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Optimized retroviral transduction protocol for human progenitor cells utilizing fibronectin fragments

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Background

Retroviral transduction in the presence of fibronectin (FN) fragments has proven an efficient and clinically-applicable procedure for gene transfer into hematopoietic cells. So far, FN-based transduction protocols have been optimized primarily for transduction of stem cells, whereas for several therapeutic applications transduction of clonogenic progenitors (CFU) may be sufficient.

Methods

Transduction protocols for CFU were optimized by evaluating the effect of growth factors, timing of retroviral transduction, CD34-selection and heparin, using a neomycin-phosphotransferase (neo^R)-expressing retroviral vector.

Results

The presence of multiple growth factors during prestimulation and transduction, including the differentiating cytokines G-CSF or GM-CSF, substantially enhanced transduction of CFU. Best results were achieved when 24 h of prestimulation were followed by a 24–48 h transduction period in the presence of the CH-296 FN-fragment and IL-3, IL-11, SCF, erythropoietin (EPO), and GM-CSF. With this protocol we observed highly efficient transduction of BM-derived CFU (90.7 ± 8.8 % G 418-resistant colonies), even with retrovirus preparations of moderate infectious titer ($5 \times 10^4 - 2 \times 10^5$ CFU/mL). The number of CFU increased on average 2.6-fold (range 1.5–3.8) during the transduction procedure. Selection of $CD34^+$ cells prior to transduction did not improve transduction efficiency. Heparin, even in concentrations as low as 2.0 µg/mL, significantly inhibited transduction of CFU on FN-fragments.

Discussion

An optimized protocol for retroviral gene transfer into human clonogenic progenitor cells that allows highly efficient transduction, even with moderate titer retroviral vectors, is presented.

Keywords

fibronectin, retroviral gene transfer, hematopoietic cells, cytokines.

Introduction

Retroviral gene transfer into hematopoietic cells holds the promise of new therapeutic options for severe and potentially fatal diseases, for which the chances of cure are limited at present [1–3]. For a long time the clinical application of this technology has been hampered by low gene-transfer efficiency for the hematopoietic target cells. This has in particular been a problem with regard to long-term repopulating stem cells (LTRC). Transduction of these cells seems mandatory for strategies aiming at the lifelong cure of monogenetic diseases with manifestation in the hematopoietic system, such as Fanconi anemia, Gaucher's disease, or inherited immunodeficiency syndromes. However, transduction of more committed cell populations, such as lymphoid cells or clonogenic progenitor cells, may be sufficient for approaches aiming at control of GvHD after stem-cell transplantation [4] or protection of the hematopoietic system from chemotherapy-induced myelosuppression [5–9].

Despite recent progress in the development of alternative gene transfer systems, such as lentiviral vectors [10,11], retroviral vectors currently represent the standard

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technology for clinical studies investigating gene transfer into hematopoietic cells. Early retroviral transduction strategies for hematopoietic cells used coculture of hematopoietic target cells with stromal cells, such as retroviral producer cell-lines or primary BM fibroblasts [12,13]. Although these strategies yielded satisfactory gene-transfer efficiency, safety concerns and logistic problems minimize their feasibility in clinical practice. Nowadays, transduction of hematopoietic cells for clinical applications is generally performed with cell-free retrovirus-containing supernatant. The addition of polycations, such as protamine or polybrene, to facilitate the initial cell–virus interaction [14], and of hematopoietic growth factors, to stimulate cycling and retroviral receptor expression of hematopoietic target cells [15–17], has proven helpful. Meanwhile, efficient transduction of hematopoietic cells from human BM, cord blood and peripheral blood using cell-free retrovirus has been described by several authors [18–20].

Recently, fibronectin (FN) fragments have been shown to increase efficiency of retrovirally-mediated gene transfer into hematopoietic cells [21–26]. However, so far FN-fragments have mainly been used in transduction protocols particularly designed for the transduction of primitive stem cells, such as murine long-term reconstituting cells or human cells with the capacity to repopulate immunodeficient SCID-, NOD/SCID-, or BNX-mice [22,24–27]. Highly efficient transduction of clonogenic cells (> 50%) was only detected when retroviral supernatant with high infectious titers was used, while reduction of viral titer to 10^5 virions/mL reduced transduction rates significantly [21]. Since the large-scale production of high-titer retroviral supernatants, as necessary for clinical applications, may represent a problem with certain retroviral vectors, a procedure allowing efficient transduction of clonogenic cells even with moderate-titer retroviral preparations seems warranted.

