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RESEARCH ARTICLE

Reconstitution of a metabolic pathway with triple-cistronic IRES-containing retroviral vectors for correction of tetrahydrobiopterin deficiency

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Abstract

Background Tetrahydrobiopterin (BH_4) is an essential cofactor for catecholamine and serotonin neurotransmitter biosynthesis. BH_4 biosynthesis is carried out in a three-enzyme pathway involving GTP cyclohydrolase I (GTPCH), 6-pyruvoyl-tetrahydropterin synthase (PTPS) and sepiapterin reductase (SR). Treatment of genetic defects leading to BH_4 deficiency requires neurotransmitter replacement since synthetic cofactor does not efficiently penetrate the blood–brain barrier. Autologous fibroblasts transplanted into the brain as depository cells for drug delivery might offer an alternative. However, normal fibroblasts do not express GTPCH, and fibroblasts from PTPS patients lack two biosynthetic enzymes for BH_4 production.

Methods We engineered primary fibroblasts by the use of triple-cistronic, retroviral vectors for cofactor production.

Results Constitutive SR activity in these cells enabled BH_4 biosynthesis by transducing GTPCH and PTPS cDNAs together with a selective marker coupled in a single transcript with two IRES-elements in tandem. Upon reaching a critical concentration (>400 pmol/mg protein) of intracellular BH_4, the fibroblasts efficiently released cofactor even under non-dividing conditions.

Conclusion The use of triple-cistronic vectors for single transduction to reconstitute metabolic pathways or to treat multi-genetic diseases may be useful for engineering, for instance, depository cells for various organs, including the nervous system. Copyright © 2000 John Wiley & Sons, Ltd.

Keywords tetrahydrobiopterin deficiency; neurotransmitter depletion; hyperphenylalaninemia; gene transfer; retrovirus

Introduction

Tetrahydrobiopterin (BH_4) is an essential cofactor for tyrosine and tryptophan hydroxylases, the rate-limiting enzymes for catecholamine and serotonin neurotransmitter biosynthesis. BH_4 is also required for the nitric oxide synthase enzymes present in various organs, and for the catabolic phenylalanine hydroxylase in liver [1]. The BH_4-biosynthetic pathway starts with the conversion of guanosine triphosphate (GTP) to dihydroneopterin triphosphate by the enzyme GTP cyclohydrolase I (GTPCH). The second enzymatic step involves 6-pyruvoyl-tetrahydropterin synthase (PTPS) to convert dihydroneopterin triphosphate to 6-pyruvoyl tetrahydropterin. The

last step of the three-enzyme pathway is carried out by sepiapterin reductase (SR). SR is considered to be constitutively expressed [2], while PTPS is not, although enzyme activity is present in many cell types such as fibroblasts [A. Résibois and B. Thöny, unpublished observation]. GTPCH is constitutively present in at least liver and brain; however, it has to be stimulated with cytokines, for instance, in macrophages and skin fibroblasts [3–5].

Human GTPCH and PTPS are each encoded by single genes containing six exons, and map to chromosomes 14q21–q22.2 and 11q22.3–q23.3, respectively [6–10]. The mammalian GTPCH is thought to be a homodecameric complex composed of subunits with a molecular mass of 25 kDa, based on biochemical evidence [11] and sequence similarity to the *Escherichia coli* enzyme, whose structure was solved by X-ray crystallography [12,13]. This multimeric GTPCH enzyme complex exhibits a face-to-face association of two pentamers with ten equivalent active sites. The human PTPS is a homohexameric complex composed of two trimers with a subunit molecular mass of 16 kDa. Six equivalent active sites with a Zn^{2+} ion as an integral part of each reaction center are located at the trimer interfaces [14–17]. Interestingly, the C-terminal domain of the GTPCH monomer is topologically identical to a folded subunit of the PTPS, despite lack of significant sequence homology [18]. Upon recombinant expression for instance in bacterial cells both proteins, GTPCH and PTPS, spontaneously assemble into the correct homomultimers to form the active enzymes.

Owing to disorders in BH_4 metabolism, deficiencies of biogenic amine neurotransmitters and hyperphenylalaninemia are accompanied by severe and progressive mental retardation [19,20]. Autosomal recessive mutations in the respective biosynthetic genes have been reported for GTPCH and PTPS [21; for updates see also Ref. 22]. For patients with BH_4 deficiency, hepatic hyperphenylalaninemia is controllable with oral doses of 2–5 mg synthetic BH_4/kg/day. However, such relatively low doses of BH_4 do not allow the cofactor to penetrate the blood–brain barrier efficiently [23,24]. This problem can be overcome to some extent by administering higher doses of BH_4, up to 20 mg kg/day, together with corresponding neurotransmitter precursors [25,26]. Although such a combined therapy is mandatory to avoid neurological damage, it turned out not to be sufficient in every case [19]. We thus sought to develop alternative approaches, such as gene therapy, to treat BH_4 and neurotransmitter deficiency in the central nervous system (CNS).

To treat a neuro-metabolic disease like PTPS deficiency in the brain, we are pursuing an *ex vivo* approach using *in vitro* modification of primary skin fibroblasts, followed by transplantation of the genetically engineered cells. Skin fibroblasts are easily obtained and can be maintained and genetically modified under cell culture conditions. Furthermore, several studies for the combination of transgene-containing cells and intracerebral implantation have been evaluated so far. For instance, primary skin fibroblasts retrovirally transfected with the tyrosine hydroxylase gene for treatment of Parkinson's disease, or the gene for nerve growth factor or acetylcholine to treat Alzheimer's disease were implanted intracerebrally and successfully used in rat models (for reviews see Refs. [27,28]). In initial studies we showed that primary fibroblasts from PTPS deficient patients can be manipulated to synthesize BH_4 *in vitro* by retrovirus-mediated transfer of the human PTPS-cDNA, followed by stimulation of the GTPCH with cytokines [29]. Furthermore, with retrovirus-mediated double transduction of the human GTPCH and PTPS genes, cytokine stimulation in PTPS-deficient fibroblasts became obsolete. We also observed that BH_4 was synthesized and efficiently released by these *in vitro* manipulated cells [30].

