Hematopoiesis & Growth Factors

original paper

haematologica 2001; 86:693-699
http://www.haematologica.it/2001_07/0693.htm

Quantification of human cells in NOD/SCID mice by duplex real-time polymerase-chain reaction

ANDREAS NITSCHE,* MICHAEL BECKER,° ILSE JUNGHAHN,° JUTTA AUMANN,° OLFERT LANDT,# IDUNA FICHTNER,° BURGHARDT WITTIG,@ WOLFGANG SIEGERT*

*Klinik für Innere Medizin m.S. Hämatologie und Onkologie, Charité Campus, Virchow Klinikum, Humboldt Universität zu Berlin; °Department of Experimental Pharmacology, Max-Delbrück-Center for Molecular Medicine, Berlin; #TIB Molbiol, Berlin; @Institut für Molekularbiologie und Biochemie und Centrum Somatische Gentherapie, UKBF, FU Berlin, Germany

Correspondence: Prof. Dr. W. Siegert, Medizinische Klinik II, Charité Campus, Charité Mitte, Schumanstr. 20/21, 10117 Berlin, Germany. Phone: international +49-30-450-513144. Fax: international +49-30-450-513952. E-mail: wolfgang.siegert@charite.de

Background and Objectives. The aim of this study was the development of a fast and reliable polymerase chain reaction (PCR) assay which quantifies the proportion of human cells in immunodeficient chimeric mice, for example transplanted with human hematopoietic stem cells.

Design and Methods. We developed a TaqMan chemistry-based, real-time duplex PCR assay to quantify human and murine DNA in a single-tube reaction in parallel (HUmu PCR). Two independent sets of primers and exonuclease probes, located in the tumor necrosis factor-α gene of both species, were selected to amplify specifically human and murine genomic DNA. Serial dilutions of defined numbers of human cells in mouse cells served to construct calibration curves. The test was applied to NOD/SCID mice transplanted with $CD34^+$ cells isolated from human cord blood and compared to FACS analysis.

Results. Analysis of DNA from human cells diluted stepwise into a fixed number of murine cells – and *vice versa* – led to calibration curves with good correlation for human and murine cells (r^2>0.99) with a detection limit of 2% human cells. Results obtained with the HUmu PCR paralleled those of FACS analysis. However, in contrast to FACS analysis, which requires fresh single cell suspensions, the HUmu PCR can be carried out on already stored samples, even from solid organs and, moreover, the quantity of material required for analysis is very low.

Interpretation and Conclusions. The HUmu PCR presented here is the first real-time PCR assay for simultaneous quantification of human and murine cells. It is extremely fast, accurate and is an interesting alternative method for quantifying the proportion of human DNA in organs of chimeric mice.

Key words: real-time PCR, Duplex-PCR, NOD/SCID mice, chimerism

Non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice lack T-cells, B-cells and have reduced functionality of NK-cells and macrophages. Consequently, they suffer from severe immunodeficiency and accept xenografts of human hematopoietic stem cells without rejection.[1] The NOD/SCID mouse system has, therefore, been used as an *in vivo* system to characterize early hematopoietic stem and progenitor cells derived from various sources such as bone marrow,[2] peripheral blood or cord blood[3] and led to the definition of an immature hematopoietic precursor cell which is capable of repopulating the murine bone marrow with human hematopoietic cells.[4] This cell was termed the SCID repopulating cell (SRC).[5]

As immunodeficient mice are increasingly being used to investigate human hematopoiesis, there is need for a simple and rapid method to follow the development of human cells in the chimeric mouse and to determine the proportion of human cells among mouse cells. Furthermore, it is necessary that only minute amounts of blood or bone marrow are required for this assay in order to allow the investigation of various parameters from one individual mouse. In the recent past, the quantification of human cells in mouse blood or bone marrow was performed by FACS analysis, e.g. by the detection of cells expressing human CD45 or HLA-I antigens.[6] Beside FACS analysis, polymerase chain reaction (PCR)-based

techniques have also been used to amplify human specific DNA regions.[7] Quantification is usually achieved by determination of band intensities of the human specific PCR product in ethidium bromide stained gels.