We report on studies in which we have systematically modified FN-based gene-transfer protocols in order to specifically optimize gene transfer into human clonogenic progenitor cells. In particular, the effects of hematopoietic growth factors, timing of retroviral transduction and CD34-selection of target cells on transduction efficiency were investigated. As the result, we report a clinically-applicable transduction protocol that allows highly efficient transduction of human clonogenic progenitor cells, even with moderate-titer retroviral vectors. In addition, we have investigated heparin as an inhibitor of FN-enhanced retroviral gene transfer and demonstrated complete inhibition of the beneficiary effect of FN when heparin is present at concentrations as low as 2 μg/mL.

Methods

Retroviral vectors and producer cell lines

The structure of the N_2/Zip TK-NEO (TKNEO) vector has been described previously [20]. In this vector, the neomycinphosphotransferase (*neo*^{R}) gene is expressed from the herpes simplex virus thymidine kinase promoter. The packaging cell-line GP+*env*Am 12 [28] producing TKNEO retroviral particles was cultured in IMDM (Sigma Aldrich Chemie, Deisendorf, Germany) containing 10% FCS (Sigma) and 1% penicillin–streptomycin (Sigma). Virus-containing supernatant was collected after adding 10 mL of fresh IMDM supplemented with 20% FCS to confluent plates of TKNEO producer cells overnight. The next day, the medium was harvested, filtered through 0.45 μm filters (Gelman Sciences, Ann Arbor, MI, USA) and stored at −80°C until used. Two preparations of virus-containing supernatant differing slightly in infectious titer (2×10^5 and 5×10^4 G418-resistant colony-forming units (CFU)/mL) were used. Titration was performed according to standard procedures using resistance of TKNEO transduced NIH/3T3 fibroblasts to 0.75 mg of G 418 (GIBCO BRL, Paisley, Scotland) [28]. Control supernatant for mock transduction was harvested from a non-transduced GP+*env*Am 12 packaging cell-line, using the same procedure as that used for the production of retroviral supernatant from the TKNEO producer cell-line.

Retroviral transduction protocol

BM samples from healthy donors were collected in tubes containing sterile, preservative-free sodium sulfate heparin according to protocols approved by the local ethics committee. Low-density mononuclear cells (MNC) were prepared by centrifugation on Ficoll-Hypaque (Pharmacia Biotech Europe, Freiburg, Germany). Plastic-adherent cells were removed from low-density BM cells, by incubation on non-tissue culture treated dishes for 4–16 h in IMDM and 20% FCS at 37°C and 5% CO_2. For some experiments cells were enriched for CD34$^+$ cells using the MidiMacs

purification system according to the manufacturer's instruction (Miltenyi Biotec, Bergisch Gladbach, Germany). Purity of the CD34-enriched cell fraction (range 55–80%) was controlled by FACS analysis using the anti-CD34 Ab HPCA-2 (Becton Dickinson, Erembodegem-Aast, Belgium). Adherent negative low-density MNC or CD34-enriched cells were prestimulated for 24 h in IMDM, 20% FCS at 37°C and 5% CO_2 using different combinations of the following recombinant human cytokines: 50 ng/mL IL-3, 20 ng/mL IL-6 (both kindly provided by Novartis Pharma, Basel, Switzerland), 50 ng/mL IL-11 (Genzyme Diagnostics, Cambridge, MA, USA), 20 ng/mL rh stem cell factor (SCF; Pepro Tech, London, UK), 100 ng/mL rh GM-CSF (Novartis Pharma), 1000 U/mL G-CSF (Amgen München, Germany), 6 U/mL erythropoietin (EPO; Boehringer, Mannheim, Germany). After prestimulation, cells were harvested by vigorous pipetting to remove loose plastic-adherent cells. Subsequently, prestimulated cells were transduced with retroviral supernatant (1×10^5 MNC/mL or 2×10^4 $CD34^+$/mL) on culture dishes coated with BSA (control dishes) or FN-fragment CH-296 (Retronectin; Bio Whittaker Europe, Taufkirchen, Germany) in the presence of the same growth factors as used for prestimulation.

