificity and sensitivity of the B19 PCR

Theoretically, unspecific results using the TaqMan-technology are very unlikely justified by the fact that in addition to the primer-pair the fluorescent-labelled probe has to bind to the template DNA as well in order to achieve a positive fluorescence signal. In order to confirm the specificity of the B19 TaqMan PCR, plasma samples from 100 blood donors

were analysed. In none of the 100 plasma samples a specific amplification signal was obtained using a total of 45 cycles in the TaqMan-PCR.

The detection limit of the B19-TaqMan-PCR was evaluated using B19 positive standard plasma showing a concentration of 10^7 geq/ml. Pre-diluted standard plasma was 2-fold diluted using B19-negative plasma, reaching final concentrations ranging from 2.500 down to 5 geq/ml. Twenty-four aliquots (140µl) of each virus dilution were prepared in 3 independent sets of 8 and stored at –80°C until used. DNA-isolation was performed by 3 operators, each one extracting viral DNA from 8 aliquots of the 10 dilutions-points. Extraction and amplification were shared out over a couple of days, using different lot-numbers of the Viral RNA Mini Kit and PlatinumTaq-DNA Polymerase ®. Up to a concentration of 156 geq/ml a positive B19-TaqMan-PCR result was obtained in 100% (24/24) of the reactions (figure 1). The 95% cut-off value was calculated by Probit analysis and was determined to be 104 geq/ml, showing a 95% confidence interval of 70 to 195 geq/ml.

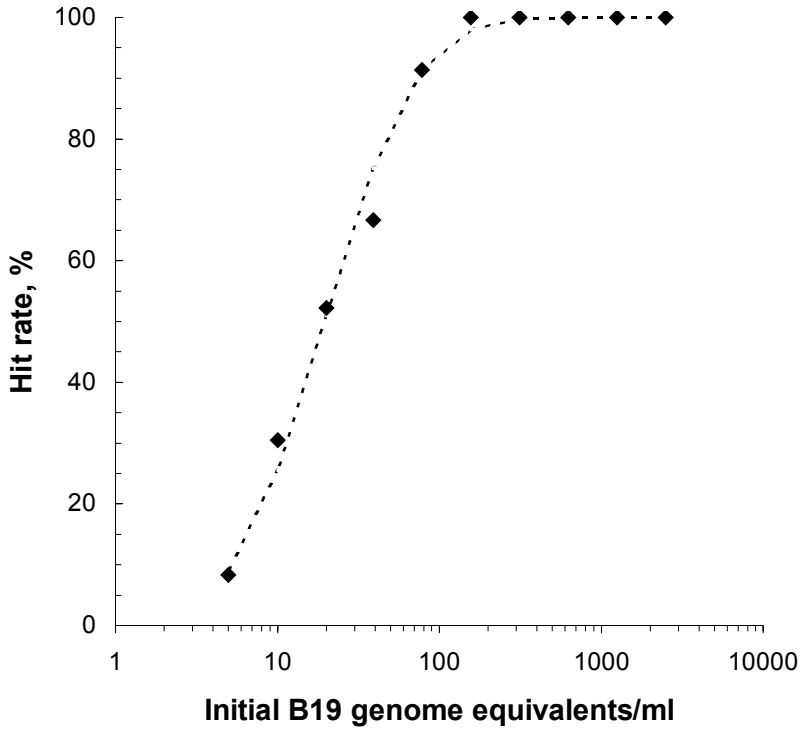

Figure 1. Percent of positive results (Hit rate) found in dependence of the initial concentration of B19-DNA. Dotted line: Interpolation for the calculation of the 95% cut-off value as determined by probit regression analysis.

Precision of the assay

For the determination of the intra- and inter-assay precision of the method, data sets from the utilised dilution series for determining the sensitivity limit were used. Eight replicates of each dilution were performed by one operator in one experiment under the same conditions. The remaining aliquots were processed by 2 operators over a period of 4 days. The results for the mean threshold cycle and coefficient of variation of the highest and lowest concentration, found positive in 24 of 24 replicates, are given in Table 1.

Table 1: Intra- and inter-assay precision of the assay

	Initial B19-DNA [geq/ml]	mean threshold cycle	% coefficient of variation
Intra-assay precision (n=8)[a]	2500	31.72	1.53
	156	36.21	1.31
Inter-assay precision (n=16)[b]	2500	31.60	1.24
	156	35.97	2.80

[a] Samples were processed under same operating conditions in one single experiment or
[b] within different experiments carried out on different days by different operators using different lot-numbers of used extraction kit and Taq DNA polymerase.

Performance of the standard curve

B19-DNA extracted from standard plasma containing 10^7 geq/ml was 10-fold diluted with water and used for generation of a standard curve. In figure 2 the obtained mean threshold cycles of each dilution from 8 different experiments are plotted against the initial template concentration corresponding to the B19-DNA level in the plasma sample. The shown standard deviations display the high coefficient of regression (> 0,99) found in all single experiments.

Testing of B19 positive plasma samples

To evaluate the installed assay regarding different isolates of Parvovirus B19-DNA, twelve B19 positive samples were tested. A positive result was obtained in each case.

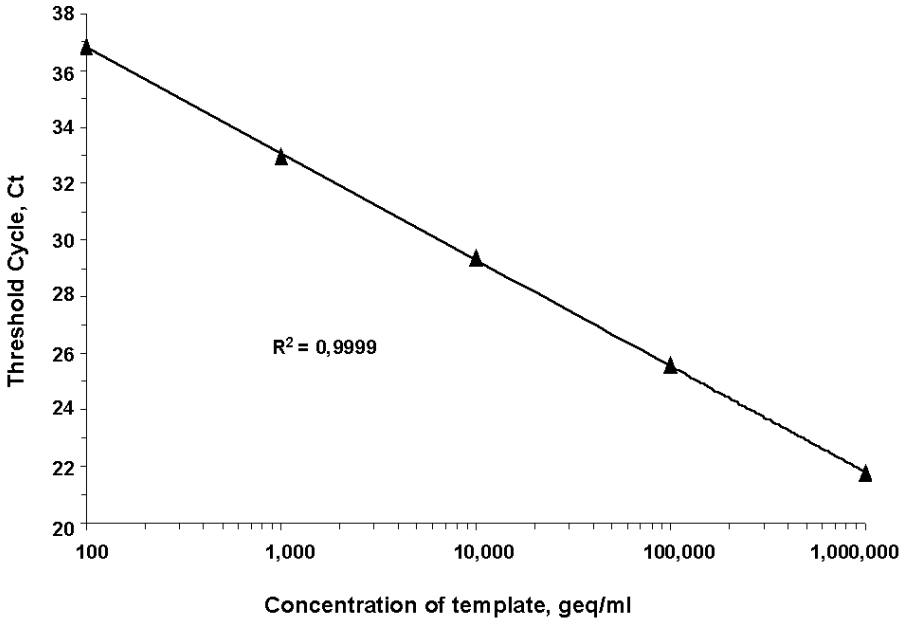

Figure 2. Mean threshold cycles of each concentration of the standard curve calculated from 8 independent experiments. Observed standard deviations from the highest to the lowest input-concentration were as follows: 0.12, 0.22, 0.23, 0.35, and 0,37.

DISCUSSION

We developed and evaluated a B19 NAT assay based on the TaqMan-PCR technology. The advantages of the newly developed assay are its sensitivity and the possibility of distinguishing high from low virus titers of B19 in plasma samples by reliable quantification of B19-DNA. As the TaqMan-PCR works in a closed tube format, jointly with the use of an uracil-N-glycosylase system the chances of false-positive results caused by carry-over contamination are strongly decreased.

A key issue, which defines the validity of a NAT procedure, is the use of an internal control. This is especially important in a blood bank setting. This issue was addressed by the design of a 180 base pair spanning B19-variant DNA fragment that was cloned into a pGEM-T-plasmid. The PCR product, which is generated from the B19-variant DNA is identical to the wild-type fragment with the exception that the area where the hybridisation probe anneals is inverted. This variant DNA fragment assures that the characteristics of wild type DNA and internal control are as homologous as possible. Constructing the described sequence of the internal control as an oligonucleotide is an easy to handle alternative to the construction of an internal control DNA fragment by

PCR-based site-directed mutagenesis as previously described in several publications [16,21,22]

Sequence diversities of the B19-parvovirus have been recently described. The majority of these variabilties are characterised by polymorphism's in the VP regions and in the C-terminal region of NS1 [23,24,25]. Primers and wildtype-probe were chosen according to sequence alignments using the NCBI WebPage. The NS1-region amplified is highly conserved ensuring detection of various isolates of B19. This was confirmed by testing twelve B19 positive plasma samples, all giving a positive result. However, future isolates of B19-DNA could demonstrate base mismatches located in the amplified region of NS1, reducing the amplification efficiency of the described assay, especially in case of base substitutions located in the annealing sequence of the used wildtyp-probe [26]. Thus, even with regard to correct quantification, it would be useful to keep at least one additional PCR-assay, targeting an alternative region of the B19 genome. For example Aberham et al. described a quantitative, internally controlled real-time TaqMan-PCR assay for the detection of Parvovirus B19 DNA, targeting a conserved area in the VP1 region of B19 [16].

The data presented, demonstrates that the established B19-TaqMan-PCR assay is a sensitive and well standardised method that can be applied for large scale screening of single blood donations as well as plasma pools used for further manufacture into components. Since a quantitative assay is a prerequisite for the screening of plasma for production pools an absolute standard curve ranging from 10^2 to 10^6 geq/ml was constructed. The risk of false negative results due to inhibition or another failure of the amplification reaction is minimised by the use of an internal control. It was also shown, that purification of Parvovirus B19 DNA is possible using a silica membrane based extraction method for viral RNA. This circumstance allows the use of only one extraction step for screening blood donations for HCV-RNA, HIV-RNA and Parvovirus B19-DNA.

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Chapter 3

Quantitative Tissue Factor Gene Expression Analysis in Whole Blood: Development and Evaluation of a Real-Time PCR Platform*

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Up-regulation of tissue factor (TF) expression in circulating blood cells, especially monocytes, plays a key role in the pathogenesis of various thromboembolic diseases [1-4]. Measurement of TF expression in monocytes therefore might be helpful in the diagnosis of a hypercoagulable state [5-7]. Monocytic TF expression can be measured on both the protein and RNA levels. Testing on the RNA level seems to be more appropriate than antigen testing to detect a procoagulant state because monocytes that already express functionally active TF on their membrane surfaces are rapidly cleared from the circulation [8].

The aim of the present study was to develop a protocol for quantitative determination of TF mRNA transcripts in monocytes and other circulating blood cells. The method should be rapid, robust, and applicable in whole blood without the need for cell isolation. We developed a one-step quantitative reverse transcription (qRT)-PCR assay based on the real-time TaqMan® technology and evaluated the preanalytical conditions required for the use of TF mRNA measurements on a routine clinical basis as well as several normalization strategies, including normalization based on CD14 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reference gene expression. Finally, we measured baseline TF expression in healthy individuals and in thrombophilic patients.

RNA calibrators for quantification were prepared by in vitro transcription of DNA hybrids that combined the T7 promoter sequence with the following sequences of TF, CD14, or GAPDH cDNA: for TF, bp 265–848 (GenBank accession no. M16553); for CD14, bp 18–564 (GenBank accession no. M86511); and for GAPDH, bp 8–525 (GenBank accession no. M33197). Generated RNAs were treated with DNase I (Roche) to remove the added DNA templates and purified by use of the RNeasy Mini Kit (Qiagen). RNA was quantified

by photometric measurement (A_{260} reading of 1 = 44 mg/L). Oligonucleotide primers and probes for TF and CD14 qRT-PCR were designed by use of Primer Express software, Ver. 1.5 (Perkin-Elmer), and were purchased from Eurogentec. All sequences shown in Table 1 were chosen to prevent amplification of genomic DNA. Primer and probe sequences for amplification of GAPDH mRNA were taken from the "TaqMan Gold RT-PCR Kit" protocol (Perkin-Elmer).

Table 1. List of primers and probes used for TF- and CD14-amplification

qPCR	Oligonucleotide	Sequence[a]	Position[b]
TF	**forward primer**	5'-AATGTGGAGAGCACCGGTTC-3'	415 – 434
	reverse primer	5'-CGTTCATCTTCTACGGTCACATTC-3'	563 – 540
	probe	5'-CACCTTACCTGGAGACAAACCTCGGA-3'	473 – 498
CD14	**forward primer**	5'-AGAGCCTGTCCGGAGCTCA-3'	34 – 52
	reverse primer	5'-TCACAAGGTTCTGGCGTGG-3'	152 – 134
	probe	5'-ACGCGCGCTCCATGGTCGATA-3'	88 – 68

[a] TF- and CD14-probes were labeled with the fluorescent reporter dye FAM (6-carboxy- fluorescein) at the 5' end and the quencher TAMRA (6-carboxytetramethyl-rhodamine) at the 3' end. To enable multiplex reaction, a different reporter dye (JOE) was labeled at the 5' end of the GAPDH-probe, while also TAMRA was used as a quencher at the 3' end

[b] Positions according to GenBank accession numbers M16553 (TF) and M86511 (CD14)

PCRs were performed in a final volume of 20 µL using the QuantiTect Probe RT-PCR-Mix (Qiagen). Optimum reaction conditions were as follows: 1x Mastermix (including PCR buffer, deoxynucleotide triphosphates, 4 mM $MgCl_2$, and Rox reference dye); TF or CD14 forward and reverse primers (200 and 150 nM, respectively); GAPDH forward and reverse primers (100 nM); TF or CD14 probe (200 nM); GAPDH probe (100 nM); 1 U of QT Probe RT Mix; and 5 µL (TF/GAPDH PCR) or 1 µL (CD14/GAPDH PCR) of calibrator or sample preparation. Calibrators and samples were run in duplicate.

Thermal cycling was performed in a 96-well spectrofluorometric thermal cycler (Prism SDS 7700; Applied Biosystems) with the following profile: 50 °C for 20 min for the reverse transcription reaction; 95 °C for 15 min; and 40 cycles of denaturation for 20 s at

95 °C and annealing/extension for 60 s at 60 °C. The TF qRT-PCR performed in this way exhibited a linear range extending from 10^2 to 10^7 copies/reaction. The amplification efficacy *(E)* of 0.99 as determined from the slope *(s)* of the calibration curve according to the equation $E = 10^{-1/s} - 1$ was not influenced by coamplification of GAPDH or CD14 mRNA, making multiplex approaches possible. CV were calculated based on concentrations as determined by additional processed calibration curves. Intraassay CVs were <19%. Including the RNA isolation process, interassay CVs ranged between 3.1% and 21%. Variations of this magnitude are regular for quantitative PCR, and our results were well within accepted values for such assays.

Accurate analysis of in vivo gene expression might be complicated by unintended ex vivo gene expression or degradation of gene transcripts. This is especially a problem when working with monocytes because monocytes are highly reactive and TF expression can be induced by contact with foreign surfaces and extended blood storage [9]. To minimize these ex vivo changes, we tested a blood sampling system (PAXgene™ Blood RNA tubes; PreAnalytiX) that includes a stabilizing additive in the blood collection tube [10]. Whole blood from one healthy donor was drawn in parallel into PAXgene and EDTA tubes. Tubes were stored at room temperature or 4 °C, and total RNA was isolated after 0, 1, 3, 5, and 7 days of storage. TF, CD14, and GAPDH RNA concentrations remained stable up to 5 days of storage in PAXgene tubes at 4°C.