With the aim of developing a more reproducible, accurate and practical PCR assay, we established a duplex real-time PCR which simultaneously detects and quantifies the amount of human and mouse cells in cell mixtures (HUmu PCR). The recently introduced real-time monitoring of PCR reactions has set new standards in quantitative PCR.[8] About 4,500 real-time PCR instruments are in use to date worldwide. In contrast to conventional PCR, real-time monitoring facilitates quantification of PCR products in the early, exponential part of the PCR reaction. Therefore, results obtained from real-time PCR are much more accurate and reliable than conventional end-point PCR determinations. Finally, real-time PCR assays do not require any post-PCR handling and hence combine accuracy with speed and a minimized risk of carry-over contamination.[9]

Design and Methods

Extraction of DNA

DNA from blood, bone marrow or spleen was prepared using the Qiagen Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations for blood and tissue, respectively. Briefly, the DNA of lysed cells was adsorbed onto a silica matrix, washed and eluted with 100 µL elution buffer by centrifugation.

Selection of primers and probes

Primers and exonuclease probes were selected to bind specifically and exclusively to either the human or the murine tumor necrosis factor-α (TNF-α) genomic DNA sequence (Table 1). The amplicon size of the human TNF-α is 495 bp whereas that of the murine TNF-α amplicon is 264 bp. The exonuclease probe for the HU-assay (HU probe) was 5'-labeled with the reporter fluorescent dye FAM (6-carboxy-fluorescein), and the mu probe with BO-TMR (6-((4,4-difluoro-1,3-dimethyl-5-(4-methoxyphenyl)-4-bora-3a,4a-diaza-s-indacene-2-propionyl)amino)-hexanyl-tetramethylrhodamine).

Both probes carry the non-fluorescent quencher dye DABCYL (4-(4'-(dimethylaminophenylazo)-benzoic acid)), attached to a linker-arm modified nucleotide near the 3' end. Probe extension during PCR was blocked by a 3' phosphate.

Quantitative HUmu TaqMan PCR

PCR was performed in a Perkin Elmer 7700 Sequence Detection System (TaqMan) in 96-well microtiter plates in a final volume of 50 µL. Various compositions of reaction mixtures were tested. Optimum reaction conditions for the simultaneous detection of both, human DNA (HU-assay) and murine DNA (mu-assay) were obtained with 5 µL 10x PCR buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 4.5 mM $MgCl_2$, 1.0 mM dNTP, 2 U Platinum Taq DNA polymerase (all Life Technologies, Eggenstein, Germany), 600 nM primer HU se, 600 nM primer HU as, 100 nM HU probe, 200 nM primer mu se, 200 nM primer mu as and 100 nM mu probe. Finally, 1 µM ROX (6-carboxy-X-rhodamine). Ten microliters of template DNA were added to the reaction mixture. Amplifications were performed starting with a 3 minute template denaturation step at 94°C, followed by 45 cycles of denaturation at 94°C for 20 seconds and combined primer annealing/extension at 64°C for 30 seconds. The total running time is approximately 80 minutes. The fluorescence increase of FAM and BO-TMR was automatically measured during PCR.

Table 1. Oligonucleotides used in the HUmu PCR.

Name	*5'→3' sequence*	*Localization*
HU se	AGGAACAGCACAGGCCTTAGTG	1768-1789
HU as	AAGACCCCTCCCAGATAGATGG	2261-2241
HU probe	**FAM**-CCAGGATGTGGAGAGTGAACCGACATG(**D**)**p**	1878-1904
mu se	GGCTTTCCGAATTCACTGGAG	6455–6475
mu as	CCCCGGCCTTCCAAATAAA	6718–6700
mu probe	**BO-TMR**-ATGTCCATTCCTGAGTTCTGCAAAGGGA(**D**)**p**	6482–6509

Abbreviations. ***D****: DABCYL,* ***p****: phosphate group attached to 3'-terminus. *Nucleotide positions are based on sequence of the human (accession number M26331) and mouse tumor necrosis factor-α gene (accession number Y00467).*

FACS analysis

Single-cell suspensions were prepared, cell counts were performed and viability of the cells was determined by trypan blue exclusion. Flow cytometric analysis was performed as described previously.[7] In order to quantify only cells of human origin, human-specific monoclonal antibodies to CD45 (clone HI 30, Pharmingen) and HLA-class-I (clone G46-2.6, Pharmingen) were used. Cell analysis was performed with a FACSCalibur system (Becton Dickinson; Heidelberg, Germany) using CellQuest software. Each measurement was based on 10,000 events. Dead cells were excluded by outgating of propidium-iodide-stained cells.