After 2 h an equal volume of fresh retroviral supernatant containing no additional cytokines was added. Virus supernatant was replaced with fresh virus (including cytokines) after 24 h; any non-adherent cells were re-added with the fresh virus supernatant. Again, fresh supernatant without cytokines was added 2 h later. Cells were incubated in the presence of retrovirus-containing supernatant for a total of 48 h. Control transductions were performed with GP+*env*Am 12 supernatant (mock) or medium. In some of the experiments the time-course of transduction was slightly modified, as stated below. Finally, hematopoietic cells were harvested using treatment with 0.5 % trypsin/EDTA (Sigma) and vigorous pipetting to recover adherent cells. Adherent and non-adherent cells were pooled and cultured in clonogenic progenitor assays.

In some experiments 5 μg/mL protamine sulfate (Sigma), 8 μg/mL polybrene (Sigma) or 0.05–500 μg/mL heparin (Novo Nordisk, Mainz, Germany) were added to the retroviral supernatant prior to retroviral transduction. Polybrene was only added at 0 h and 24 h during the transduction period.

Coating of culture dishes with FN-fragment CH-296

The recombinant FN-fragment CH-296, which contains the connecting segment, the central cell-binding domain and the heparin-binding domain of plasma-FN, was immobilized on 10 or 35 mm non-tissue culture treated dishes (Becton Dickinson, Lincoln Park, NJ, USA) at a concentration of 4 μg/cm^2. This was followed by blocking of the plates with 2% BSA (Fluka Chemie, Buchs, Switzerland) as previously described [23]. Control dishes were coated with 2% BSA.

Clonogenic progenitor assays

The assays were performed using standard procedures as described by Toksoz *et al.* [29]. After retroviral transduction 1×10^4 MNC or 1×10^3 CD34-enriched cells were plated in 1 mL of IMDM/0.9% methylcellulose (Fluka) containing 50 ng/mL IL-3, 10 ng/mL SCF, 6 U/mL EPO, 25% FCS, 10% human plasma, 10^{-5} M β-mercaptoethanol, and 1% penicillin–streptomycin. Culture dishes were incubated at 37°C and 5% CO_2, and colonies (> 50 cells) were scored on Day 13 as CFU-GM (containing granulocytes and macrophages), or BFU-E plus CFU-Mix (containing erythroid cells alone, or in combination with myeloid elements, respectively). BFU-E and CFU-Mix were summarized in one category as most colonies appearing to be BFU-E (> 70 %) also contained some myeloid cells when picked from the methylcellulose, blown out on a slide, stained and analyzed morphologically at the single-cell level under the microscope.

Analysis of retroviral transduction efficiency

The efficiency of transduction was analyzed by determining the percentage of colonies resistant to 1.5 mg/mL G418 (GIBCO BRL). Colonies of mock-transduced control cells consistently demonstrated < 1% background colony growth after exposure to 1.5 mg/mL G418.

Statistics

All experiments were performed in triplicate and results are shown as mean ± SEM, unless indicated otherwise. Differences between transduction rates of various cytokine combinations were evaluated using analysis of variance in combination with Scheffé test. Correlation between transduction and expansion of clonogenic progenitors was examined utilizing Spearman's correlation coefficient. A two-sided Student's *t* test was performed to

compare transduction efficiencies on FN-fragment, polybrene and protamine. The general level of significance was postulated $\alpha = 0.05$ unless indicated otherwise.

Results

Effect of hematopoietic growth factors on transduction efficiency

The effect of hematopoietic growth factors on the transduction efficiency of clonogenic progenitor cells was analyzed for various combinations of the cytokines IL-3, IL-6, IL-11, SCF, G-CSF, GM-CSF and EPO. For the first set of experiments a prestimulation period of 24 h, followed by a transduction period of 48 h, was used. Fresh retroviral supernatant was added at hours 24, 26, 48, and 50 of growth factor stimulation. In comparison with transduction protocols using single growth factors, a significant increase in gene-transfer efficiency was demonstrated when combinations of four or five growth factors were used (Figure 1). Combinations including G-CSF or GM-CSF were superior to combinations without these factors and the highest transfer efficiency for the neo^R marker gene was demonstrated in the presence of the growth factors IL-3, IL-11, SCF, GM-CSF ± EPO. Utilizing these factor combinations 88.0 ± 5.0 % (n = 3) and 90.7 ± 8.8 % (n = 3) of clonogenic progenitors, respectively, became resistant to G418 after retroviral transduction. In contrast to the transduction rates observed with these cytokines, transduction protocols using single growth factors, or certain double growth factor combinations, yielded significantly lower transduction rates ($p < 0.05$). This also included the combination IL-6 and SCF (38.0 ± 3.3 %; $p < 0.05$) that has already been applied successfully to transduction of more primitive hematopoietic cells [30].