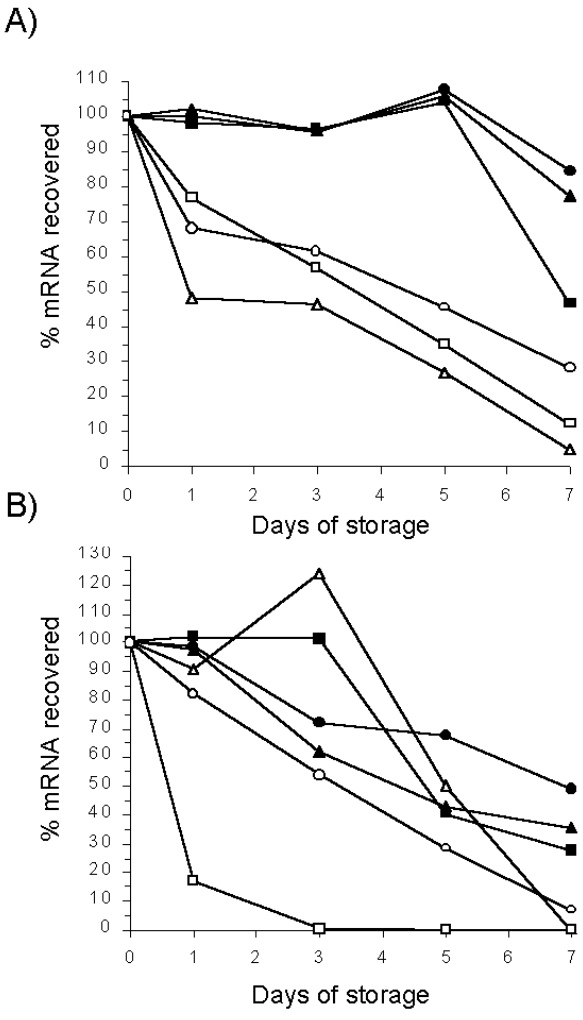

Figure 1. Influence of sample handling on RNA stability. Whole blood was collected into PAXgene blood sampling tubes (closed symbols) or EDTA tubes (open symbols) and stored at 4°C (A) or at room temperature (B) over a total of 7 days. At the indicated time points the yield of RNA transcripts was measured by quantitative RT-PCR. TF (▲); CD 14 (■), GAPDH (◆).

Storage at room temperature reduced this time span to 24 h. In EDTA-anticoagulated blood, the RNA expression profile dramatically changed within the first hours independent of the storage temperature (for details see Fig. 1). On the basis of these findings, we conclude that quantification of TF mRNA is reliable and reproducible only if the cellular RNA profile undergoes immediate stabilization after blood sampling. Methods that require isolation of peripheral blood mononuclear cells before RNA determination do not necessarily reflect the in vivo situation.

Induction of monocytic TF expression by lipopolysaccharide (LPS) plays an important role in the pathogenesis of sepsis-associated thrombotic complications and has been investigated extensively in several in vitro studies [8, 11-13]. We therefore used the LPS model to evaluate the newly established PCR methods. As shown in Fig. 2A, increasing amounts of TF mRNA were quickly generated, and peak concentrations were reached between 1 and 2 h after exposure to LPS, followed by a progressive decrease thereafter. This pattern of TF mRNA expression agrees well with previously published data [11-13]. The LPS receptor CD14 has been proposed to be a suitable marker for the number of monocytic cells in blood [14]. This requires that CD14 mRNA expression is relatively constant and not regulated by different triggers. Studying CD14 expression patterns in the LPS model, we found LPS-independent but time-dependent changes in CD14 mRNA concentrations (Fig. 2B). Moreover, our data obtained on blood samples from healthy individuals demonstrate high interindividual variability of CD14 mRNA concentrations when normalized to the number of monocytes (range, 215–684 copies/monocyte), indicating that CD14 cannot be used as a marker for the number of monocytes.

The use of monocytic TF mRNA as a diagnostic marker requires the definition of a reference interval. We established a reference interval from TF mRNA and monocyte measurements of 50 healthy blood volunteers (mean age, 33.0 years; range, 19–65 years). The percentage of monocytes in whole-blood samples was determined by flow cytometry using a FACScan and CellQuest software, Ver. 3.3 (Becton Dickinson). Monocytes were identified by their specific forward and side scatter patterns and CD14 expression. The mean (SD) transcription rate of 0.0041 (0.0021) TF mRNA copies/monocyte indicates very low basal TF expression that is 5 orders of magnitude lower than CD14 expression. The data showed a log-normal distribution, and a 95% reference interval of 0.0018–0.0095 TF transcripts/monocyte was calculated according

to IFCC guidelines [15]. As a consequence, a patient will be scored positive if monocytic TF expression exceeds 0.0115 transcripts/monocyte (upper limit of the reference interval + 2 SD).

Monocytes are the major, but may not be the only source of TF mRNA in whole blood [1,4]. At present it is a matter of discussion whether platelets contain TF mRNA and whether granulocytes can be induced to express TF mRNA [16,17]. To consider these potential TF mRNA sources, we also normalized TF mRNA results to the volume of whole blood. Using this mode of normalization, we calculated a 95% reference interval of 685-3155 TF transcripts/mL. On the basis of these data, a patient will be scored positive if TF expression exceeds 4745 TF transcripts/mL.

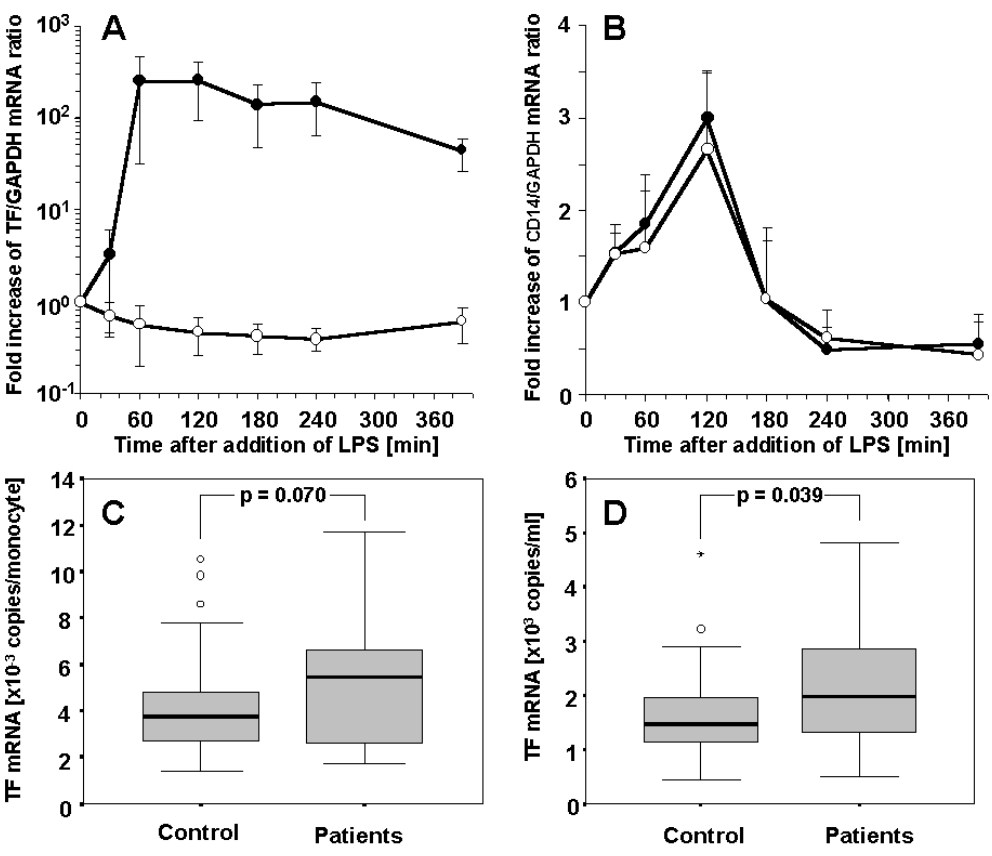

Figure 2. In-vitro and in-vivo studies of TF expression in blood cells. (A), EDTA-anticoagulated whole blood (n=10) was stimulated with LPS (100 µg/L) at 37°C. Aliquots of 2.5 mL were drawn from the whole blood samples at the indicated time points after addition of LPS and transferred to PAXgene Blood RNA tubes . The expression of TF mRNA (A) and CD14 mRNA (B) was then measured. O, results obtained with unstimulated samples (n=3). Results are expressed as mean values ± s (*error bars*). TF mRNA was measured in blood samples obtained from 50 healthy blood donors and 27 thrombophilic patients. Results were normalized to the number of monocytes (C) or to the volume of whole blood (D). *Boxes* represent the interquartile range, with the median represented by the *line* inside the box. *Whiskers* indicate the highest and lowest values, excluding outliers (O) and extreme outliers (*).

The TF transcription profile was also analyzed in blood samples taken from 27 thrombophilic patients (16 females and 11 males; mean age, 49.5 years; range, 18–83 years). All patients had a positive history of at least one idiopathic deep venous thrombosis but showed no acute event in the last 3 months. The results shown in panels C and D of Fig. 2 show that these patients had statistically significant higher TF expression rates than the reference group. These findings suggest that increased TF expression in whole blood contributes to the procoagulant state in thrombophilic patients and support the findings of other groups [6,18]. Further studies on larger series of patients, however, are required to clarify whether increased TF mRNA concentrations in circulating blood cells are a thrombophilic risk factor.

In summary, the TF multiplex approach described here allows rapid and quantitative determination of TF mRNA concentrations in whole blood without the need for cell isolation. Ex vivo changes in the expression pattern of blood cells were avoided by the use of blood-sampling tubes that contain RNA preservation and cell-fixing additives. Together with the established reference interval, this easy-to-perform method could provide a useful basis for further studies in clinical settings in which TF expression plays an essential role.

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Chapter 4

Desmopressin Acetate (DDAVP) Administration Induces Tissue Factor-dependent Procoagulant Activity in Monocytes*

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ABSTRACT

Monocytes are a potential cellular target for the hemostatic agent desmopressin (DDAVP). Since activated monocytes typically express tissue factor (TF), we examined the effect of DDAVP on the expression of TF in monocytes. Ten healthy volunteers were injected with 0.3 µg/kg body weight DDAVP and blood was collected before and up to 6 hours after DDAVP. A significant decrease ($p<0.027$) in monocyte counts was observed after DDAVP reaching a nadir at 1 hour. TF mRNA levels measured by quantitative real-time RT-PCR showed a progressive and significant increase ($p<0.001$), peaking at 1 hour after DDAVP. Increased TF gene activities were associated with increased TF activities as measured through the rate of factor Xa formation. Comparing the in vivo response to DDAVP with the in vitro response to LPS similar patterns of response were observed, but on a 100-fold lower level. In vitro exposure of monocytes with DDAVP failed to induce TF expression making a direct activating mechanism unlikely. We suggest an indirect mechanism that involves activation of monocytes through DDAVP stimulated endothelial cells in a P-selectin/PSGL-1-dependent manner. In conclusion, our data demonstrate induction of TF-dependent procoagulant activity on monocytes as an additional hemostatic mechanism of DDAVP.

INTRODUCTION

Administration of desmopressin acetate (1-desamino-8-D-arginine vasopressin, DDAVP), a synthetic analogue of vasopressin, induces a rapid rise in plasma levels of factor VIII (FVIII) and von Willebrand factor (vWF) through activation of endothelial cells [1-3]. DDAVP was therefore introduced by Mannucci and coworkers as a pharmacological alternative to plasma-derived factor VIII preparations in the treatment of patients with mild hemophilia A and von Willebrand´s disease [4]. In addition to these established indications DDAVP is now successfully used in the treatment of various acquired and inherited bleeding disorders including those where vWF and FVIII levels are within the normal range or even increased such as several platelet disorders [5-7]. Interestingly, DDAVP shortens the bleeding time in Bernard-Soulier patients [8-11], and in patients with severe von Willebrand´s disease treated with cryoprecipitate [12]. As Bernard-Soulier patients are characterized by a defect in the glycoprotein-Ib-IX-complex, the primary vWF receptor on the platelet surface, and patients with severe von Willebrand´s disease do not have vWF stores that could be mobilized by DDAVP, concerns were raised whether the hemostatic effect of DDAVP is sufficiently explained by its effect on the FVIII-vWF-axis [13].

Monocytes, a blood cell type showing a wide phenotypic overlap with endothelial cells and in particular endothelial cells of microvascular origin [14] have been discussed to be an additional cellular target of DDAVP. Hashemi et al. have shown that DDAVP stimulates monocytes to release platelet-activating factor (PAF) that triggers the release of vWF from endothelial cells and activates platelets [15,16]. Consistent with these findings increased levels of PAF have been found within leukocytes after administration of DDAVP to patients with hemophilia A or vWD [17]. Additional evidence that DDAVP activates monocytes is given by monocyte-platelet interaction studies [18]. Using an in vitro model, Pereira et al. demonstrated enhanced binding of formaldehyde fixed platelets to DDAVP stimulated monocytes when compared with non-stimulated cells. The increase in platelet binding correlated with increased expression rates of the P-selectin ligand (PSGL-1) that acts as a P-selectin counter receptor and is expressed by activated monocytes [19].

Typically, activated monocytes express functional active tissue factor on their plasma membrane. This has been shown for various activators such as endotoxin,

immune complexes, proinflammatory cytokines, and platelets [19-22]. Tissue factor (TF) binds factor VII (FVII) and activated FVII (FVIIa) to form the TF/FVIIa complex that activates the coagulation cascade by conversion of factors IX and X into the active enzymes [23]. If DDAVP activates monocytes, a procoagulant shift should be induced through induction of TF expression. To test this hypothesis, ten healthy volunteers were treated with DDAVP and time-dependent changes in monocytic TF expression levels were recorded on the transcriptional and translational level.

DDAVP action is mediated through the type 2 vasopressin receptor (V2R) [24]. This receptor subtype is expressed in the kidney collecting duct where it mediates the antidiuretic effect of DDAVP and on microvascular endothelial cells where it mediates the release of vWF and t-PA [13]. Although vasopressin receptors have been identified on human peripheral blood mononuclear cells [25], expression of the type 2 variant has not been conclusively demonstrated [13]. To determine whether the DDAVP action on monocytes occurs through a direct, possibly V2R-dependent mechanism, or by an indirect mechanism we performed in vitro incubations of whole blood and isolated monocytes with DDAVP.

PATIENTS AND METHODS

Study population and administration of DDAVP

Ten healthy volunteers (mean age, 27.2 years, range 20 - 41 years) received an intravenous infusion of DDAVP (Minirin®, Ferring Arzneimittel, Kiel, Germany) in a dose of 0.3 µg/kg b.w. diluted in 50 ml 0.9 % NaCl over 15 minutes. All subjects were in good health, as documented by history, physical examination, and hematologic and biochemical screening. Blood samples for hematologic and coagulation parameters, mRNA isolation, and monocyte isolation were taken from the antecubital vein some minutes before DDAVP-administration, immediately after end of DDAVP infusion and every 1 hour after initiation of DDAVP-treatment over a time-period of 6 hours. The study was approved by the institutional ethics committee for human subjects. Informed consent was obtained from all volunteers.

Hematologic parameters and monocyte isolation

Global white cell counts were measured in blood samples collected in tubes containing EDTA using a Sysmex K-4500 (Sysmex, Norderstedt, Germany). The percentage of monocytes was determined by flow cytometry using a FACScan and CellQuest software version 3.3 (Becton Dickinson (BD), Basel, Switzerland). Fifty microliters of EDTA-anticoagulated whole blood was labeled with fluorescent dye conjugated mouse monoclonal antibodies (10 µl each, BD) against human CD45 (phycoerythrin-(PE)-conjugated) and human CD14 (fluorescein-(FITC)-conjugated). As negative control, 10 µl each mouse IgG_1 (PE) and IgG_{2a} (FITC) antibodies (BD) were added to 50 µl whole blood. All samples were incubated for 20 min at room temperature in the dark. Afterwards, 500 µl FACS lysis solution (BD) were added and samples measured directly after complete lysis of red cells. At least 20,000 CD45 positive events were recorded and monocytes were gated by their specific forward and side-scatter pattern. Furthermore, the monocyte population was identified by high CD14 expression.