Mice

A NOD/LtSz-scid/scid mouse colony (originally obtained from Dr. Leonard Schultz, Jackson Laboratories; Bar Harbor, ME, USA) was expanded and maintained under pathogen-free conditions as described

previously.[7] Mice aged 6-8 weeks were irradiated with a dose of 200 cGy of γ irradiation and transplanted with human cells within 3 to 5 h after irradiation. For transplantation, 0.2 mL samples of $CD34^+$ cord blood cells (purity > 95%) at graft doses of 5×10^5 or 1×10^6 cells were injected into the lateral tail vein. To support engraftment mice were additionally transplanted with a stable transfected rat fibroblast cell line (Rat-hIL-3) secreting human interleukin-3 (IL-3).[10] As controls, NOD/SCID mice not transplanted with human cells were treated and investigated alike. At weekly intervals 100 µL of peripheral blood were drawn from each mouse. Ten weeks post-transplantation, the mice were killed by cervical dislocation, and blood as well as bone marrow cells and the spleen were collected for analysis.

Results

TaqMan assay for quantification of human and mouse DNA (HUmu PCR)

First, the primer/exonuclease probe sets for the HU assay and the mu assay (Table 1) were tested separately. Both assays proved to be species-specific in single reactions (data not shown). When combining both single assays in a duplex reaction, we still obtained species-specific fluorescence signal production and DNA amplification. As shown in Figure 1, fluorescence signal production was specific for human DNA (Figure 1A) and mouse DNA (Figure 1B) and was not inhibited by the concurrent amplification of DNA from the other species. The C_T values were almost identical for human DNA and the same amount of human DNA in a mixture with the same amount of mouse DNA. Gel electrophoretic analysis supported these findings (Figure 1C).

Calibration of the HUmu PCR

Human and mouse bone marrow cells were counted and diluted stepwise. DNA was prepared and DNA equivalents corresponding to 1×10^6, 1×10^5, 1×10^4, 1×10^3 and 1×10^2 cells were used as templates in the HUmu PCR. We obtained a linear correlation between DNA amount, respectively cell numbers, utilized in the HUmu PCR and the C_T values over the whole detection range between 1×10^6 and 1×10^2 cells (data not shown).

Because often only low amounts of cells from mouse blood are available for monitoring the contingent of human cells, we scaled down the HUmu PCR to detect $<1\times10^5$ cells corresponding to <50 µL of peripheral mouse blood. Therefore calibration curves were constructed with DNA amounts corresponding to 80%, 50%, 40%, 30%, 20%, 10%, 5%, 2%, 1%, 0.5% and 0.1% human cells in a constant amount of mouse DNA from 1×10^4 cells. For calibration of mouse cell numbers, reverse dilutions of mouse bone marrow cells in human bone marrow cells were prepared. Both experiments were repeated 4 times, beginning with cell counting, and each sample was measured in triplicate.