Utilizing the growth factor combination IL-3, IL-11, SCF, EPO, GM-CSF, similar transduction rates for myeloid progenitor cells (CFU-GM; 70.9 ± 6.4 %) and erythroid plus mixed progenitor cells (BFU-E/CFU-Mix; 73.0 ± 4.9 %) were observed (Figure 2). BFU-E and CFU-Mix were summarized in one category as most red cell-containing colonies also contained some myeloid cells (see Methods above). Similarly, there were no significant differences when transduction rates for CFU-GM and BFU-E/CFU-Mix were compared in the presence of IL-3, IL-11, SCF, G-CSF (CFU-GM 70.2 ± 15.7%; BFU-E/CFU-Mix 76.0 ± 18.8%). In contrast, transduction in the presence of SCF alone (CFU-GM 41.0 ± 3.6%; BFU-E/CFU-Mix

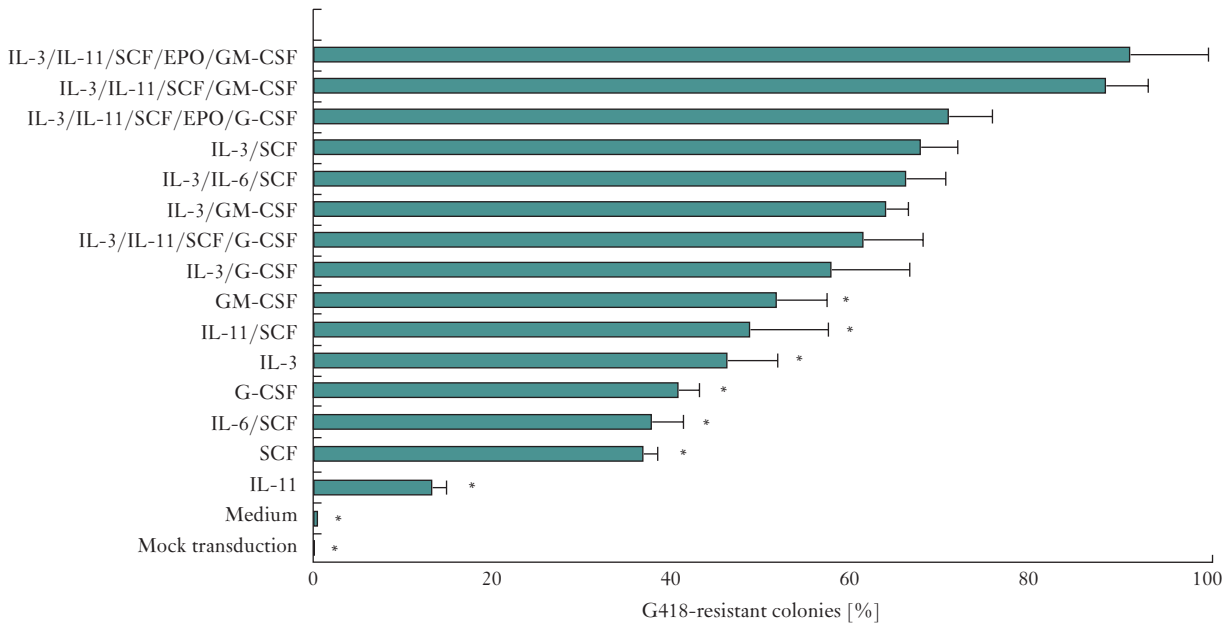

Figure 1. *Transduction of clonogenic progenitor cells on fibronectin: influence of growth factors. Following a 24 h prestimulation period MNC were repeatedly transduced with TKNEO-containing retroviral supernatant on fibronectin fragments for a total of 48 h. The same growth factors were present during prestimulation and transduction. Percentage of G418-resistant progenitor-derived colonies (mean ± SEM) is given for 3–6 independent experiments. *denotes significant difference in comparison to IL-3, IL-11, SCF, EPO, GM-CSF using analysis of variance in combination with Scheffé test ($p < 0.05$).*