Peripheral blood mononuclear cells (PBMCs) were isolated from 8 ml of whole blood using cell preparation tubes (CPT, BD).

Determination of FVIII-activity and vWF antigen

All measurements of coagulation parameters were performed in citrated platelet-poor plasma. FVIII activity was determined by an one-stage clotting assay using FVIII-deficient plasma (Dade-Behring, Marburg, Germany) prepared by immunoadsorption. The assay was performed on an automated clotting analyzer (Amax, Pharmacia, Germany). Plasma levels of vWF-antigen were determined using a sandwich ELISA (Vidas vWF, bioMérieux, Marcy-l´Etoile, France).

TF mRNA analysis

To minimize modulation of the mRNA profile ex vivo, isolated PBMCs (adjusted to aliquots of 2 x 10^6 cells) were lysed using 600 µl cell lysis buffer (buffer RLT, RNeasy Mini Kit, Qiagen) containing 1% (v/v) ß-mercaptoethanol. The cell lysates were stored at -80°C until isolation of RNA. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer´s instructions. Isolated RNA was eluted in 50 µl of RNase free water.

TF- and GAPDH-mRNA were quantified using a multiplex real-time reverse transcription (RT) PCR protocol as previously described [26]. The quantitative TF-RT-PCR performed in this way exhibited a linear range extending from 10^2 - 10^7 TF mRNA copies/reaction and reproducibility with coefficients of variation < 20%. In vitro transcripted TF RNA and GAPDH RNA were used as standard material. Results were normalized to the number of monocytes.

Factor Xa generation assay

The TF-dependent procoagulant activity of monocytes was measured using a factor Xa generation assay. Isolated PBMCs were resuspended in cell buffer (21 nM HEPES, 137 mM NaCl, 5 mM KCl, 0.75 mM Na_2HPO_4, 5.5 mM glucose, 2 mM $CaCl_2$, pH 7.4) to reach a final concentration of 2.5 x 10^6 monocytes/ml and 200 µl of this cell solution were added to a 96-well microtiter plate (Polysorb, Nunc, Roskilde, Denmark). Cells were overlaid with 100 µl $CaCl_2$ (25 mM), and 100 µl of defibrinated normal human plasma. Defibrinated plasma was used as a source for FVII/FVIIa and factor X and was prepared by addition of 1 ml reptilase (0.7 U; Pentapharm, Switzerland) to 5 ml of plasma. After incubation for 5 min fibrin was removed by centrifugation for 5 min at 10,000 x g. The fibrinogen free supernatant was aliquoted at 500 µl and stored at -80 °C until used. After incubation of the reaction mixture for 10 minutes at 37°C, cells were depleted by centrifugation (5 min, 150 x g) and 80 µl of cell free supernatant were added to a well of a 96-well microtiter plate containing 80 µl Tris/EDTA (20 mM/4 mM) and 40 µl of the chromogenic substrate S2765 (2 mM; Chromogenix, Mölndal, Sweden). FXa generation was monitored by measuring the change of absorbance at 405 nm. A calibration curve was constructed using recombinant TF (Innovin, Dade-Behring, Marburg, Germany). This TF reagent was assigned a value of 1,000 arbitrary units (U) of TF-dependent procoagulant activity. The assay performed in this way was sensitive up to 0.005 U/10^6 cells. The interassay variability ranged from 6.9 % to 11.3 % for TF activities between 1 and 0.005 U/10^6 cells, respectively.

FACScan analysis of monocytic TF antigen expression

Either 50 µl of whole blood or a cell suspension at a concentration of 10^7 cells/ml (PBMCs) were incubated with 10 µl FITC-labelled mouse monoclonal anti human TF

antibody (American Diagnostica Inc, Greenwich, CT) and 10 µl PE labelled anti-CD14 antibody for 60 minutes at 4°C. As negative control, 10 µl each mouse IgG_1 (FITC) and IgG_{2a} (PE) antibodies (BD) were used. After incubation, cells were washed with ice-cold PBS containing 0.1 % BSA (wt/vol). After washing, cells were resuspended in PBS containing 1 % BSA and analyzed. Monocytes were gated by their specific forward and side-scatter pattern. Furthermore, the monocyte population was identified by high CD14 expression. A total of 20,000 events was recorded for each file. Specific TF antibody binding was expressed as mean fluorescence intensity (MFI) of the monocyte population.

In vitro stimulation experiments

Anti-coagulated whole blood from 5 healthy volunteers was used to perform DDAVP stimulation experiments with monocytes. Individual blood samples were sub sampled into 1 ml aliquots and DDAVP was added to achieve final concentrations from 10 to 100 ng/ml. A non-stimulated sample series was processed in parallel as a negative control. After the incubation levels of TF-mRNA and cell surface expressed TF antigen were measured as outlined before.

To determine the stimulation potential of monocytes, whole blood was drawn from study participants at least 24 hours before administration of DDAVP. PBMCs were isolated, adjusted to 2 x 10^6 cells in 1 ml RPMI and incubated for 3 h in the absence or presence of 1 µg/ml LPS. All incubation steps were performed at 37°C in 15 ml polypropylen tubes. After the incubation expression levels of TF-mRNA were measured by quantitative TaqMan-PCR. Results were normalized to the number of monocytes present in the sample preparation.

Statistical analysis

Data were analyzed using SPSS 9.1 for Windows software. The student's t-test was used to determine statistical significance between various data sets. Statistical significance was judged at confidence levels greater than 95% ($p<0.05$). All results were expressed as mean ± standard deviation.

RESULTS

Hematologic and coagulation parameters in DDAVP-treated subjects

Administration of DDAVP was associated with a rise in FVIII activity and vWF plasma levels. It also induced a change in leukocyte counts, involving monocytopenia and neutrophilia (Fig. 1). Mean values of leukocyte counts increased from 6.75 x 10^3/µl before DDAVP to a maximum of 9.2 x 10^3/µl reached at 3 h after DDAVP ($p < 0.001$). Monocyte counts (mean value before DDAVP: 0.6 x 10^3/µl) decreased immediately after DDAVP administration reaching a nadir (mean value: 0.2 x 10^3/µl) at 1 h after DDAVP ($p = 0.027$). Two to 3 hours after DDAVP administration monocyte counts returned to baseline levels. Platelet counts were not influenced by DDAVP administration.

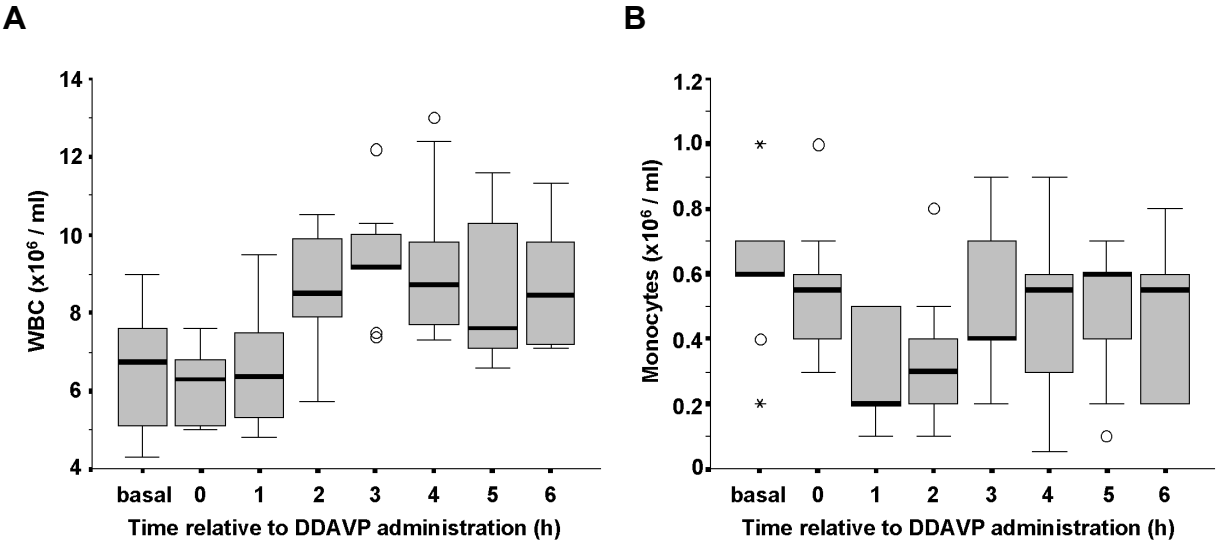

Figure 1. Time course of leukocyte and monocyte counts after DDAVP administration. (A) DDAVP administration induced a significant increase ($p < 0.001$) in leukocyte counts starting 2 hours after DDAVP and reaching a maximum 3 h after DDAVP. (B) Immediately after DDAVP infusion the number of monocytes decreased reaching a minimum 1 h after DDAVP ($p = 0.027$). Boxes represent the quartile range, with the median represented by the line. Whiskers at the top and the bottom of the box show the highest and lowest values, excluding outliers (o) and extreme outliers (*).

In vivo kinetics of tissue factor expression after DDAVP administration

Starting from a baseline value of 0.0071 $\pm$ 0.00354 (mean $\pm$ SD) TF copies/monocyte TF mRNA levels rapidly increased reaching a peak of 0.0366 $\pm$ 0.00363 (mean $\pm$ SD) TF copies/monocyte at 1 h after DDAVP administration. The results shown in figure 2A represent the increase over time after DDAVP administration relative to baseline levels.

With a p-value of 0.001 the increase to 377 % was statistically significant. At three hours after DDAVP administration TF mRNA levels were returned to baseline values. Among the study population, only 1 volunteer showed no detectable increase in TF mRNA levels.

Basal levels of monocytic TF activity measured through the rate of FXa-formation were below the detection limit of the assay of 5 mU/10^6 cells. DDAVP administration induced a biphasic but statistically not significant response with a moderate increase to 8.9 $\pm$ 1.3 (mean $\pm$ SD) mU/10^6 cells at 2 hours after DDAVP and a second increase between 4 and 5 hours after DDAVP (16.4 + 0.4 mU/10^6 cells, p = 0.08) (Fig. 2B). Statistical significance was not reached, because of the high interindividual variability in TF activity levels.

However, TF-antigen levels on monocytes measured by FACS analysis showed no significant increase after administration of DDAVP. Binding patterns of the corresponding control antibody were similar to that of the TF-specific antibody excluding any changes of MFI (FITC) caused by changes of TF antigen expression on the monocytic surface.

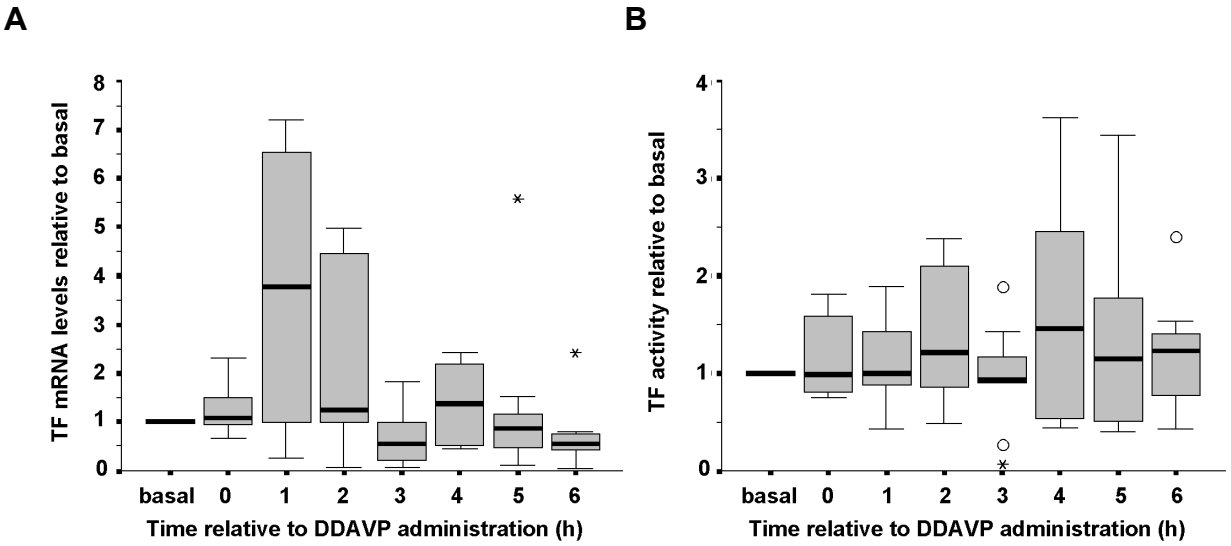

Figure 2. Changes in TF expression levels over time after DDAVP administration. (A) Blood samples were taken at the indicated time points and TF mRNA levels were quantified using the real time TaqMan PCR approach. Results were normalized to the number of monocytes. (B) Surface expressed TF activity was analyzed by a FXa-generation assay. For details of box plots see figure 1.

In-vivo DDAVP-induced TF expression versus ex-vivo LPS-induced TF expression

The effect of LPS (1 µg/ml) upon the rate of TF transcription was examined in PMBCs obtained before administration of DDAVP. LPS treatment induced an increase that ranged from 178-fold to 410-fold (mean: 311-fold) after 3h of incubation. Figure 3 shows a linear plot of TF mRNA levels of in vitro stimulated monocytes against the maximal rate of TF mRNA stimulation measured after in vivo DDAVP stimulation. Although LPS-stimulation induced a 50 to 150 fold higher increase in TF expression levels than the DDAVP stimulated response, the relative increase was identical. Low responders to LPS reacted also mild to DDAVP and vice versa in high responders.

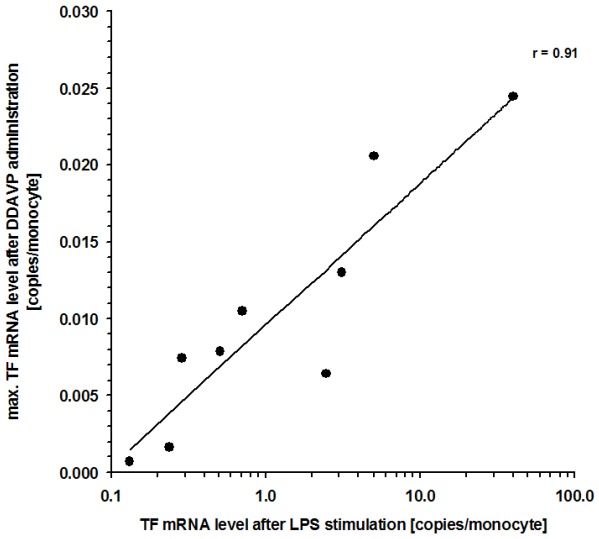

Figure 3. Monocytic TF expression induced by in-vitro lipopolysaccharide stimulation compared with ex-vivo results after DDAVP administration. PBMCs were isolated from study patients 2 - 3 days before DDAVP administration and incubated with 1 µg/ml lipopolysaccharide for 3 hours at 37°C. Following incubation TF mRNA levels were quantified (X-axis) and compared with the TF stimulation rate at 1 hour after DDAVP administration (Y-axis) from the same study patient.

TF mRNA and TF antigen analysis in whole blood cells and monocytes after in vitro DDAVP stimulation

To assess direct effects of DDAVP on monocytes, whole blood or isolated PBMCs obtained from 5 volunteers were incubated with DDAVP (10 - 100 ng/ml, 8.45 - 84.5 nM) at 37°C and the time-dependent expression of monocytic TF measured. There was no time- or concentration-dependent increase in TF mRNA levels whereas the response to LPS was regular. In addition, TF antigen was not modulated independent of the presence or absence of DDAVP (data not shown).

To study whether monocytes are activated by an activator that is released into plasma in response to DDAVP, we incubated purified PBMCs with plasma that was obtained from individuals immediately and at 1 hour after DDAVP administration (DDAVP conditioned

plasma). PBMCs were isolated from the same individuals after a washout period of 7 days, mixed with DDAVP conditioned plasma to reach a final concentration of 2.5 x 10^6 cells/ml, and incubated at 37°C. Normal human plasma was used as negative and LPS containing plasma (100 ng/ml) was used as positive control. There was no difference in TF gene activities (shown in figure 4), monocytic TF activity and TF-antigen levels between DDAVP conditioned plasma and normal control plasma.