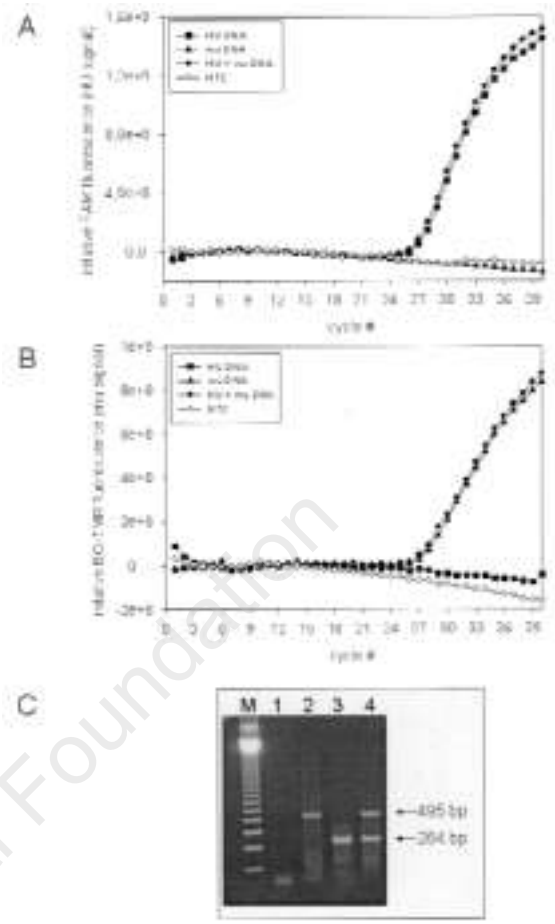

Figure 1. Specificity of the HUmu PCR. DNA was prepared from 1×10^6 human bone marrow cells, 1×10^6 murine bone marrow cells and a mixture consisting of 1×10^6 human and murine bone marrow cells each. One tenth of the volume of these samples was subjected to the duplex HUmu PCR. A: filled squares: FAM signal from human DNA, filled triangles: no FAM signal from murine DNA, filled circles: FAM signal from the mixture of human and murine DNA. B: Inverse situation for the BO-TMR signal: filled squares: no BO-TMR signal from human DNA, filled triangles: BO-TMR signal from murine DNA, filled circles: BO-TMR signal from the mixture of human and murine cells. C: Species-specific amplification was confirmed by gel analysis which demonstrates the different sizes for the human (495 bp) and the mouse (264 bp) amplicon. 1: no template control, 2: human DNA, 3: murine DNA, 4: mixture of human and murine DNA.

Results are shown in Figure 2. Panels A and C show the calibration curves for dilutions of human cells in a DNA equivalent corresponding to 1×10^4 murine

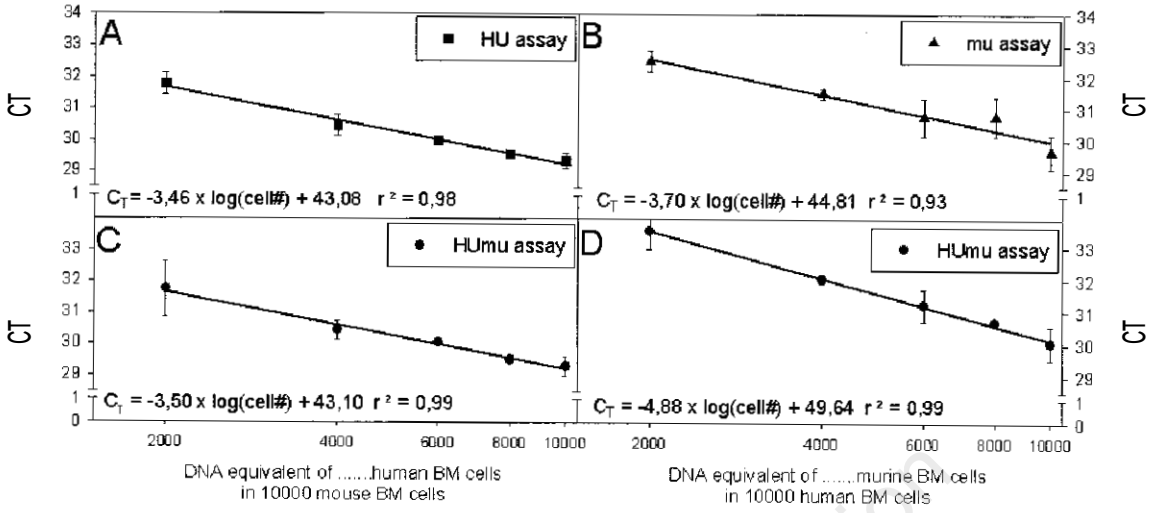

Figure 2. Comparison of calibration curves obtained from single (A and B) assays and the duplex assay (C and D). C_T describes the threshold cycle number, at which fluorescence exceeds ten-fold the standard deviation of the fluorescence detected during PCR cycles 3-15. Open symbols indicate cell amounts for which no signal could be obtained after 40 PCR cycles. Further explanations are given in the text.

cells, amplified with the single HU assay (Figure 2A) and the duplex HUmu PCR (Figure 2C). While the single HU assay could detect minute amounts of human DNA corresponding to 0.5% of human cells in a linear range up to 80% human cells, the lower detection limit of the duplex HUmu assay was 2% of human cells in 1×10^4 murine cells. Both assays displayed a similar high correlation ($r^2>0.98$).