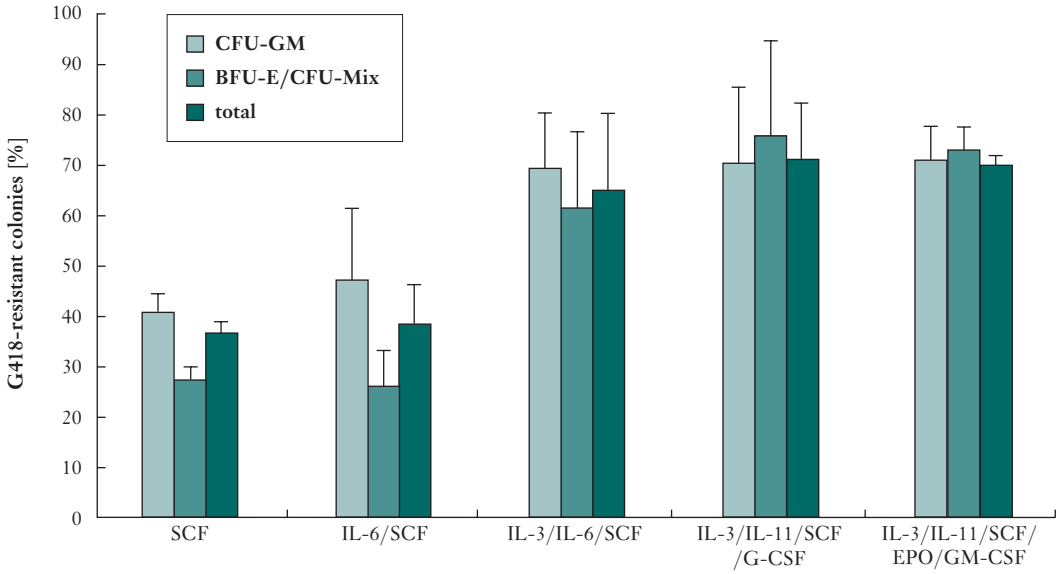

Figure 2. *Transduction of clonogenic progenitor subpopulations on fibronectin: MNC were transduced with TKNEO-containing retroviral supernatant in the presence of various growth factor combinations. Percentage of G418-resistant progenitor-derived colonies (mean ± SEM) is given for 3–6 independent experiments.*

27.3 ± 3.1%) or IL-6, SCF (CFU-GM 47.5 ± 14.0%, BFU-E/CFU-Mix 25.8 ± 7.1%), favored transduction of myeloid progenitor cells over progenitors with erythroid potential.

The presence of multiple growth factors not only improved gene-transfer efficiency, but also led to expansion of clonogenic progenitors during the prestimulation and transduction period. There was significant correlation between transduction efficiency and expansion of CFU ($r = 0.69$; $p = 0.004$), and the highest expansion was seen with the same growth factor combination that also worked best for retroviral transduction (Table 1). In comparison to input numbers, clonogenic progenitor cells increased 2.6 ± 0.7-fold with the growth factor combination IL-3, IL-6, SCF, EPO, GM-CSF and 2.1 ± 0.2-fold with IL-3, IL-11, SCF, EPO, G-CSF. This corresponded to the generation of 248 ± 77 or 152 ± 16 transduced clonogenic progenitor cells per 100 CFU present at the beginning of the prestimulation period.

CD34-selection of hematopoietic target cells

Certain experimental settings have identified the virus:target-cell ratio as a critical parameter for efficient retroviral transduction [31]. We therefore compared transduction of progenitor cells within a population of MNC with transduction of progenitor cells within a population enriched for $CD34^+$ cells. This selection procedure reduces the amount of cells present during the transduction procedure 20–100-fold, resulting in a substantially increased virus:target-cell ratio. Transduction rates, however, were not significantly different for experiments using MNC or CD34-enriched cells. Using the established IL-3, IL-11, SCF, EPO, GM-CSF multiple growth factor combination, transduction of CFU within a population of MNC yielded 73.7 ± 1.3% G 418^R colonies, while 71.3 ± 1.3 % G 418^R colonies were observed when transduction was performed with CD34-enriched cells ($n = 3$).

Timing of retroviral transduction

We evaluated the influence of timing of retroviral transduction relative to the cytokine-stimulation period. For retroviral transduction of primitive $CD34^+38^-$ cells in the presence of FN-fragments and cytokine combinations, such as IL-6, SCF or SCF, G-CSF and thrombopoietin, a 48 h prestimulation period has been required for maximal efficiency [30]. This was confirmed by our own experiments.