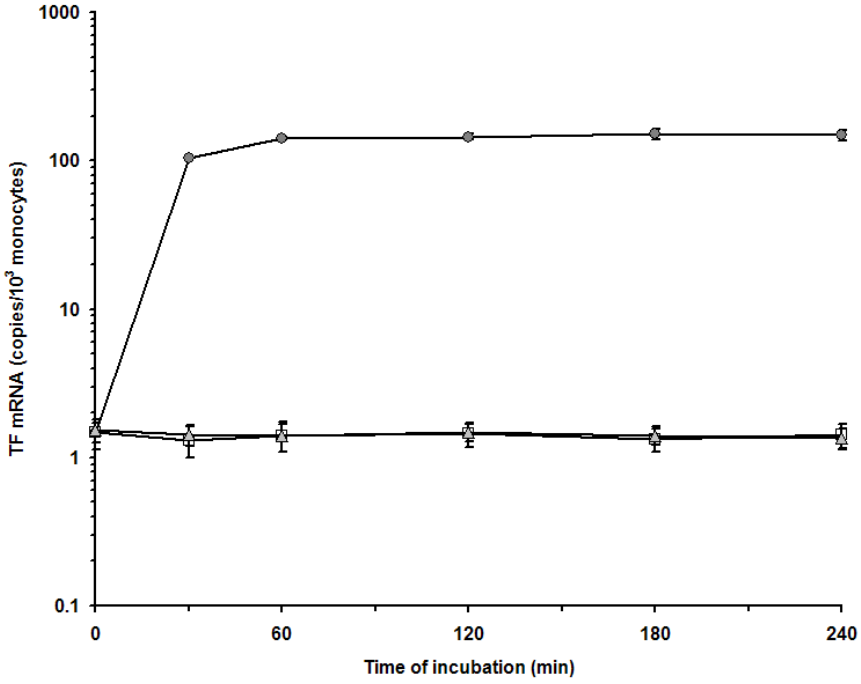

Figure 4. In vitro stimulation of isolated monocytes with plasma obtained after DDAVP administration. Plasma was collected 1 hours after DDAVP treatment and incubated with PBMCs at a final concentration of 2.5 x 10^6 cells. At the indicated time points TF mRNA levels were analyzed. Normal human plasma and plasma supplemented with LPS (100 ng/ml) were used as negative (▲) and positive (●) controls. Results are expressed as mean ± SD.

Discussion

In the present study, we showed that administration of DDAVP induced a TF-dependent procoagulant activity on monocytes, the effect activates coagulation and involves the interaction between DDAVP-activated endothelial cells and monocytes.

The effect of DDAVP on FVIII and vWF levels only partially explains the hemostatic action of the drug [13]. Searching for additional cellular effects of DDAVP we focussed our interest on monocytes. Activation of monocytes in response to DDAVP has been shown by in vitro studies. Incubation of whole blood or purified monocytes with DDAVP induced the release of platelet activating factor and enhanced the binding of activated platelets to monocytes in a P-selectin/PSGL-1-dependent manner [15,16,18]. At present, however, there is only one in vivo study available that reports enhanced levels of intracellular platelet activating factor within leukocytes after administration of DDAVP to patients with hemophilia A or von Willebrand disease [17]. Thus, the contribution of activated monocytes to the hemostatic action of DDAVP remains unclear.

Typically, activation of monocytes induces TF expression. This is documented for a variety of activators such as endotoxin, immune complexes, certain cytokines, and activated platelets [23,27]. Monocytic TF belongs to the so called intravascular or blood-borne TF moiety and is able to activate coagulation [28]. Assuming that monocytes are activated in response to DDAVP one should expect induction of TF expression. To test this hypothesis, healthy volunteers were treated with DDAVP and monocytic TF expression levels measured.

All of the ten individuals responded regularly to DDAVP as indicated by a rapid rise in plasma levels of vWF and FVIII. Leukocyte counts showed a biphasic change involving monocytopenia followed by neutrophilia. This pattern is nearly identical to the changes in leukocyte counts observed in the human endotoxemia model where the decrease in monocytes was associated with a clear pattern of TF mRNA expression [21]. Identical results were obtained in our DDAVP treated study group. TF mRNA expression was induced after DDAVP, showed peak levels between 1 and 2 hours after DDAVP followed by a progressive decline and immediate normalization thereafter.

Studying TF activity on monocytes through FXa formation and TF antigen expression by FACS analysis we found only a moderate and statistically not significant increase in TF. This again is in agreement with the findings in human endotoxemia and

is most likely caused by adhesion of activated monocytes to endothelium. It has been shown that expression of functional active TF promotes adhesion of monocytes to the endothelial surface [29]. In this case, preferentially, monocytes with poor TF antigen expression will remain in the blood circulation. Such an explanation would also imply that the induction of mRNA expression of TF in the retained monocytes might be even higher than shown in our data and that we are detecting monocytes with a relatively poor response to DDAVP.

Our results indicate inter-individual differences in monocytic TF expression upon DDAVP stimulation. A high degree of inter-individual variation has also been reported for endotoxin stimulation [30]. To study whether both variations correlate we compared the in-vivo response to DDAVP with the ex-vivo response to LPS. There was a strong positive correlation between both agonists suggesting strongly related intracellular activation pathways.

Activation of endothelial cells through DDAVP is mediated through interaction with the vasopressin receptor type 2. At present, however, there are conflicting data whether monocytes express functional active V2R. While Block et al. give indirect evidence for the presence of V2R through binding of labelled DDAVP to monocytes [31], others were not able to detect this receptor subtype in monocytes by RNA expression studies [25]. In our in vitro studies there was no TF expression detectable after stimulation of monocytes with DDAVP. In addition, plasma obtained from DDAVP-treated patients was not able to induce TF expression in monocytes. These data make it most likely, that monocyte activation occurs through a mechanism that requires interaction of monocytes with DDAVP-activated endothelial cells rather than through a direct V2R-dependent mechanism. We propose a cellular model that involves activation of monocytes on the surface of DDAVP-activated endothelial cells (Fig. 5). DDAVP induced exocytosis of Weibel-Palade-bodies translocates P-selectin in the endothelial cell plasma membrane. P-selectin mediates leukocyte rolling and upon interaction with its counter receptor PSGL-1 on monocytes upregulates the de novo synthesis of TF in monocytes as previously described by Celi and others [20]. Thus, DDAVP activated endothelial induce the TF pathway on the surface of monocytes as an additional hemostatic mechanism.

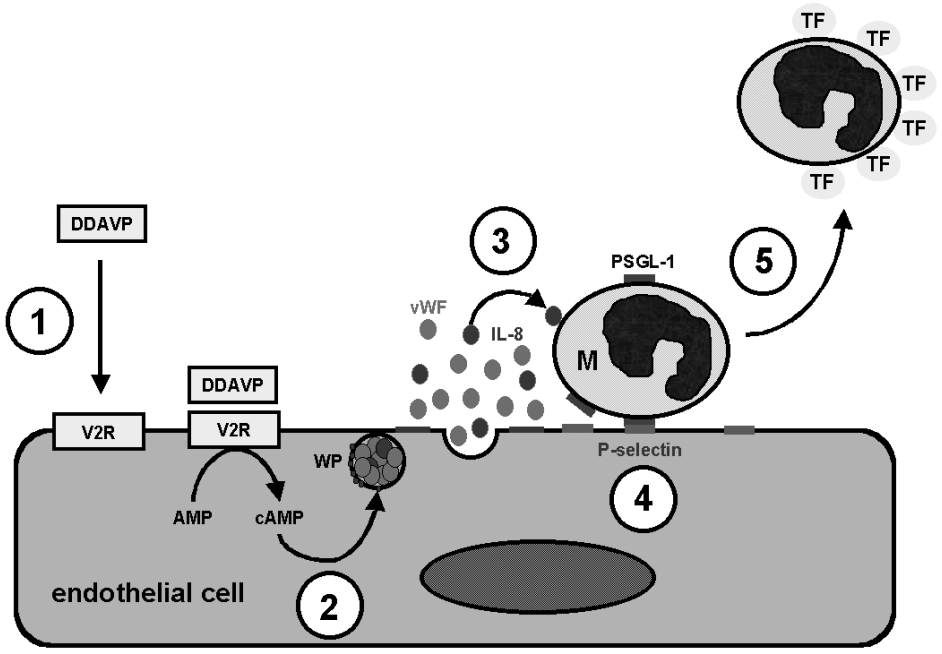

Figure 5. A proposed model of monocytic TF induction by DDAVP. Binding of DDAVP (1) to the vasopressin 2 receptor (V2R) activates endothelial cells in a cAMP-dependent manner (2) resulting in exocytosis of Weibel-Palade-bodies (WPB). Von Willebrand factor (vWF) and other components of WPBs such as IL-8 are secreted (3) while P-selectin, a membrane component of WPBs, is inserted in the plasma membrane (4) of the stimulated endothelial cell. Through P-selectin/P-selectin ligand (PSGL-1) interaction monocytes (M) adhere to the endothelial cell surface and become activated to express tissue factor (TF) (5). Tissue factor expression is further augmented by secreted IL-8 (3).

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Chapter 5

Gene Expression Analysis in Platelets from a Single Donor: Evaluation of a PCR-Based Amplification Technique*

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ABSTRACT

BACKGROUND: Genetic analysis of platelet mRNA may facilitate the diagnosis of disorders affecting the megakaryocytic-platelet lineage. Its use, however, is limited by the exceptionally small yield of platelet mRNA and the risk of leukocyte contamination during platelet preparation. **METHODS:** We depleted platelet suspensions of leukocytes by filtration and used a PCR-based RNA amplification step [switching mechanism at the 5' end of RNA templates (SMART)]. We tested the reliability and precision of the RNA amplification procedure by use of real-time PCR to measure quantities of specific transcripts: von Willebrand factor (vWF), A-subunit of coagulation factor XIII (F13A), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Microarray analysis was performed on platelet RNA with and without amplification**. RESULTS:** Microgram quantities of platelet-specific cDNAs were produced from as little as 50 ng of total platelet RNA or 40 mL of whole blood. At cycle numbers <16, amplification of all transcripts tested was exponential with slightly more efficient amplification of low-abundance transcripts. Expression profiling of 9850 genes gave identical results for 9815 genes (1576 positive/8239 negative). Eight transcripts failed to be amplified by the SMART procedure. Expression of vWF, F13A, and GAPDH transcripts showed only minor day-to-day variations in three healthy individuals. **CONCLUSION:** The proposed protocol makes extremely small amounts of platelet RNA available for gene expression analysis in single patients.

INTRODUCTION

Platelets are anucleated but contain small amounts of mRNA. This mRNA is of megakaryocytic origin and is translationally active [1]. Recently, the platelet transcriptome was profiled by microarray analysis [2,3]. At the same time, the platelet proteome was defined by highresolution 2-dimensional polyacrylamide gel electrophoresis [4]. Combining of these data has enabled the molecular anatomy of human platelets to be resolved as a tool for better understanding of normal and pathologic platelet function [5,6].

Analysis of the platelet transcriptome in patients, however, is limited by the exceptionally small yield of platelet mRNA. Thus, more than $1x10^{12}$ platelets, representing more than 5000 mL of whole blood, are usually required to obtain 1-4 µg of poly(A)$^+$ RNA [7]. Platelet apheresis offers the only chance of obtaining such a high number of platelets from a single donor, but this approach cannot be performed in patients with platelet disorders [8].

Techniques that amplify the starting mRNA may overcome these limitations [9-11]. At present, two amplification strategies are typically used for RNA amplification; both include reverse transcription, which is followed either by exponential PCR amplification [12-15] or by T7-based linear in vitro transcription [16-19]. To minimize the amount of whole blood needed for platelet gene expression analysis in a single patient, we decided to use an exponential PCR amplification method, the switching mechanism at the 5' end of RNA templates (SMART) technique [12]. The main concern about amplification procedures, especially with PCR-based methods, is that they provide genetic information with high yield but an altered gene expression profile [10,17,20,21].

We carefully evaluated this aspect by comparative microarray analysis and by quantitative PCR. In the microarray experiments, original and amplified platelet RNA was profiled across 9850 genes. To determine the bias between transcript ratios, selected transcripts were quantified by real-time PCR during the amplification process. von Willebrand factor (vWF) was selected for two reasons: (a), with a length of 8.3 kb, vWF mRNA is one of the longest human messages; and (b), it is expressed exclusively in platelets and endothelial cells. We selected RNA coding for subunit A of coagulation factor XIII (F13A) as a second platelet typical RNA. This 3.8-kb spanning RNA represents the group of medium-sized RNAs and is highly expressed in platelets [2,3,5

6]. Recently, expression of fibrinogen α-chain (FGA; 2.2 kb) has been found in platelets by array analysis. It has been ranked in position 632 of the most abundant platelet messages, lying between F13A (rank 199) and vWF (rank 2465) [5]. On the basis of these data, we decided to use FGA as an additional candidate RNA representing platelet RNAs that are expressed in low to moderate concentrations. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen because this 1.3-kb gene is highly expressed in nearly all cells, including platelets.

Because the concentration of mRNA in platelets is much lower than in leukocytes, a small number of leukocytes can distort platelet gene expression profiles [22]. To address this problem, we included a leukocyte depletion step in the platelet preparation protocol and monitored its efficiency by analyzing leukocyte-specific gene products. Finally, we tested the reproducibility and sensitivity of our approach and the day-to-day variations by measuring the amounts of vWF, F13A, and GAPDH RNA over time in three healthy individuals.

MATERIALS AND METHODS

Isolation and preparation of platelets

For large-scale RNA preparation, platelets were collected from healthy blood donors by platelet apheresis on an Amicus Crescendo cell separator (Baxter). Each leukocytereduced apheresate contained >$2x10^{11}$ platelets in 200–300 mL of autologous plasma. Contaminating leukocytes (>$1x10^6$ per unit of apheresate) were completely removed by filtration (PXL2; Pall Biomedizin) as judged by flow cytometric analysis (LeucoCountTM; Becton Dickinson). The platelets were washed with Tyrode's buffer and collected by centrifugation at 1000*g* for 15 min, after which the platelet pellets were frozen in liquid nitrogen and stored at -80 °C. To obtain RNA from a single donor blood, we collected 20–80 mL of citrate-anticoagulated blood from healthy volunteers. Platelets were separated from erythrocytes by centrifugation at 150*g* for 20 min at room temperature. The upper two thirds of the plateletrich plasma (PRP) was resuspended in twice a volume of Tyrode's buffer, filtered through a Purecell PL leukocyte removal filter (Pall Biomedizin), and processed as described above.

RNA extraction

For microarray analysis, total RNA was isolated from six single donor platelet apheresis concentrates after leukocyte depletion with the use of TRIzol reagent (Invitrogen) as described previously [3]. From each platelet concentrate with total platelet numbers in the range of 2.4–2.8x10^{11} (mean, 2.7x10^{11}) we could obtain 18.5–28.2 µg (mean, 22.5 µg) of total RNA. When we worked with whole blood, we directly extracted mRNA from isolated platelets with oligo(dT)-coupled paramagnetic beads [Dynabeads Oligo(dT)25; Dynal] according to the procedure described by Jakobsen et al. [23]. Bead-coupled platelet mRNA was eluted in 10 µL of RNase-free water.

cDNA generation and amplification

Platelet RNA was transcribed to cDNA and subsequently amplified by SMART technology (BD Biosciences Clontech) according to the manufacturer's instructions. Samples were amplified in a PTC-200 thermal cycler (MJ Research, Biozym Diagnostika, Hess) by use of the following program: 95 °C for 1 min, then 2–18 cycles at 95 °C for 15 s, 58 °C for 30 s, and 68 °C for 6 min. After every two cycles, 5 µL of the reaction mixture was transferred to a fresh tube and kept at 4 °C; the remaining mixture was subjected to additional cycles.