Panels B and D present calibration results for the dilution of mouse bone marrow cells in a constant number of 1×10^4 human bone marrow cells. Like the HU assay, the mu assay showed a broader linear range from 1% to 80% of murine cells in 1×10^4 human cells, whereas the lowest amount detected by the duplex assay was 2%. However, duplex amplification led to a steeper slope, the correlation being high (single assay: $r^2=0.95$, duplex assay: $r^2>0.96$). When experiments were repeated four times and samples were measured in quadruplicate, we observed a mean variability of the duplex HUmu PCR of 15% for the detection of human DNA and 20% for the detection of murine DNA. The variability of the single assays was lower (<10%).

Beside human and murine bone marrow cells, human cord blood mononuclear cells, human buffy coat cells, the T-cell line HSB-2 and the murine cell lines CMT93 and B16-FO were tested and produced calibration curves similar to those obtained from bone marrow cells (data not shown).

Monitoring of human blood cell engraftment in NOD/SCID mice

NOD/SCID mice were transplanted with $CD34^+$ cells purified from human cord blood. At weekly intervals 100 µL peripheral blood were drawn from each mouse: 50 µL were subjected to DNA preparation and 50 µL were analyzed by FACS. The HUmu PCR was used to measure 10µL of DNA, the human and mouse cell numbers were determined by extrapolation from the calibration curves and the ratio of human cells to mouse cells was calculated. Results obtained by sequential analysis of blood of two mice are shown in Figure 3. Both methods, FACS analysis and HUmu PCR, led to comparable results. The proportion of human cells in mouse blood reached maximum values 6 and 7 weeks after transplantation (mouse A1: FACS 25.8% and HUmu PCR 21.5%; mouse B3: FACS 15.7% and HUmu PCR 14.1%).

We also determined the proportion of human cells in bone marrow and spleen of 4 transplanted and two control mice ten weeks after transplantation. Results of FACS analysis and the HUmu PCR are compared in Figure 4. Black bars indicate the percentage of human cells measured by FACS analysis, open bars indicate results from the HUmu PCR. Neither method detected human cells in the non-transplanted control mice C5 and C6. The greatest difference between FACS and HUmu PCR results was found in the bone marrow of mouse A2 (approximately 9%). In a total of 50 chimeric

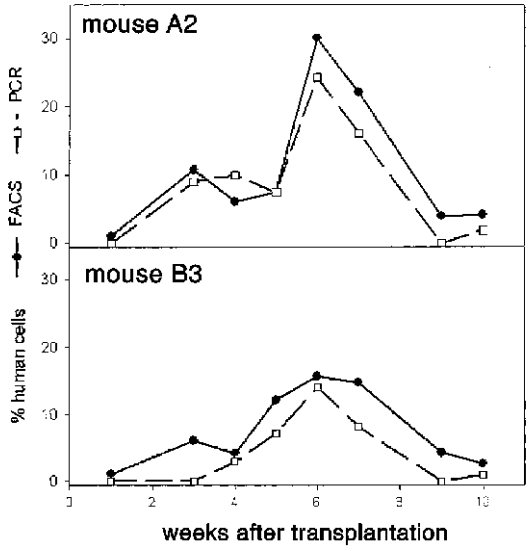

Figure 3. Monitoring of human cells in blood of NOD/SCID mice transplanted with human CD34+ cells from cord blood. Changes over time of the number of human cells quantified by FACS analysis (black circles) compared to results obtained by the HUmu PCR (open squares) are shown for two representative mice.