With the factor combination IL-6, SCF a maximal transduction efficiency of 37 ± 6% was observed when double transduction was performed after 48 and 50 h of cytokine stimulation. In contrast, only 18 ± 4% or 20 ± 7% G 418^R colonies were detected, when target

Table 1. Transduction and expansion of clonogenic progenitor cells on fibronectin

Growth factor combination	Transduction[1]	Expansion[2]	Transduced CFU per 100 CFU input[3]
IL-3/IL-11/SCF/EPO/GM-CSF	90.6 ± 8.8	2.6 ± 0.7	248.6 ± 77.2
IL-3/IL-11/SCF/EPO/G-CSF	71.8 ± 4.7	2.1 ± 0.2	152.5 ± 15.8
IL-3/SCF	67.7 ± 4.2	1.8 ± 0.2	127.5 ± 22.4
IL-3/IL-11/SCF/GM-CSF	88.0 ± 5.0	1.4 ± 0.4	127.4 ± 39.5
IL-3/IL-6/SCF	64.8 ± 4.3	1.8 ± 0.2	124.4 ± 22.0
IL-3/GM-CSF	63.7 ± 2.6	1.8 ± 0.3	115.6 ± 24.2
IL-3/IL-11/SCF/G-CSF	61.3 ± 6.6	1.5 ± 0.3	96.0 ± 25.0
IL-3	46.3 ± 5.5	2.0 ± 0.1	93.3 ± 13.7
IL-3/G-CSF	57.7 ± 8.7	1.3 ± 0.2	73.4 ± 10.2
IL-11/SCF	48.7 ± 8.9	1.1 ± 0.1	54.5 ± 9.0
IL-6/SCF	38.0 ± 3.3	1.3 ± 0.1	48.5 ± 6.2
GM-CSF	51.7 ± 5.5	0.8 ± 0.1	42.6 ± 3.0
SCF	36.7 ± 1.7	1.1 ± 0.3	38.3 ± 8.2
G-CSF	41.0 ± 2.1	0.8 ± 0.1	33.1 ± 5.8
IL-11	12.3 ± 1.9	1.1 ± 0.1	12.7 ± 1.0

Transduction of mononuclear cells was performed with TKNEO-containing retroviral supernatant in the presence of various growth-factor combinations. Transduction efficiency is shown as percentage of G418-resistant CFU[1], expansion rates in multiples of CFU input[2] and number of transduced CFU per 100 CFU input[3], calculated as the product of transduction × expansion are given for 3–6 independent experiments as mean ± SEM.

cells were double-transduced after 24 and 26 h, or multiple transductions were performed after 24, 26, 48, and 50 h of cytokine stimulation, respectively. Optimal transduction in the presence of IL-3, IL-11, SCF, EPO, GM-CSF was achieved with a shorter prestimulation period of 24 h (Table 2). No further improvement was noted when extending the prestimulation period to 48 h or using multiple transductions after 24, 26, 48, and 50 h of cytokine stimulation. In independent experiments 55 ± 7%, 52 ± 9% and 51 ± 9% G 418^R colonies were detected when the retrovirus was added after 24 and 26 h only, 48 and 50 h only, or 24 and 26 plus 48 and 50 h, respectively (n = 3).

Comparison with supernatant transduction protocols utilizing protamine or polybrene

Enhancement of retroviral transduction of target cells in the presence of polycations, such as protamine or polybrene, is a well-established procedure and has been extensively used for the transduction of hematopoietic cells. In order to compare the efficiency of FN-fragments with the best currently-available alternative methods of cell-free supernatant transduction, we transduced clonogenic cells in the presence of protamine (5 μg/mL) or polybrene (8 μg/mL), using the growth factor combinations IL-3, IL-11, SCF, EPO, GM-CSF or IL-6, SCF. Transduction in the presence of FN-fragments was

Table 2. Transduction of clonogenic progenitor cells on fibronectin: timing of transduction

Time of tranduction	IL-3/IL-11/SCF/EPO/G-CSF				IL-6/SCF			
Relative to cytokine stimulation	1	2	3	a ± SEM	1	2	3	a ± SEM
24 and 26 h	53	61	67	55 ± 7	21	10	24	18 ± 4
48 and 50 h	44	60	71	52 ± 9	48	26	38	37 ± 6
24, 26 and 48, 50 h	47	62	69	51 ± 9	24	6	29	20 ± 7

MNC or CD34-enriched cells were transduced with TKNEO-containing retroviral supernatant following different intervals of cytokine stimulation. The percentage of G418-resistant CFU (mean ± SEM) for individual experiments is given and summarized as mean(a) ± SEM.

significantly more efficient than protocols applying polybrene or protamine. In three independent experiments polybrene- or protamine-aided transduction yielded only 44 ± 5% or 28 ± 11% of the transduction efficiency observed with the CH-296 based protocol ($p < 0.05$).