Quantitative real-time PCR

One-step quantitative real-time reverse transcription-PCR (RT-PCR) was used for measuring the concentrations of RNA transcripts coding for vWF, FGA, F13A, and GAPDH. Oligonucleotide primers and probes for quantification of vWF and FGA transcripts were designed by use of Primer Express software, Ver. 1.5 (Perkin-Elmer). Primer and probe sequences for amplification of GAPDH mRNA were taken from the TaqMan Gold RT-PCR Kit protocol (Perkin-Elmer). Oligonucleotides for quantification of vWF, FGA, and GAPDH transcripts were purchased from Eurogentec. Detailed sequence information is provided in Table 1. For quantification of F13A transcripts, we used a predeveloped primer/probe set from Applied Biosystems (Assay-on-DemandTM product, containing a 5'-6-carboxyfluorescein-labeled MGB® probe with a black hole quencher at the 3' end). Corresponding sequence information was not available. All

sequences were chosen to prevent amplification of genomic DNA by overlapping or spanning exon/intron boundaries.

Table 1. Oligonucleotide primers and probes used for quantitative PCR.

Target mRNA	Oligonucleotide	Sequence[a]	Position[b]
FGA	Forward primer	5'-AAGTACAGCATATCCAGCTTCTGC-3'	473 - 496
	Reverse primer	5'-CCTCGACAAGATCGGATCTTAA-3'	575 – 554
	Probe	5'-TCAATGTCCACCTCCAGTCGTTTCA-3'	551 - 527
vWF	Forward primer	5'-TCTGTGGATTCAGTGGATGCA-3'	5720-5740
	Reverse primer	5'-CGTAGCGATCTCCAATTCCAA-3'	5804-5784
	Probe	5'-CGCCAGGTCCAACAGAGTGACAGTGT-3'	5752-5777
GAPDH	Forward primer	5'-GAAGGTGAAGGTCGGAGTC-3'	81 – 99
	Reverse primer	5'-GAAGATGGTGATGGGATTTC-3'	306 – 287
	Probe	5'-CAAGCTTCCCGTTCTCAGCC-3'	277 - 258

[a] FGA- and vWF-probes were labeled with the fluorescent reporter dye 6-carboxyfluorescein (FAM) at the 5'end and the quencher 6-carboxytetramethylrhodamine (TAMRA) at the 3'end. To enable multiplex reactions, a different reporter dye (JOE) was labelled at the 5'end of the GAPDH-probe, but TAMRA was still used as quencher at the 3'end.
[b] Nucleotide positions based on the GenBank accesssion numbers J00127 (FGA), NM_000552 (vWF), and NM_002046 (GAPDH).

Quantitative RT-PCR reactions were performed with the QuantiTect Probe RT PCR Kit (Qiagen). Reactions were performed in a final volume of 20 µL containing 1x master mixture (including PCR buffer, deoxynucleotide triphosphates, 4 mM MgCl2, and ROX reference dye), FGA or vWF forward and reverse primers (150 nM each), GAPDH forward and reverse primers (100 nM each), FGA or vWF probe (200 nM), GAPDH probe (100 nM), 0.2 µL of QT Probe RT Mix, and 1 µL of each dilution of calibrators and

samples. For quantification of F13A transcripts, 1 µL of the primer/probe mixture was added to each reaction. Samples were amplified in a 96-well spectrofluorometric thermal cycler (ABI Prism SDS 7700; Applied Biosystems) using the following program: 50 °C for 30 min, 95 °C for 15 min, 40 cycles at 94 °C for 15 s, and 60 °C for 1 min.

To measure amplified cDNA, we performed real-time PCR using probes and primers identical to those used for the quantitative RT-PCR. Multiplex reactions were performed in a final volume of 25 µL containing 1x PCR buffer [20 mM Tris-HCl (pH 8.5), 50 mM KCl], 4 mM MgCl2, 200 mM each deoxynucleotide triphosphate, 0.5 µL of ROX reference dye, 1.25 U of PlatinumTaq DNA polymerase, and 1 µL of each calibrator or sample preparation. Oligonucleotide concentrations were the same as those described for RT-PCR reactions. PlatinumTaq DNA polymerase and ROX reference dye were purchased from Invitrogen. Thermal cycling using the Prism SDS 7700 was performed with the following profile: 95 °C for 5 min followed by 40 cycles consisting of 20 s of denaturation at 95 °C and 60 s of annealing and extension at 60 °C.

All calibrators and samples were run in duplicate.

RNA synthesis and calibrator preparation

RNA calibrator for absolute quantification of vWF and GAPDH transcripts were prepared by in vitro transcription. Starting points were PCR-amplified cDNAs coding for vWF and GAPDH that were cloned in the *Srf*I site of pPCR-Script Amp SK (+) (Stratagene). These constructs were used to generate DNA templates containing a T7 RNA polymerase promoter sequence at their 5' end with the following sequences of vWF and GAPDH downstream: for vWF, bp 5633–5919 (GenBank accession no. NM_000552); and for GAPDH, bp 8–525 (GenBank accession no. M33197). In vitro transcription was performed with these modified DNA templates using T7 RNA polymerase (Invitrogen) according to the manufacturer's instructions. After digestion of DNA templates with DNase I (Roche), RNAs were purified by use of the RNeasy Mini Kit (Qiagen). RNA was quantified by photometric measurement (*A*260 reading of 1 = 44 mg/L). All RNA stock solutions were stored at -70 °C. Total placental RNA (BD Biosciences Clontech) was used for relative quantification of F13A transcripts. One unit was defined as the number of F13A transcripts present in 10 pg of the placental RNA.

Calibrators for quantification of SMART-generated vWF and GAPDH cDNA products were prepared from cDNA plasmids containing the sequences described above. The plasmids were isolated from transformed XL10-Gold (Stratagene) cultures and quantified by photometric measurement (*A*260 reading of 1 = 50 mg/L). The DNA concentration is expressed in molecules/mL. All DNA stock solutions were stored at -70 °C. For relative quantification of SMART-generated F13A sequences, total placental RNA was transcribed to cDNA by use of Super- Script RNase H^- reverse transcriptase (Invitrogen).

Comparative microarray analysis

To compare gene expression profiles from platelet RNA with and without amplification, we used total RNA isolated from single donor platelet apheresis concentrates in six individual experiments. Unamplified samples were fluorescently labeled by use of 10 µg of platelet RNA, Cy3- or Cy5-dCTP (Amersham Biosciences), and the LabelStar reagents (Qiagen). Amplified samples were generated each from 0.5 µg of total platelet RNA by use of the SMART fluorescent probe amplification reagents (BD Biosciences Clontech) according to the manufacturer's protocol. The SMART PCR products were further processed by random primed labeling with aminoallyl-dUTP. Aminoallyl-labeled DNA was then labeled with Cy3 or Cy5 by use of monoreactive dyes (Amersham Biosciences). The differential labeling of the six RNA samples was performed as follows: RNA samples 1, 2, and 3 were labeled with Cy3 without amplification and labeled with Cy5 after SMART amplification; RNA samples 4, 5, and 6 were labeled with Cy5 without amplification and with Cy3 after SMART amplification.

Before the hybridization on microarray glass slides representing 9850 human genes (MWG-Biotech AG), the Cy3- and Cy5-labeled samples were combined as follows: slide 1, unamplified sample 1 (Cy3) + unamplified sample 4 (Cy5); slide 2, unamplified sample 2 (Cy3) + unamplified sample 5 (Cy5); slide 3, unamplified sample 3 (Cy3) + unamplified sample 6 (Cy5); slide 4, amplified sample 1 (Cy5) + amplified sample 4 (Cy3); slide 5, amplified sample 2 (Cy5) + amplified sample 5 (Cy3); slide 6, amplified sample 3 (Cy5) + amplified sample 6 (Cy3). Arrays were hybridized and washed according to the manufacturer's protocol and scanned by a laser scanner (GMS 417' MWG-Biotech). Computer-assisted data evaluation was performed with use of the

ArrayVision software (Imaging Research, Inc.) as described previously [3]. In brief, negative hybridization signals revealed intensity values <3000, the gray area range was 3000–5000, and positive hybridization signals showed intensity values >5000. Mean values were calculated from the six unamplified and the six amplified samples for each gene spot on the microarray. The mean values from the unamplified and the amplified samples were compared in a scatter plot.

RESULTS

Platelet preparation and precision of RNA isolation

Platelets were prepared from 40 mL of citrate-anticoagulated blood from 16 healthy volunteers. Platelet count ranged from 105 000 to 259 000/μL. Leukocyte counts ranged from 3600 to 6100/μL. After preparation of PRP and leukofiltration, no leukocytes were detectable by flow cytometric analysis. Mean (SD) platelet loss attributable to preparation of PRP and leukofiltration was 72 (3)%. To determine the precision of mRNA isolation with oligo(dT)-coupled magnetic beads, we aliquoted platelets obtained from one donor by platelet apheresis after filtration ($1x10^{10}$ platelets each), and mRNA was extracted in triplicate on 3 different days. vWF and GAPDH mRNA was analyzed by quantitative RT-PCR. The mean (SD) the threshold cycle (Ct) values were 30.1 (0.65) and 19.1 (0.27) for vWF and GAPDH mRNA, respectively; the CVs indicate the low intraassay variation. For the run-to run-variation, the mean (SD) Ct values were 30.21 (0.65) and 19.52 (0.45) for vWF-and GAPDH mRNA, respectively.

Efficiency of amplification

One of the most critical steps in PCR-based RNA amplification is the maintenance of the original message profile. To obtain optimum results, PCR should be in the exponential phase of the reaction, and irrespective of length and abundance, all mRNAs should be amplified with equal efficiency. To establish optimum PCR conditions, purified total platelet RNA at concentrations of 50 ng, 100 ng, and 1 μg was subjected to SMART-PCR amplification. During the process of PCR amplification, aliquots were repeatedly taken from each sample, and the concentrations of vWF, F13A, and GAPDH cDNA were measured. Copy numbers exponentially increased from cycles 10 to 18 for all RNA species tested when 50 or 100 ng of initial RNA was used (Fig. 1). At a starting RNA

concentration of 1 µg, the reactions became nonexponential after 16 amplification cycles for F13A and GAPDH, whereas vWF amplification remained in the exponential phase. Interestingly, we failed to detect message for FGA in our samples. We confirmed this result by testing 10 additional unamplified and SMART-amplified platelet RNA samples from 10 healthy individuals who tested positive for vWF transcripts.

The amplification efficiencies were calculated for each initial RNA concentration according to the formula: $E = e^s - 1$, where E is the efficiency of the PCR and s is the slope of the corresponding interpolation curve (Fig. 1). Efficiencies for vWF RNA amplification were 0.81, 0.84, and 0.80 when we used 50 ng, 100 ng, and 1 µg of total RNA, respectively, as the initial starting concentration. In contrast to the nearly identical amplification efficiencies observed for vWF, the amplification efficiencies of F13A (0.66, 0.65, and 0.36) and GAPDH (0.72, 0.76, and 0.65) transcripts decreased with increasing concentrations of starting RNA (50 ng, 100 ng, and 1 µg). Overall, the amplification efficiency was higher for vWF amplification than for GAPDH or F13A amplification.

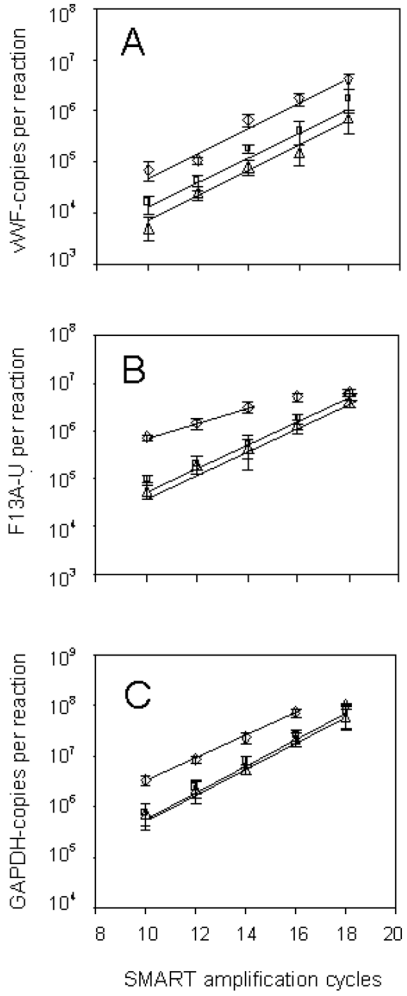

Figure 1. Gene-specific monitoring of SMART amplification by quantitative real-time PCR. Total platelet RNA at concentrations of 0.05 µg (▲), 0.1 µg (■), and 1 µg (◆) was amplified by the SMART method. At the indicated amplification cycle numbers (x axis), vWF (A), F13A (B), and GAPDH cDNA (C) concentrations were measured by quantitative real-time PCR. Results are shown as mean (SD; error bars) of three independent experiments.

Precision of amplification results

The assay-to-assay variation, which defines the precision of the amplification process, was calculated by analysis of cDNAs generated from identical RNA samples (0.5 µg) using 14, 18, and 22 amplification cycles three times on 3 different days. The mean Ct values of the multiplex vWF/GAPDH PCR decreased with increasing SMART amplification cycles. The corresponding SD increased with increasing SMART amplification cycles, but they did not exceed 0.73 for both transcripts tested. Considering that these values include the intraassay variability of the PCR, they are remarkably low. We determined the intraassay variability of the multiplex PCR by processing each cDNA calibration dilution six times in one experiment. The maximum SD values calculated on the basis of Ct values were 0.19 and 0.23 for vWF and GAPDH, respectively.

Gene expression profiles of unamplified and smart-amplified platelet rna

The platelet gene expression profile was determined in six microarray hybridization experiments with either unamplified or SMART-amplified platelet RNA obtained from six single donor platelet concentrates. Mean hybridization signals were calculated for each gene and compared between the unamplified samples (directly labeled with Cy3 or Cy5 by reverse transcription) and the amplified samples (SMART-amplified and labeled with Cy3 or Cy5). The scatter plot of mean signal intensity values of the unamplified vs the amplified samples revealed a strong linear relationship (R^2 = 0.915; Fig. 2). Positive hybridization signals were detected for 1576 genes (16%) in all samples. The most prominent genes corresponded to those described previously [3], such as platelet factor 4, RANTES, glycoprotein Ibβ, and others. For 8239 genes (83.6%), we found signal intensities in the gray area or negative range in all samples analyzed. The CVs were in the range of 0.001–1.6 [mean (SD), 0.34 (0.17)] for the unamplified samples and 0.001–1.4 [0.31 (0.16)] for the amplified samples. The hypothetical protein KIA0433 revealed that the highest CV values were 1.6 in the unamplified and 1.4 in the amplified samples. This was attributable to the highly positive signal intensity values in unamplified and amplified RNA sample 5, whereas the signals were negative or in the gray area range in all other RNA samples. Similar results could be seen for the ribosomal protein L29 ($CV_{unamplified}$ = 1.22; $CV_{amplified}$ = 1.39), the ubiquitin-specific protease 1 (1.26 and 1.12, respectively), the acid fibroblast growth factor-like protein GLIO703 (1.22 and 1.20,

respectively), and others. In summary, 10 genes had CV values ≥1 in both sample types. Furthermore, 28 genes had CV values ≥1 in at least one of the two sample types (25 genes only in the unamplified samples; 3 genes only in the amplified samples). In all cases, the high CV values resulted from one or two positive signals among the six analyzed RNA samples, indicating an interindividual difference in gene expression.