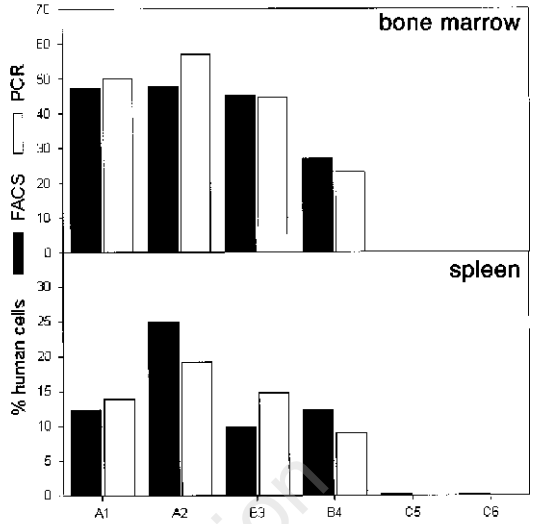

Figure 4. Determination of human cell engraftment in bone marrow (upper panel) and spleen (lower panel) in 6 mice ten weeks after transplantation. Black bars represent results obtained by FACS analysis, open bars results obtained by the HUmu PCR.

specimens we observed differences of up to 50% between FACS analysis results and HUmu PCR results in some samples. The average difference was 21%, including samples near the detection limit of 2%.

Discussion

The determination of the extent of human blood cell engraftment in mice transplanted with hematopoietic stem cells can be achieved by quantification of cells carrying human antigens (FACS analysis) or by PCR amplification of human-specific genome regions. FACS analysis is a sensitive method which can provide important phenotypic information in addition to the enumeration of human cells by multi-parameter analysis. However, for FACS analysis samples need to be fresh and in cell suspensions; therefore tissue samples are often not suitable for FACS analysis. Immunocytochemistry can be performed on tissues, but this detection procedure is relatively time consuming and precise quantification of particular cell types is difficult.

Hence, during the last years several PCR assays have been developed to detect and quantify the proportion of human cells present in chimeric mice.[4,11] These assays are very sensitive and require only low amounts of material. Unfortunately, the PCR assays utilized to date require laborious post-PCR handling because quantification occurs by determination of PCR product intensities either in ethidium bromide stained agarose-gels or after hybridization of Southern blots with human DNA specific oligonucleotides. Moreover, these assays determine only the amount of human DNA present in the sample. The HUmu PCR we established is rapid and reliable, since it amplifies both human and mouse DNA simultaneously in a duplex reaction and quantifies the DNA amount in real-time mode. The costs are comparable to the those of FACS analysis, considering the fluorescence dye labeled monoclonal antibodies required for FACS. Compared to the presently used PCR assays, the HUmu PCR is fast and results can be obtained in less than 90 minutes. Including DNA preparation for 96 samples the assay takes about 180 minutes. FACS analysis of 96 samples cannot be performed faster. Finally, there is no need for skilled personnel to determine the proportion of human cells, as is required in FACS analysis. We preferred the amplification of DNA rather than RNA in order to allow the analysis of various tissues independently of specific gene expression in different tissues. The duplex HUmu PCR reliably detects the DNA of 2×10^2 cells (2%) of one species, human or

mouse, in a background of DNA corresponding to 1×10^4 cells of the other species. This detection limit was improved to the detection limit of FACS analysis (0.5–1.0%), when the HU PCR and the mu PCR were performed separately, a result in agreement with general considerations of duplex PCR. We, therefore, recommend the application of the duplex assay only when a detection limit of >2% is sufficient or the chimeric DNA material is rare and two independent PCRs can not be performed. Recently used PCR assays displayed detection limits of <0.1% human DNA in murine DNA.[7] Consequently, these assays are superior when only minimal amounts of human DNA have to be detected, but are not suitable for quantitative determinations even when high amounts of human DNA are present. In this case conventional PCR is always in or near the plateau phase. However, the HUmu PCR provides a sensitivity sufficient for most investigations and needs only low amounts of blood or bone marrow. In addition, the concurrent quantification of human and murine DNA provides a higher accuracy and an internal amplification control.

The use of defined cell numbers instead of plasmids[9] for calibration of the quantitative assay seems to afford a high reproducibility. We prefer this calibration strategy, because it considers the loss of DNA during preparation and the quality of DNA exactly as occurs in regular quantification experiments from mouse blood or bone marrow. Our results indicate, that even when different cell types of human and mouse origin were prepared, calibration curves are indistinguishable. However, to ensure accurate calibration, we propose that reliable calibration should always be performed with the cells of interest.