Inhibitory effect of heparin on FN-enhanced retroviral gene transfer

Co-localization of retrovirus and hematopoietic target cell has been suggested as one functional mechanism underlying the enhanced gene-transfer efficiency in the presence of FN-fragments [22]. In this co-localization-model, binding of the retrovirus occurs within the c-terminal high-affinity heparin-binding domain of FN [23]. Therefore, it appeared reasonable to investigate heparin as an inhibitor of FN-enhanced retroviral gene transfer and several investigators have indeed observed an inhibitory effect of heparin (D. Carstanjen, H. Hanenberg, unpublished observation). In order to evaluate the relevance of this observation for clinical gene-transfer protocols, we transduced clonogenic progenitor cells in the presence of different concentrations of unfractionated heparin (Figure 3). These experiments demonstrated the inhibitory effect of very low concentrations of heparin on FN-enhanced gene transfer. Even at 2 µg/mL heparin (equivalent to 0.3 U/mL), the beneficial effect of CH-296 on retroviral transduction of clonogenic cells was completely inhibited.

Discussion

Efficient gene transfer and maintained gene expression in the hematopoietic target cell constitutes a prerequisite for meaningful clinical studies in the field of hematopoietic-cell gene therapy. Most efforts so far have focused on transduction of long-term reconstituting stem cells. Disappointing results in the majority of clinical studies, however, have reduced much of the early enthusiasm for hematopoietic-cell gene therapy. Recently, more promising results have been described in primate models and clinical studies [32–34], but stable and efficient gene transfer into long-term repopulating stem cells remains problematic.

For certain applications more differentiated target cells may be an interesting alternative: transduction of clonogenic progenitor cells, for example, represents a valid strategy to protect patients from the myelotoxicity associated with cytotoxic chemotherapy. Here we report a highly-efficient retroviral transduction protocol for human clonogenic progenitor cells, allowing the transduction of up to 90% of these cells — even with moderate-titer retroviral vectors. In addition, a more than 2.5-fold expansion of clonogenic cells occurs during the

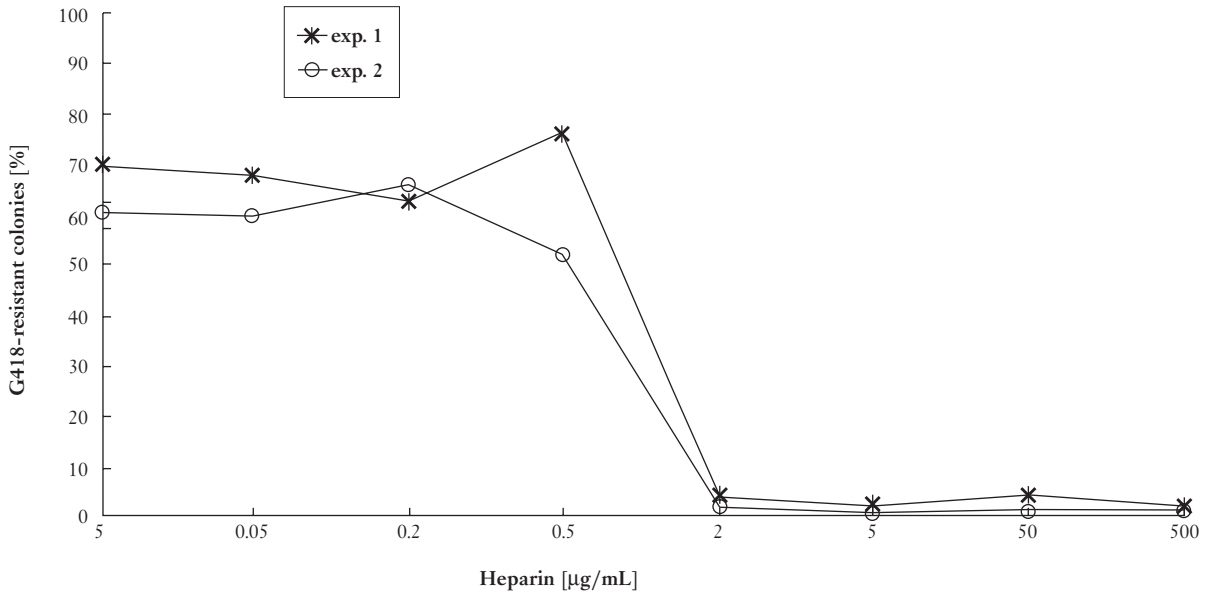

Figure 3. *Transduction of clonogenic progenitors on fibronectin: influence of heparin. MNC were transduced with TKNEO-containing retroviral supernatant in the presence of various concentrations of heparin. Percentage of G418-resistant progenitor-derived colonies is given for two independent experiments.*