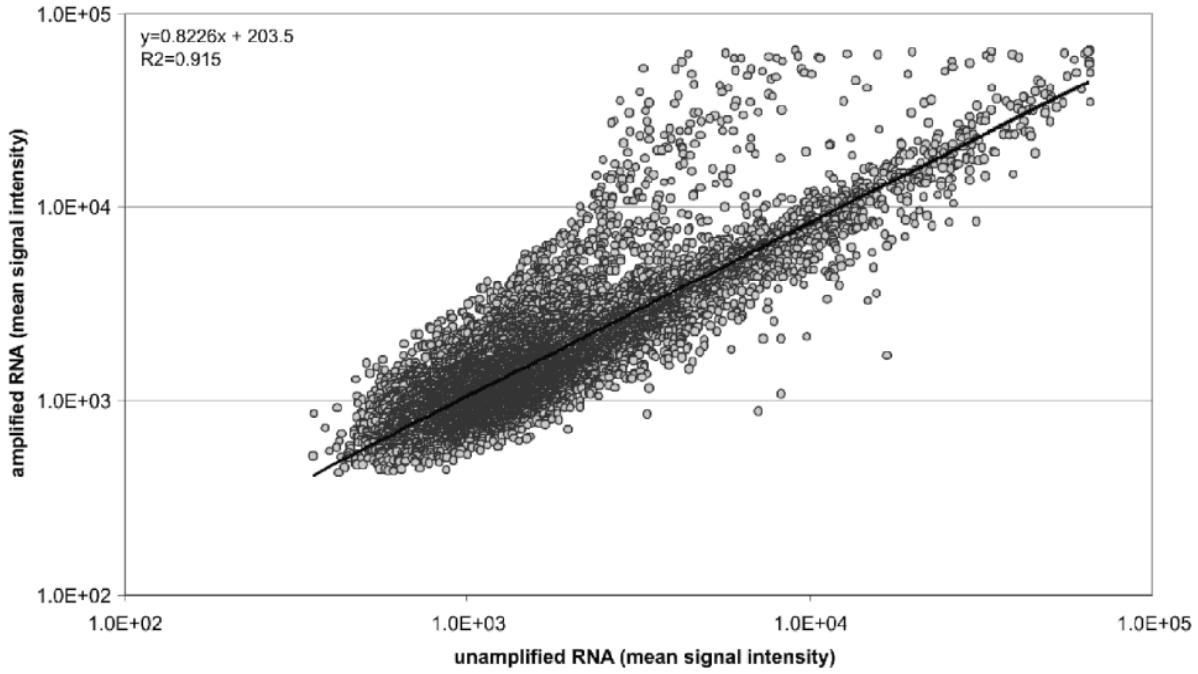

Figure 2. Scatter plot of mean signal intensity values from comparative microarray analysis. Glass slide microarrays representing 9850 human genes were used to characterize the mRNA profile in human platelets. Mean values of hybridization signals were calculated from six individual experiments for each individual gene and compared between unamplified (x axis) and SMART-amplified samples (y axis), indicated by gray circles in the scatter plot. The line indicates the linear relationship between the microarray hybridization results from unamplified and amplified samples (R2 = 0.915) calculated by the equation: y = 0.8226x + 203.5.

Only 35 of the 9850 genes (0.4%) revealed discrepant results when we compared signal intensities for the unamplified and the SMART-amplified samples. A full list of these genes is given in table 2. Among these genes we identified 27 with negative signals in the unamplified samples and unambiguously positive signals in the amplified samples, such as the matrix metalloproteinase 14, troponin C2, and others. This may reflect the higher sensitivity of the SMART amplification and may not be regarded as a “true” discrepant result. Positive signals in the unamplified samples but negative signals

in the SMART-amplified samples were found for eight genes, such as serine racemase, actin-binding LIM protein 1, and others. Microarray analysis without amplification revealed positive hybridization signals for these genes in all RNA samples, but the signals were negative when we used the amplification technique. These should be regarded as true discrepant results because the SMART technique in combination with microarray hybridization analysis failed to detect the gene transcripts.

Table2 . Genes with discrepant results in microarray analysis of unamplified and amplified platelet RNA.

Gene symbol	Gene description	Mean signal intensity ± SD* unamplified	Mean signal intensity ± SD* amplified
Negative in the unamplified but positive in the amplified samples			
NEUROD2	Neurogenic differentiation 2	2,407 ± 507	**63,947 ± 939**
TNNC2	Troponin C2, fast	2,327 ± 722	**57,109 ± 8,175**
HSPA2	Heat shock 70kd protein 2	2,783 ± 824	**35,871 ± 14,445**
CDR1	Cerebellar degeneration-related protein (34kd)	2,300 ± 257	**32,709 ± 12,089**
TNNI2	Troponin I, skeletal, fast	1,419 ± 375	**32,256 ± 14,665**
GNAO1	G protein, alpha activating activity polypeptide O	2,959 ± 1,077	**30,992 ± 16,214**
PAX2	Paired box protein 2, isoform e	2,316 ± 303	**30,821 ± 16,343**
RPL10	Ribosomal protein L10	2,453 ± 316	**30,150 ± 12,014**
TPH1	Timeless drosophila homolog	2,455 ± 434	**29,111 ± 12,980**
KIAA0938	Hypothetical protein KIAA0938	2,682 ± 631	**28,167 ± 19,529**
EMK1	ELKL motif kinase 1, isoform a	2,518 ± 399	**27,703 ± 12,678**
FOSB	Murine osteosarcoma viral oncogene FBJ, homolog b	2,631 ± 921	**27,543 ± 18,342**
PRSS7	Serine protease 7 (enterokinase)	1,545 ± 642	**26,230 ± 10,018**
MMP14	Matrix metalloproteinase 14	1,180 ± 190	**24,490 ± 7,945**
PTPRF	Protein tyrosine phosphatase, receptor type F	1,494 ± 591	**24,453 ± 3,613**
KIF5C	Kinesin family member 5c	1,272 ± 287	**24,315 ± 12,943**

Gene symbol	Gene description	Mean signal intensity ± SD* unamplified	Mean signal intensity ± SD* amplified
ABCC9	ATP-binding cassette, sub-family c, member 9	1,834 ± 231	**23,766 ± 16,136**
GSCL	Goosecoid-like	1,911 ± 294	**23,429 ± 13,377**
FLJ23469	Hypothetical protein FLJ23469	1,394 ± 284	**21,009 ± 10,010**
C21orf108	Chromosome 21 open reading frame 108;	1,932 ± 210	**20,648 ± 14,880**
NFIL3	Nuclear factor, interleukin 3 regulated	1,755 ± 244	**19,096 ± 10,226**
FLJ21213	Hypothetical protein FLJ21213	1,583 ± 156	**19,054 ± 5,660**
HMMR	Hyaluronan-mediated motility receptor, isoform a	1,332 ± 192	**17,058 ± 9,371**
MTMR3	Myotubularin related protein 3	1,356 ± 455	**15,399 ± 5,967**
ACP5	Tartrate resistant acid phosphatase 5	1,172 ± 167	**12,074 ± 4,117**
TFG	TRK-fused gene	1,072 ± 404	**11,277 ± 5,096**
FLJ23412	Hypothetical protein FLJ23412	1,723 ± 239	**11,261 ± 2,208**
Negative in amplified but positive in unamplified samples			
SRR	Serine racemase	**20,020 ± 11,132**	2,169 ± 306
PEMT	Phosphatidylethanolamine n-methyltransferase	**12,962 ± 8,004**	1,095 ± 197
ABLIM	Actin-binding LIM protein 1, isoform S	**19,958 ± 7,083**	2,130 ± 548
BM029	Uncharacterized bone marrow protein BM029	**21,987 ± 14,091**	896 ± 170
FLJ10849	Hypothetical protein FLJ10849	**12,825 ± 4,171**	2,594 ± 1,102
FLJ22347	Hypothetical protein FLJ22347	**13,784 ± 5,514**	2,114 ± 1,633
PRO0593	Predicted protein of HQ0593	**8,633 ± 3,603**	1,864 ± 733
KIAA0963	Hypothetical protein KIAA0963	**21,292 ±12,600**	1,753 ± 520

*mean signal intensity and standard deviation (SD) of unamplified (direct labeling by reverse transcription) and SMART-amplified samples: <3,000, negative, 3,000 – 4,999 gray area, ≥5,000, positive; bold numbers indicate values in the positive range and negative values are given as plain numbers.

Minimum sample size

The minimum sample size was defined as the volume of whole blood needed as starting material to produce more than 5 µg of platelet cDNA with detectable vWF transcripts by SMART PCR amplification using a maximum of 18 cycles. The minimum platelet number needed for RNA extraction and cDNA generation was first determined by testing different numbers of platelets obtained by platelet apheresis. When we started with $2x10^6$ platelets, GAPDH or vWF transcripts were not detectable by quantitative PCR even after 22 amplification cycles. When we increased the platelet number to $2x10^7$, we obtained measurable GAPDH transcripts but vWF remained undetectable. At starting concentrations of $2x10^8$ platelets or more, both marker transcripts became detectable in a platelet concentration-dependent manner. Because $2x10^8$ platelets are equivalent to 2–4 mL of whole blood, we started the evaluation of the minimum volume of whole blood with a minimum of 5 mL. However, because of losses of thrombocytes during sample preparation and leukofiltration, 40 mL of whole blood was required to achieve reliable and reproducible results with a mean yield of 25–30 µg of cDNA. Typical results obtained with blood samples from healthy volunteers (platelet counts, 143.000-398 000/µL) are listed in Table 3.

Table 3. RNA amplification results for three exemplary blood samples.

sample #	1	2	3
Platelets/40 mL whole blood	6.12×10^9	11.76×10^9	8.16×10^9
Platelets after sample preparation	3.76×10^9	6.48×10^9	4.28×10^9
Amplified cDNA, µg	34	29	26
vWF transcripts/µg cDNA	9×10^6	237×10^6	80×10^6
GAPDH transcripts/µg of cDNA	3000×10^6	3972×10^6	2738×10^6

Inter- and intraindividual variability of vWF, F13A, and gapdh transcripts in platelets

Blood samples (40 mL of whole blood) from three healthy volunteers were obtained weekly over a period of 3 weeks. Platelet counts in citrate-anticoagulated blood ranged from 138 000 to 146 000/μL, 159 000 to 169 000/μL, and 217 000 to 265 000/μL in individuals 1, 2, and 3, respectively. After mRNA isolation, vWF, GAPDH, and F13A were quantified by RT-PCR. The results (Fig. 3) demonstrated interindividual variations for all three transcripts with the lowest concentrations seen in individual 1. These differences remained stable over time, indicating a low intraindividual variability.

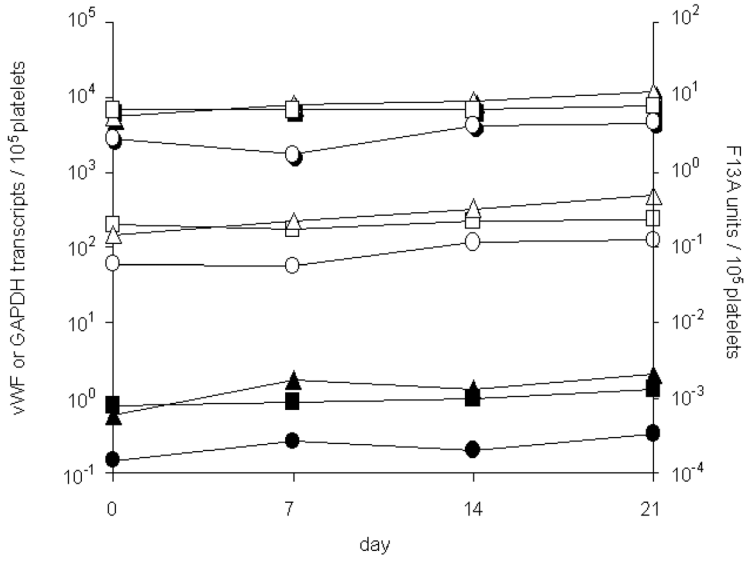

Figure 3. Intra- and interindividual variability of platelet vWF, F13A, and GAPDH gene expression. Whole blood (40 mL) was drawn weekly over a period of 3 weeks from three healthy volunteers (circles, individual 1; triangles, individual 2; squares, individual 3). After preparation of mRNA, vWF (closed symbols), GAPDH (open symbols), and F13A transcripts (open symbols with shadows) were quantified by RT-PCR. Results are shown as copy numbers (vWF and GAPDH) or units (F13A).

DISCUSSION

Our aim was to establish a protocol that makes analysis of the platelet transcriptome applicable to single patients with suspected platelet disorders.

Contaminating leukocytes are a potential problem in platelet RNA analysis because the content of mRNA in leukocytes is >10 000-fold higher than in platelets [22]. To overcome this problem, several techniques, including laser-assisted microdissection and manipulation, serial filtration, and antibody-mediated depletion of leukocytes by magnetic beads, have been used [2,3,22]. Filtration is the only technique that is used on

a routine basis [24,25], and filters adapted for the processing of small volumes of PRP are commercially available. Taking these advantages into account, we studied the efficiency of leukocyte filtration by flow cytometry analysis and determination of CD45 and CD14 transcripts. After one filtration, leukocytes were not detectable by flow cytometry analysis, and negative array results were obtained for CD45 and CD14 transcripts. These results support previously published data obtained on large volume preparations of platelets [3,26]. When we used a more sensitive quantitative RT-PCR approach, however, low concentrations of CD45 transcripts were detectable, indicating that the filtered PRP was not completely devoid of leukocytes. Analyzing the efficiency of leukocyte filtration, we found that one filtration step reduced the amount of CD45 transcripts by three orders of magnitude, corresponding to a reduction in the leukocyte/platelet ratio from 1:2000 to 1:2 000 000. Adding more filtration steps did not significantly reduce the concentration of CD45 mRNA but led to additional platelet loss. On the basis of these results, we included one filtration step in our experimental protocol.

The quality of the mRNA preparation is of critical importance when working with a tissue source that contains only minute amounts of mRNA. We used oligo(dT)-coupled magnetic beads, which directly bind platelet mRNA representing 1–3% of total RNA. To control our mRNA isolation procedure, we isolated mRNA at various time points from one subsampled platelet preparation. Subsequently, the numbers of vWF and GAPDH transcripts were measured. The SD for the Ct values never exceeded 0.73, demonstrating the high reproducibility and precision of this procedure.

The use of an amplification procedure before gene expression analysis requires maintenance of the original message profile. In our microarray investigation, we compared mRNA profiles from 10 μg of unamplified and 0.5 μg of SMART-amplified platelet RNA and found that 91.5% of the gene expression information was maintained. A total of 508 genes revealed negative signals in the unamplified samples but were positive (27 genes) or in the gray area range (481 genes) in the SMART-amplified samples. These results may be regarded as indicative of the higher sensitivity of the SMART technique, but not as true discrepancies. However, we observed a false-negative microarray result in the SMART-amplified samples for eight transcripts. Obviously, amplification of these transcripts failed for unknown reasons. Their lengths

varied between 1.0 and 6.8 kb; therefore, length can probably be ruled out as a main reason for the amplification failure. One can speculate that failure of template switching at the 5' end might occur because of secondary structures in these transcripts. Such failures must be considered for future studies on individual mRNA profiling of platelet RNA.

SMART amplification revealed consistent results when we compared microarray data from six individual experiments. The CV for the majority of genes (8913 of 9850; 90.5%) was <0.5, and only 13 genes had CV values <1. Mean CV values did not differ between amplified and unamplified samples. This confirmed the maintenance of gene expression profiles after SMART amplification. The observed differences may not result from the amplification technique but may represent individually different gene expression patterns.

Comparability of array results depends on the array format used [27]. The array results presented here are consistent with our previously published data obtained with an identical platform (MWG Biotech) [3]. When we compared our results with the data published by Gnatenko et al. [2] and McRedmond et al. [6],we had to consider that these results were generated with a different microarray platform (Affymetrix). Among the 50 most abundant platelet transcripts identified by Gnatenko et al. [2] and McRedmond et al. [6], 25 are arrayed on both platforms. For 22 of these genes, we confirmed strongly positive hybridization signals. Only three genes gave discrepant results: one gave signal intensities in the gray area (cofilin 1, nonmuscle) and two in the negative range (progesterone receptor component and nonmuscle myosin light chain).