The results obtained from the FACS analysis were in good agreement with the results obtained with the HUmu PCR. The discrepancy we found could be explained by the determination of different cell components, antigens and DNA.

Summing up, the HUmu PCR is a suitable PCR method for the quantification of human DNA in the background of murine DNA. Although it can not replace the multi-parameter FACS analysis, HUmu PCR may be useful to support FACS analysis results or for the investigation of non-hematopoietic tissues, such as liver, spleen, lung or brain.

Contributions and Acknowledgments

AN established the PCR with MB, co-ordinated the entire project and wrote the manuscript. AN and MB analyzed the PCR data. IJ was responsible for cell enrichment of CD34 cells from cord blood and transplantation of mice, JA performed the engraftment quantification by FACS analysis. AN and OL designed the PCR assays. IF was responsible for the mouse related data analysis. BW and WS, as senior scientists, initiated the project, supervised it and wrote the manuscript with AN. All the authors critically revised the manuscript and gave the final approval for its submission. We gratefully acknowledge the expert technical assistance of Delia Barz, Monika Becker and Margit Lemm.

Funding

This work was supported by a grant from the Deutsche Krebshilfe (10-1362-Si I).

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Dr. Vittorio Rosti, who acted as an Associate Editor. The final decision to accept this paper was taken jointly by Dr. Rosti and the Editors. Manuscript received January 8, 2001; accepted May 16, 2001.

Potential implications for clinical practice

As long as immunodeficient mice are not used in routine analysis of blood stem cell preparations, there is no direct clinical use.

References

1. van der Loo JC, Hanenberg H, Cooper RJ, Luo FY, Lazaridis EN, Williams DA. Nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mouse as a model system to study the engraftment and mobilization of human peripheral blood stem cells. Blood 1998; 92:2556-70.
2. Cashman JD, Lapidot T, Wang JC, et al. Kinetic evidence of the regeneration of multilineage hematopoiesis from primitive cells in normal human bone marrow transplanted into immunodeficient mice. Blood 1997; 89:4307-16.
3. de Wynter EA, Coutinho LH, Pei X, et al. Comparison of purity and enrichment of CD34+ cells from bone marrow, umbilical cord and peripheral blood (primed for apheresis) using five separation systems. Stem Cells 1995; 13:524-32.
4. Piacibello W, Sanavio F, Severino A, et al. Engraftment in nonobese diabetic severe combined immunodeficient mice of human CD34(+) cord blood cells after ex vivo expansion: evidence for the amplification and self-renewal of repopulating stem cells. Blood 1999; 93:3736-49.
5. Wang JC, Doedens M, Dick JE. Primitive human hematopoietic cells are enriched in cord blood compared with adult bone marrow or mobilized

peripheral blood as measured by the quantitative in vivo SCID-repopulating cell assay. Blood 1997; 89: 3919-24.
6. Cashman JD, Eaves CJ. Human growth factor-enhanced regeneration of transplantable human hematopoietic stem cells in non-obese diabetic/severe combined immunodeficient mice. Blood 1999; 93:481-7.
7. Mobest D, Goan SR, Junghahn I, et al. Differential kinetics of primitive hematopoietic cells assayed in vitro and in vivo during serum-free suspension culture of $CD34^+$ blood progenitor cells. Stem Cells 1999; 17:152-61.
8. Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. Genome Res 1996; 6:986-94.
9. Nitsche A, Steuer N, Schmidt CA, Landt O, Siegert W. Different real-time PCR formats compared for the quantitative detection of human cytomegalovirus DNA. Clin Chem 1999; 45:1932-7.
10. Goan SR, Schwarz K, von Harsdorf S, et al. Fibroblasts retrovirally transfected with the human IL-3 gene initiate and sustain multilineage human hematopoiesis in SCID mice: comparison of CD34-enriched vs CD34-enriched and in vitro expanded grafts. Bone Marrow Transplant 1996; 18:513-9.
11. Ramirez M, Rottman GA, Shultz LD, Civin CI. Mature human hematopoietic cells in donor bone marrow complicate interpretation of stem/progenitor cell assays in xenogeneic hematopoietic chimeras. Exp Hematol 1998; 26:332-44.