transduction procedure, resulting in an output of up to 250 genetically-modified progenitor cells per 100 progenitor cell input at the beginning of prestimulation. The observation that retroviral gene-transfer efficiency and expansion are correlated comes as no surprise as both parameters are strongly linked to the proliferation rate of hematopoietic target cells.

The protocol described here combines growth-factor stimulation of hematopoietic target cells with transduction in the presence of FN-fragments, a strategy that already has been used by several investigators to optimize gene transfer into primitive hematopoietic target cells [22,25,27, 30,35,36]. Transduction protocols for primitive hematopoietic cells and, in particular, long-term reconstituting stem-cells usually avoid the use of late-acting and differentiating growth factors [32,34,35,37]. In contrast, transduction efficiency of the more differentiated clonogenic progenitor cells is significantly augmented by G-CSF, GM-CSF, EPO and/or IL-3 exposure of target cells prior to and during the transduction procedure. Similarly, optimal timing of retroviral transduction differs between clonogenic and more primitive cells.

Optimal transduction of primitive $CD34^+38^-$ cells has been shown to require 48 h of cytokine stimulation, whereas transduction at 24 h decreases gene-transfer efficiency [30]. Our data demonstrate that clonogenic cells can efficiently be transduced after 24 h of adequate cytokine stimulation.

CD34-enrichment of target cells represents an efficient method of increasing the virus:target-cell ratio before retroviral transduction. Depending on the content of $CD34^+$ cells in the initial specimen and the quality of the enrichment procedure, cell numbers can be reduced between 10- and over 100-fold. For clinical gene-therapy trials this constitutes a major logistical and financial advantage. In the context of comparatively low-titer retroviral vectors, such as the one used in our study, an increased virus:target-cell ratio would have also been expected to improve gene-transfer efficiency. Thus, the missing effect of CD34-enrichment on transduction efficiency observed in our study appears surprising. The data are in accordance, however, with the experiences of other investigators.

FN-fragments enhance retroviral gene transfer by binding and co-localizing target cells and retroviral particles. In this co-localization model, binding of the retrovirus is achieved by the carboxyterminal high-affinity heparin-binding domain of FN. A role of heparin as an inhibitor of retroviral binding in this context seemed a reasonable speculation. Indeed, heparin has already been demonstrated to act as an inhibitor of retroviral binding to FN and as a potent inhibitor of FN-enhanced gene transfer at concentrations of 100-500 U/mL (D. Carstanjen, manuscript in preparation). We now demonstrate inhibition of gene transfer by unfractionated heparin down to concentrations of 0.5 to 2 μg/mL(− 0.07–0.3 IU/mL). This finding could have several implications for clinical gene-transfer protocols.

As fibroblast cell lines may produce heparin or related proteoglycans, these substances may also be present in the supernatant of retroviral producer cell-lines. According to our own experience and that of several other groups, this does not seem to present problems with the commonly-used producer cell lines; e.g. GP+*env*AM12-based producer lines have been tested negative for heparin production with a detection threshold of 2.8μg/mL (D. Carstanjen, unpublished observation). On the other hand, heparin or proteoglycan interference with FN-enhanced gene transfer might be considered when unexpectedly low transduction rates are observed in the context of new and less well characterized producer cell-lines. It may also be advisable to restrict heparin and proteoglycans in the process of collecting and preparing target cells for retroviral gene transfer, unless elimination of these substances before transduction is ensured by subsequent procedures.

In summary, we have presented an optimized protocol for retroviral gene transfer into human clonogenic progenitor cells, utilizing prestimulation with multiple growth factors as well as transduction in the presence of FN-fragments. This protocol allows highly efficient transduction of clonogenic cells, even with moderate-titer retroviral vectors. It may be clinically-useful in situations in which expression of a therapeutic transgene in the hematopoietic system is required only for a limited time, such as the transfer of the drug-resistance genes *MDR-1*, mutant dihydrofolate reductase, or *O^6-MGMT*, in order to reduce myelosuppression after intensive cytotoxic chemotherapy.

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