Quantification of vWF, F13A, and GAPDH as marker transcripts to determine how accurately PCR-generated cDNA reflects the original platelet mRNA population revealed exponential amplification between cycles 10 and 18 for all transcripts when 50 or 100 ng of total platelet RNA was used as starting material. However, amplification efficiency was slightly higher for vWF than for F13A and GAPDH. Most likely, this is attributable to the more than 200-fold lower concentration of vWF transcripts compared with GAPDH transcripts in the starting mRNA, which led to the C_0t effect [28,29]. The bias toward more efficient amplification of low-abundance transcripts during SMART amplification has already been described in other tissues [15,18]. However, when we compared platelet RNA with SMART-amplified RNA by microarray analysis, the correlation was

excellent. Presumably, the semiquantitative microarray method is not sensitive enough to detect such differences, which can be seen with quantitative real-time PCR [29].

Using quantitative PCR, we were not able to detect FGA transcripts in any of the amplified or unamplified samples tested. This is in contrast to recently published array data [5], but agrees well with data obtained by in situ hybridization and PCR testing [30]. Both sensitive techniques failed to demonstrate FGA mRNA in platelets or megakaryocytes. The reason for this discrepancy remains unknown, but it emphasizes that array results need to be validated by independent methods such as RT-PCR.

The platelet count is regulated in a narrow range with minimal day-to-day individual variation [31]. At present, however, no data are available on intra- and interindividual variability in platelet gene expression. In prelimi- nary experiments, we measured changes in vWF, F13A, and GAPDH mRNA concentrations over time in three healthy individuals. The interindividual differences in the expression of all three transcripts remained stable over 3 weeks, indicating that there is only minor intraindividual variation.

In conclusion, the assessed protocol allows production of micrograms of platelet-specific cDNA from blood volumes as low as 40 mL. SMART amplification does not cause distortion of the gene expression profile as estimated by microarray analysis. Preliminary results on samples from three healthy volunteers indicated interindividual differences of platelet gene expression profiles that seemed to be consistent over time. Therefore, this protocol makes platelet RNA isolated from single patients available for gene expression studies that may detect target genes involved in the development of inherited megakaryocytic/platelet disorders.

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Chapter 6

Molecular analysis of thrombophilic risk factors in patients with dural arteriovenous fistuals*

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ABSTRACT

Dural arteriovenous fistulas (DAVFs) are direct artery-to-cerebral venous sinus shunts. Our recent finding of a significantly increased prevalence of factor V (FV) Leiden in patients with DAVFs prompted us to evaluate prothrombinG20210A, MTHFRC677T, ß-fibrinogenG455A, PAI-1 4G/5G and FXIIIVal34Leu as additional risk factors for thrombophilia in 26 patients with DAVFs and a group of age- and gender-matched controls. There was no significantly increased prevalence of these risk factors in DAVF patients. We conclude that FV Leiden is of pathogenetic significance in the aetiology of a subgroup of DAVFs whereas the other thrombophilic risk factors are not likely to be involved.

INTRODUCTION

Dural arteriovenous fistulas (DAVFs) are direct arterytocerebral venous sinus shunts, the etiology of which remains controversial. Initially thought to be of congenital origin, more recent data emphasize the role of cerebral sinus thrombosis and elevated sinus pressure. It has been proposed that impaired venous outflow favors the development of indigenous dysplastic dural vessels within the sinus establishing a direct artery-to-sinus communication and causes opening of preexisting microarteriovenous fistulas in the sinus walls [5,9,11]. In addition, we recently reported a significantly increased prevalence of resistance to activated protein C and factor V (FV) Leiden in white patients with DAVFs [8],suggesting a link between thrombophilia and the development of DAVFs. Therefore, we extended the search for thrombophilic risk factors to

prothrombinG20210A, methylentetrahydrofolate reductase (MTHFR) C677T, ß-fibrinogenG455A, plasminogen activator inhibitor-1 (PAI-1) 4G/5G and factor XIII (F XIII)Val34Leu in a case-control study.

PATIENTS AND METHODS

Patients

We recruited 26 patients with DAVFs, seen in the Departments of Neurology and Neuroradiology, Alfried-Krupp-Hospital, for endovascular treatment between May 1997 and January 2001,and 26 age- (± 1 years) and gender-matched healthy control subjects without any evidence of cerebrovascular or cardiac disease or venous thrombosis. All individuals gave their written informed consent to the study. There were 13 males and 13 females in the patient and in the control group. Mean age was 57 ± 12 years in patients and 58 ± 12 years in controls. Smoking and estrogen intake as risk factors were equally distributed in each group. The DAVFs were located as follows: occipital (10), temporal (4), cerebellar tentorium (4), cerebral falx (1), orbita (1), carotid-cavernous sinus (2), transverse sinus (1) ,spinal (3). Data of the FV Leiden mutation have been previously reported for 22 of these patients [8].

Methods

DNA from peripheral blood leukocytes was extracted using the QIAamp DNA Blood Mini Kit (Qiagen,Hilden,Germany) according to the manufacturer's protocol. Factor V G1691A (FV Leiden) [3], prothrombinG20210A, MTHFRC677T [4] and ß fibrinogenG455A [15] were determined as previously described. FXIIIVal34Leu was analysed by allelic discrimination using the TaqMan technique. Oligonucleotide sequences were 5'-GAAGATGACCTGCCCACAGTG-3' (forward-primer), 5'-ATGCT-CATACCTTGCAGGTTGA-3' (reverse-primer), 5'-FAM-AGCTTCAGGGCTTGGTG-CCC-TAMRA-3' (wildtype-probe) and 5'-TET-TCAGGGCGTGGTGCCCC-TAMRA-3' (mutation probe). Fluorescence intensities were measured using the sequence detection system 7700 (Perkin Elmer, Weiterstadt, Germany). PAI-1 4G/5G was investigated by restriction fragment length polymorphism using a mutant forward primer 5'-CAGAGAGAGTCTGGCCACGTG-3' and reverse primer 5'-TGTCTAG-GACTTGGGACCAACAG-3'. Amplification products were digested with 1 U of Bsi I (New

England Biolabs,I nc. Beverly,MA,USA) and loaded on an ethidium-bromide stained 2% small DNA agarose-gel (Biozym,Hessisch Oldendorf,Germany).
For statistical analysis,the Student's t-test and the Fisher's exact test were used.

RESULTS

The results of prothrombinG20210A, MTHFRC677T, ß-fibrinogenG455A, PAI-1 4G/5G, FXIIIVal34Leu are summarised in Table 1. The prevalence of homozygous and heterozygous alleles of the mutations or polymorphisms was not different between the DAVF patients and controls. Among the additional 4 DAVF patients not reported previously [8], one was heterozygous for the FV Leiden mutation, whereas the 4 matching controls had wild-type FV. Thus, among the extended group of 26 DAVF patients analysed in the present and previous studies, 6 were heterozygous for the FV Leiden mutation (6/26; 23%). There was no preferred localisation of the DAVFs among the patients with FV Leiden compared with the DAVF patients with wild-type FV. Of the six DAVF patients with abnormal activated protein C resistance and heterozygosity for FV Leiden, two patients had a history of previous sinus thrombosis. In addition, there was another patient who had suffered from a deep leg vein thrombosis. Among the controls, no thrombotic events had occurred.

Genotype	Total (n=52) % (n)	DAVF patients (n=26) % (n)	Controls (n=26) % (n)	Statistical significance
Prothrombin				
G20210G	100 (52)	100 (26)	100 (26)	n. s.
G20210A	0	0	0	n. s.
A20210A	0	0	0	n. s.
MTHFR				
C677C	48 (25)	50 (13)	46 (12)	n. s.
C677T	44 (23)	42 (11)	46 (12)	n. s.
T677T	8 (4)	8 (2)	8 (2)	n. s.
PAI–1				
4G/4G	44 (23)	58 (15)	31 (8)	n. s.
4G/5G	40 (21)	27 (7)	54 (14)	n. s.
5G/5G	15 (8)	15 (4)	15 (4)	n. s.
β-Fibrinogen				
G455G	56 (29)	77 (20)	35 (9)	n. s.
G455A	38 (20)	23 (6)	54 (14)	n. s.
A455A	6 (3)	0	11 (3)	n. s.
FXIII				
Val34Val	58 (30)	54 (14)	62 (16)	n. s.
Val34Leu	35 (18)	35 (9)	35 (9)	n. s.
Leu34Leu	8 (4)	11 (3)	4 (1)	n. s.

Table 1. Genotype frequencies in DAVF patients and age- and gender-matched controls (n.s.: not significant).

DISCUSSION

In this case-control study we did not find an association between prothrombin G20210A,MTHFRC677T, PAI1 4G/5G, ß-fibrinogenG455A, FXIIIVal34Leu and the presence of DAVFs. Thus, there is no evidence of a major pathogenetic role of these thrombophilic risk factors in the etiology of DAVFs. This contrasts our previous finding of a significantly increased prevalence of FV Leiden in DAVF patients confirmed in the present study. Among the investigated thrombophilic risk factors, FV Leiden exhibits the highest relative-risk for venous thrombosis [13] and is also known to play a considerable role in the development of cerebral venous thrombosis [10]. The prevalence of FV Leiden in patients with venous sinus thrombosis reported in the literature [10] is comparable to that found in our studies. This strongly supports the etiologic impact of cerebral venous thrombosis in the development of DAVFs. Compared with FV Leiden,the prothrombinG20210A-mutation has a lower relative risk for first venous thrombotic events and a relative risk

for recurrent thromboembolic events similar to patients with a normal genotype [2,14]. In addition, compound heterozygotes (FV Leiden and prothrombinG20210A) may exhibit a further increase in the relative risk for these events. At present, the degree of an association between the prothrombinG20210A-mutation and cerebral venous thrombosis remains unclear [1,12]. It cannot be excluded that the number of DAVF patients studied here is too small to reveal a contribution of this mutation alone or as an additive risk factor. The polymorphisms of MTHFR, ß-fibrinogen, PAI-1 and FXIII

are believed to contribute mainly to arterial thrombotic events [6,7], via elevated concentrations of homocysteine, fibrinogen, PAI-1 and an altered clot resistance to fibrinolysis. However, we did not find an association between these genetic variants and DAVFs. This would support the hypothesis that only thrombotic events of the venous, but not the arterial system may contribute to the development of DAVFs. This hypothesis demands further confirmation by additional prospective case control

studies. In addition to the genetic factors investigated in this study, we previously ruled out a pathogenetic role of alterations of platelet counts, partial thromboplastin time, concentrations of fibrinogen, antithrombin III, protein S and cardiolipin-antibodies (IgG and IgM) in the development of DAVFs [8]. Taken together, this study corroborates our previous finding of a significantly increased prevalence of FV Leiden

in white patients with DAVFs,st rongly suggesting that FV Leiden is of pathogenetic significance at least in a subgroup of DAVFs. A major role of prothrombin G20210A, MTHFRC677T, ß-fibrinogenG455A, PAI-1 4G/5G and FXIIIVal34Leu in the pathogenesis of DAVFs was virtually ruled out. For clinical purposes, we conclude that thrombophilia screening in patients with DAVF can be restricted to FV Leiden.

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Chapter 7

Discussion

The introduction of the real-time PCR in 1992 greatly improved the reliability and speed of nucleic acid detection. Monitoring of product accumulation in real-time provided insights into even the early kinetics of a PCR reaction, allowing more accurate product quantification than possible with conventional end-point methods. The increased speed of real-time PCR is due to the removal of post-PCR handling steps, as the detection of amplification-dependend fluorescence emission is performed in a 'closed-tube' format that also reduces the risk of false positive results stemming from contaminating amplification products. These advantages make the real-time PCR to the method of choice for a variety of applications, including the testing for pathogens, SNP analysis, and gene expression profiling. This thesis deals with the implementation of real-time PCR assays that have been developed for different purposes in the field of haemostasis and transfusion medicine.

Human parvovirus B19 (B19) is an omnipresent and clinically significant pathogen. About half of 15-year-olds have specific anti-B19 antibodies [1]. Common manifestations of the infection are the mild febrile illness erythema infectiosum followed by acute arthropathy. Other severe manifestations include transient aplastic crisis and chronic anaemia in immunocompromised patients. Furthermore, infection during pregnancy is a major cause of fetal death [2]. Therefore, individual blood products given to patients that are exceptionally at risk in case of B19 infection should be free of infectious B19 particles.

Owing to its high sensitivity, the PCR is the method of choice for the screening of blood donors for B19 viruses by the detection of corresponding genome sequences. In the acute phase of B19 infection, however, virus can be present in the blood at concentrations $>10^{12}$ copies/ml [3], giving the risk of contamination and subsequent false positive test results. This is in particular true if heterogenous PCR assays are used that necessitate the opening of reaction tubes for the post-PCR detection of cumulated amplification products.

The real-time B19 PCR described in this thesis showed to be an excellent tool for the screening of blood donors for B19 genomes. Beside the advantages of the homogenous

assay format, also the implementation if a competitive internal control sequence was a significant improvement to previously described, heterogenous B19 PCR assays [4,5].

Due to its quantitative characteristics, the described assay might also be applied to the testing of plasma donations scheduled for production pools. The contamination of plasma manufacturing pools with B19 DNA has been frequently described [6-8]. To reduce the load of B19 DNA in plasma-derived coagulation factor concentrates, it has been proposed that corresponding pools should not exceed levels of 10^4 IU/ml of B19 DNA [9]. Reliable quantification of B19 DNA is a prerequisite in order to fulfil these requirements. In general, heterogenous PCR assays are limited due to the trade-off between sensitivity and the ability to provide quantitative test results [10]. In contrast, homogenous real-time PCR formats combine the advantages of high assay sensitivity with a broad dynamic range. Thus, they are more useful tools for the quantitative detection of viral genomes.

Shortly after the introduction of the described B19 Real-time PCR assay, it has been found that the genetic diversity of B19 is higher than previously expected. Thus, B19 is now classified into genotypes 1 (former B19 isolates), 2, and 3 [11,12]. Recently, the contamination of coagulation factor concentrates with genotype 2 DNA has been reported. Although genotype 2 was not found to be a frequent contaminant, the authors stated that NAT methods should be optimised to ensure reliable detection of all genotypes of Parvovirus DNA in order to further improve the viral safety of blood-products [13]. To assess the sensitivity of the described B19 Real-time PCR assay on genotype 2 and 3 templates, we performed multiple sequence alignments that showed that our assay might underestimate genotype 3 sequences. This disadvantage may be accepted for the screening of German blood donors since genotype 3 is currently mainly limited to France [11]. However, the prevalence of genotype 3 infection in Germany might increase as a result of travel and migration. Thus, continuous surveillance of Parvovirus variants is an important measure to estimate the risk of genotype 3 transmission and the need for optimised NAT assays.

Correct quantification of viral loads is generally challenged by sequence dissimilarities between different groups and subtypes of the virus of interest. For example, genome sequences within the different groups and subtypes of HIV-1 are much more distinct from each other than this is the case for the B19 genotypes [14]. In Europe, HIV-1 group

M subtype B is predominant, while non-group M infections are rare events. Thus, legal requirements for HIV-1 NAT screening procedures for blood products had been limited to the detection of all subtypes within group M of HIV-1 [15]. Recently, we described a HIV-1 group O infection in one of our blood donors that was not detected by our standard In-house HIV-1 real-time PCR assay [16].

This case and the problematic nature of Parvovirus genotype 3 DNA mentioned above emphasize the challenge that generally underlies the development of probe-mediated real-time PCR procedures for the detection of viral genomes. By providing an additional sequence-specific hybridisation step, these assays are more specific than conventional single-round PCR methods. Furthermore, this feature enables the implementation of an internal control reaction to avoid false-negative results due to poor test performance or inhibition of amplification. In case of viral nucleic acid detection, however, conserved regions within different genome sequences have not only to be considered for the primers, but also for an additional fluorogenic probe. Since nucleotide mismatches between probe and target sequences have a high impact on the level of amplification dependent fluorescence generation, the design of oligonucleotides for the detection of viral genomes by real-time PCR is more pretentious.

While obstructive for pathogen testing, the susceptibility of probe-mediated fluorescence generation to sequence variation is beneficial for genotyping purposes. In this thesis, the application of various 5'-nuclease assays for allelic discrimination have been applied for the analysis of thrombophilic risk factors in patients with dural arteriovenous fistuals (DAVFs). In addition to this relative small study, the described 5'-nuclease assays are frequently used for large scale routine testing in the scope of general diagnosis of thrombophilia. While quantitative homogenous PCR requires performance in real-time, fluorogenic amplification signals assayed for sequence specific discrimination are usually recorded in an end-point format as the valuation of these data is based merely on a qualitative survey. Therefore, amplification can be performed in conventional thermocyclers, markedly increasing throughput. The use of these 'closed-tube' assays dispenses with the need for post-PCR handling steps. Thus, the risk of inaccurate results caused by contamination or pipetting errors is markedly reduced in comparison to more labor-intensive conventional methods such as restriction fragment length polymorphism analysis or allele specific amplification.

Quantitative gene expression analysis is another application of real-time PCR described in this thesis. The general advantages of the homogenous assay format have already been mentioned above and are also significant for mRNA quantification purposes. Independent of the test system used, gene expression analysis is challenging since the original mRNA-profile in collected tissue or cell samples may rapidly alter by either unintended gene induction or mRNA degradation in vitro. Thus, reliable quantification of gene transcripts ex vivo requires accurate evaluation of preanalytical requirements. This prerequisite had to be considered with respect to the implementation of a test system for the quantitative detection of tissue factor transcripts in blood-monocytes.

Tissue factor is known to be the main initiator of the coagulation cascade and may be expressed under certain conditions on intravascular cells. We were able to demonstrate that desmopressin acetate (DDAVP) is able to induce monocytic tissue factor gene expression in vivo. All blood samples taken during the DDAVP study were immediately processed in downstream applications. Thus, the question of optimal sample storage conditions was a minor one. However, especially if several hospitals or practices are involved in the collection of blood samples for TF gene expression analysis, the storage and transport of specimen prior isolation of total RNA becomes an important aspect. To ensure the preservation of the monocytic mRNA profile ex vivo, a commercially available blood sampling system that includes a RNA stabilizing additive in the blood collection tube was evaluated. The combination of this blood sampling system and the established real-time RT-PCR assay for quantification of TF-transcripts enabled TF gene expression rates to be determined in healthy blood donors under standardized conditions. This survey of a normal range of TF transcription rates provides an essential basis for further clinical studies on intravascular TF gene expression as a potential risk factor for venous thromboembolism (VTE). We recently described significantly increased levels of monocytic TF gene expression in outpatients with a history of VTE [17]. However, when compared to previously published data on monocytic TF gene expression in other pathologic situations, this is more of a moderate difference to our control group [18]. To further examine the role of TF gene expression in VTE, also more acute events have to be investigated.

While single transcription rates are investigated primarily in the context of already clarified functions of the target gene, global gene expression profiling is studied to

understand which genes are generally involved in cellular processes under specific conditions. One limitation of microarray-based gene expression analysis is that a total of 2-4 µg of total RNA is required for each experiment. Thus, low amounts of mRNA need to be amplified prior to microarray hybridisation. We described the evaluation of the so-called 'SMART'-method for global amplification of platelet cDNA. To ensure the preservation of the original mRNA profile, the characteristics of the performed amplification procedure had to be determined. The application of real-time PCR assays for absolute quantification of chosen marker transcripts permitted precise calculation of template-depended amplification efficiencies effected by the SMART procedure. These results were essential in determining the maximum number of amplification cycles possible while preserving the original mRNA profile of the platelet sample.

Incoherent to the used global amplification procedure, there is evidence that gene expression analysis based on microarrays may lead to false positive results due to unspecific binding [19]. Recently, RNA coding for the fibrinogen alpha chain (FGA) has been detected in substantial amounts in human platelets using microarray analysis [20]. In contrast, we did not detect any FGA transcripts in different individual platelet preparations applying real-time PCR [21]. These data emphasize the need to validate individual genes expressed in microarray analysis by other independent methods like e.g. RT-PCR. To ensure maximal specificity, probe mediated real-time detection would be the method of choice.

The homogenous 5'-nuclease assays described in this thesis are valuable methods, substantially enhancing the potential of molecular analysis in haemostasis and transfusion medicine. Compared to conventional methods, possible limitations are the potentially increased susceptibility to unpredicted sequence variations and the higher costs due to required probe chemistry and devices. In general, however, the 'closed-tube' PCR format upgraded the field of molecular diagnosis and research in terms of reliability and speed. Pre-developed primer/probe sets for either quantitative expression analysis or single nucleotide polymorphism analysis are already commercially available in a genome-wide manner. Furthermore, Roche Diagnostics Corp. recently released the COBAS TaqMan analyzer, a real-time PCR instrument that was exclusively developed for the clinical laboratory. Based on this platform, certified analyte-specific reagents (ASR) for the quantification of e.g. viral genomes are already commercially available [22-

24]. Thus, one can expect that real-time PCR applications will prospectively supplant conventional PCR assays currently in use.

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Chapter 8

Summary

The introduction of the real-time PCR into the medical field significantly broadened the possibilities of both, diagnostics and research. Since first described in 1992, a variety of fluorogenic chemistry systems has been applied on different real-time PCR platforms. Due to the 'closed tube' format and the possibility to have insights into the entire kinetics of a PCR reaction, real-time PCR strongly improves the performance of nucleic acid detection regarding reliability and speed.

This thesis deals with the development and application of novel real-time PCR assays in the field of haemostasis and transfusion medicine.

Chapter 1 gives an general overview of real-time PCR and its current applications in haemotherapy. The development and validation of a real-time PCR assay for the detection of Parvovirus B19 DNA in blood donations is described in **chapter 2**. This sensitive and internal controlled PCR assay fulfils all criteria for the routine screening of predominantly B19 negative blood donations. A real-time PCR platform for the quantification of tissue factor (TF) mRNA in circulating blood monocytes is presented in **chapter 3.** To ensure reliable test results, a blood sampling system that includes a RNA stabilizing additive was evaluated. Utilizing the described TF real-time PCR assay we were able to demonstrate that desmopressin acetate (DDAVP) is able to induce monocytic tissue factor gene expression in vivo (**chapter 4**). By the application of different real-time PCR assays for absolute quantification of chosen marker transcripts, it was possible to validate the so-called SMART procedure for global amplification of platelet mRNA sequences applied for subsequent gene expression analysis (**chapter 5**). As described in **chapter 6**, different 5'-nuclease assays were used for the allelic discrimination of thrombophilic risk factors in patients with dural arteriovenous fistuals (DAVFs).

In summary, the real-time 5'-nuclease assays described in this thesis showed to be valuable methods that strongly improved the possibilities of molecular analysis in haemostasis and transfusion medicine. Generally, real-time PCR applications will continuously compensate for conventional PCR assays currently in use. However, this

process will most probably be based on pre-developed assay formats that are already available in a genome-wide manner.

Zusammenfassung

Die 1985 von Mullis et al. erstmals beschriebene Methode der Polymerase Kettenreaktion (Engl.: Polymerase Chain Reaction, PCR) hat die Arbeit molekular-genetisch ausgerichteter Laboratorien grundlegend verändert. Mit der PCR ist es möglich, eindeutig definierte Abschnitte eines DNA-Strangs in-vitro ohne die Beteiligung von Mikroorganismen zu vervielfältigen. Die PCR wird heute in allen biologischen und medizinischen Laboratorien für eine Vielzahl verschiedener Anwendungen eingesetzt. Eine Weiterentwicklung der konventionellen PCR-Methode stellt die 1992 von Higuchi et al. entwickelte Echtzeit-Detektion der exponentiell verlaufenden Produktakkumulation dar. Die Anwendung von Echtzeit-PCR-Verfahren (Engl.: Real-time PCR) erlaubt eine, im Vergleich zu End-Punkt-Methoden, zuverlässigere Quantifizierung des Ausgangsmaterials über einen weiten Konzentrationsbereich. Ein weiterer Vorteil der Real-time PCR ist die Detektion der amplifikationsbedingten Fluoreszenzgenerierung im geschlossenen System, was die Gefahr einer Kontaminationen mit verschleppten PCR-Produkten erheblich reduziert und durch den Wegfall konventioneller Detektionsmethoden auch einen erheblichen Zeitvorteil mit sich bringt.

Die ursprüngliche und auch am weitesten verbreitete Real-time PCR Methode basiert auf der Zugabe eines Fluoreszenzfarbstoffes (z.B. SYBR Green I ®), dessen spezifisches Emmisionssignal nach Einlagerung in die doppelsträngigen PCR-Produkte ein Maximum erreicht. Des weiteren wurden über die Zeit verschiedenste fluoreszenzmarkierte Sondensysteme entwickelt, welche erst nach spezifischer Hybridisierung an einen der beiden Stränge der PCR-Produkte zur Fluoreszenzgenerierung führen. Durch die Verwendung verschiedener Fluoreszenzfarbstoffe eröffnen diese sequenz-spezifischen Real-time PCR Methoden ebenfalls die Möglichkeit der Detektion verschiedener PCR-Produkte in einem multiplen Reaktionsansatz.

In der vorliegenden Arbeit wird die Entwicklung und Anwendung verschiedener Real-time PCR Verfahren im Bereich der Hämostaseologie und Transfusionsmedizin beschrieben. Die auf dem 5'-Exonuklease Prinzip (Hydrolisierungssonden) basierten Applikationen wurden zur Quantifizierung von Nukleinsäuren oder der Diskriminierung klinisch relevanter Polymorphismen/Mutationen etabliert und bildeten die Grundlage der in den einzelnen Kapiteln dieser Arbeit dargestellten Ergebnisse.

Die in **Kapitel 2** beschriebene Real-time PCR Methode zur quantitativen Detektion von Parvovirus B19 (B19) DNA erfüllt alle Voraussetzungen zur routinemäßigen Testung von Blutspendern und liefert somit einen wichtigen Beitrag zur Sicherheit von Blutprodukten und Faktorenkonzentraten. Der zur Amplifikation ausgewählte Bereich des Parvovirus Genoms ist hochkonserviert und sichert somit zumindest die Erfassung verschiedener Isolate der dominanten Genotypen 1 und 2. Die bei jeder Isolation der Nukleinsäuren und anschließenden Amplifikationsreaktion mitgeführte interne Kontrollsequenz überprüft jeden dieser Schritte hinsichtlich deren Effektivität und verhindert somit falsch negative Testergebnisse. Aufgrund der beschriebenen quantitativen Eigenschaften des Testsystems ist es möglich, B19 positive Einzelspenden mit niedriger Viruslast zu identifizieren, deren entsprechend potenziell hoher Gehalt an B19 Antikörpern zur Produktsicherheit von aus Poolplasma hergestellten Faktorenkonzentraten beitragen kann.

Während die zuverlässige Detektion viraler Nukleinsäuren in erster Linie von der Verfügbarkeit entsprechend konservierter Genombereiche abhängig ist, ist im Rahmen von Untersuchungen zur Gen-Expression verstärkte Aufmerksamkeit den präanalytischen Einflussfaktoren zu widmen. Diesem Umstand wurde bei der Entwicklung des in **Kapitel 3** beschriebenen Testsystems zur absoluten Quantifizierung monozytärer Tissue Factor (TF) mRNA in Vollblut durch Verwendung eines kommerziellen RNA-Stabilisatorsystems Rechnung getragen. Mit der ebenfalls in Kapitel 3 beschriebenen TF Real-time PCR Methode konnte bereits im Vorfeld der Etablierung des RNA-Stabilisatorsystems ein die monozytäre TF-Gen-Expression induzierender in-vivo Mechanismus von Desmopressin-Acetat (DDAVP) nachgewiesen werden (**Kapitel 4**). Da alle in dieser Studie gewonnenen Blutproben unverzüglich analysiert werden konnten, stellte sich die Problematik präanalytischer Einflussfaktoren in diesem Fall nicht unmittelbar. Zukünftig geplante, multizentrische Studien mit Fragestellung der Rolle der monozytären TF-Gen-Expression im Kontext venöser Thromboembolien allerdings verlangen nach einer adäquaten Stabilisierung des monozytären mRNA-Profils ex vivo. Auch die Erhebung eines entsprechenden Referenzwertbereiches unter standardisierten Bedingungen muss einer solchen Studie vorangestellt werden. Beidem wurde mit den in Kapitel 3 dargestellten Ergebnissen entsprochen.

Im Gegensatz zu der zielgerichteten Analyse der Expression bestimmter Gene im Rahmen einer bestimmten Fragestellung finden Genchips (Engl.: Microarrays) Anwendung bei der Untersuchung globaler Gen-Expressionsmuster. Ein Nachteil der Microarray-Technologie ist die im Vergleich zu Real-time PCR-Verfahren höhere Menge an benötigtem Ausgangsmaterial von 2-4µg an totaler RNA. Aus diesem Grund muss in Fällen, bei denen nur eine geringe Menge an zu untersuchender RNA zur Verfügung steht, eine globale mRNA-Amplifikation der eigentlichen Microarray-Analyse vorangestellt werden. **Kapitel 5** dieser Arbeit beschreibt die Evaluierung der sog. SMART-Methodik bezüglich der globalen Amplifikation revers-transkribierter mRNA zur globalen Gen-Expressionsanalyse individueller Thrombozytenpräparationen. Mit Hilfe der beschriebenen Real-time PCR Verfahren war es möglich, ausgewählte Transkripte über den globalen Amplifikationsvorgang hinweg zu quantifizieren, um Rahmenbedingungen zu definieren, welche den Erhalt des ursprünglich vorhandenen mRNA-Profils sicherstellen.
In **Kapitel 6** wird die Anwendung verschiedener homogener PCR-Verfahren zur Allel-spezifischen Diskriminierung genetisch determinierter, thrombophiler Risikofaktoren beschrieben. Der wesentliche Vorteil im Vergleich zu konventionell durchgeführten Methoden besteht hier wiederum in dem Wegfall nachgeschalteter Diskriminierungs- bzw. Detektions-Methoden, was den Probendurchsatz erhöht und die Gefahr von potentiellen Kontaminationen oder Verwechslungen reduziert.

Zusammenfassend belegen die in dieser Arbeit beschriebenen, neu entwickelten Real-time PCR Applikationen für die Indikationsgebiete Hämostaseologie und Transfusionsmedizin das breite Spektrum der Einsatzmöglichkeit dieser Methodik, das vom Mutationsscreening über die Infektionsdiagnostik bis zur Genexpressionsanalyse reicht. Mögliche Limitierungen dieser Methodik stellen die im Vergleich zu konventionellen PCR Verfahren erhöhte Anfälligkeit bezüglich viraler Sequenzvariationen und die durch die benötigen Gerätschaften und zusätzlich verwendeten Oligonukleotide verursachten Mehrkosten dar.
Schon heute werden von mehreren Herstellern Primer/Sonden-Sets zur genomweiten Gen-Expressionsanalyse bzw. SNP-Detektion angeboten. Des weiteren wird auch das Angebot komplett zertifizierter Testsysteme, wie sie beispielsweise in der molekularen

Infektionsdiagnostik eingesetzt werden, bezüglich homogener PCR-Verfahren immer umfangreicher. Aus diesem Grund ist davon auszugehen, dass zukünftig homogene PCR-Formate konventionelle Anwendungen mehr und mehr ersetzen werden.

Erklärung

Ich erkläre: Ich habe die vorgelegte Dissertation selbstständig, ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nicht veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt ist, eingehalten.

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