In order to avoid double transduction of primary fibroblasts, we intended in a next step to construct a recombinant retroviral vector that would allow efficient expression of three genes, i.e., the two BH_4 pathway enzymes GTPCH and PTPS, and a selectable marker gene. It has previously been shown that three genes can be expressed from a single transcriptional unit by the use of two internal ribosomal entry sites (IRES) [31]. Here, we report on the characterization of triple-cistronic retrovirus vectors expressing human GTPCH, human PTPS and a selectable marker gene. The genes were expressed from one transcriptional unit starting at the 5′-LTR promoter by the use of two different IRES elements in tandem. Such metabolic engineering used here to generate BH_4-producing fibroblasts as depository cells may be useful for therapy of inborn errors of metabolism.

Materials and methods

Cell lines and cell culture

Primary skin fibroblasts used in this study were derived from a control subject, KII, as previously described [32], and from two PTPS-deficient patients, HW and NG. Both patients exhibited non-detectable PTPS activity in fibroblasts, and were compound heterozygous for PTPS, carrying different codon alterations that lead to non-functional, degraded enzymes [T. Scherer-Oppliger *et al.*, unpublished]. More detailed clinical information is available on the internet (http://www.unizh.ch/%7Eblau/biodef1.html) in the BIODEF patients' database of BH_4 deficiency under ID no. 281 for patient HW, and ID no. 285 for patient NG [22]. 293T cells (293/*tsA1609*neo) were obtained from M.P. Calos (Stanford University) [33]. The NIH-3T3 (CRL1658) and COS-1 (CRL1650) cells were obtained from the American Type Culture Collection (Rockville, MD, USA). All cell types used in this study were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 50 units/ml of penicillin, and 50 μg/ml of streptomycin (cDMEM; Life Technologies, Inc.). G418 for drug selection upon viral infection was from Life Technologies, Inc., Basel, Switzerland.

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Construction of triple-cistronic retroviral plasmid-vectors containing BH_4 biosynthetic genes

The MTIN (*moloney two IRES neomycin phosphotransferase*) [S. H. Kim and S. Kim; in preparation] plasmid vector was modified to express BH_4-biosynthetic enzymes (see also Figure 1). For subcloning, the unique restriction sites in front of the two IRES-elements were used, i.e., a *Bam*HI site in front of the EMCV IRES, and a *Sac*II and an *Hpa*I site in front of the FMDV IRES. *Escherichia coli* strain XL1-Blue [34] was used routinely as a plasmid host. For generating the pHSY3207 construct, the human GTPCH and PTPS cDNAs were inserted in front of the EMCV and FMDV IRES elements, respectively. The GTPCH cDNA (provided by M. Gütlich [35,36]) was PCR amplified using the same conditions and oligonucleotides as described previously [30]. The 794 bp PCR product was cut with *Bam*HI and *Bgl*II, followed by insertion into the single *Bam*HI of the MTIN vector. The PTPS cDNA [37] was PCR-amplified using the primers BH427 (5′-TCC**CCGCGG**GGAATGAGCACGGAAGGTGG-3′) and BH428 (5′-CG**GTTAAC**CTATTCTCCTTTATAAACCACA-3′; restriction sites for cloning are in bold). The oligonucleotide primers used in this study were synthesized on a gene Assembler Plus DNA Synthesizer (Amersham Pharmacia Biotech, Switzerland). The 470 bp PCR product was cut with *Sac*II and *Hpa*I, followed by ligation into the *Sac*II-*Hpa*I cut vector to place the PTPS in front of the FMDV IRES element. The MTIN backbone plasmid was also used to construct the pHSY3212 vector containing the GTPCH and PTPS cDNAs in the reverse order as compared to pHSY3207. Thus, the PTPS cDNA was PCR amplified using the same conditions and oligonucleotides as described previously [30]. The resulting 476 bp PCR fragment was cut with *Bam*HI and *Bgl*II, and cloned into the *Bam*HI site in front of the EMCV IRES element. For inserting GTPCH in front of the FMDV IRES element, the cDNA was amplified using the following oligonucleotides: BH430 (5′-TCC**CCGCGG**GGAATGGAGAAGGGCCCTGTG-3′) and BH431 (5′-CG**GTTAAC**TCAGCTCCTAATGAGAGTCAG-3′; restriction sites for cloning are in bold). The 773 bp PCR fragment was cut with *Sac*II and *Hpa*I, followed by insertion into the *Sac*II-*Hpa*I cut plasmid to generate pHSY3212.

Transient transfection, virus production and determination of viral titers

The level of GTPCH and PTPS-transgene expression from the modified MTIN vectors was determined by transient co-transfections of COS-1 cells with the retroviral vectors plus the pRSVβgal control plasmid (expressing the bacterial β-galactosidase from the SV40 promoter) by using a modified DEAE-dextran protocol [16]. The efficiency of transfection was determined by histochemical X-gal staining that ranged from 2 to 5%. The transient 'three-plasmid expression system' from A. Kingsman and co-workers was used to generate retrovirus stocks [38]. For this, 293T cells were co-transfected with the plasmids pHIT60 and pHIT456, and MTIN-derivatives (pHSY3207 and pHSY3212) by the calcium phosphate precipitation procedure [34]. The pHIT plasmids were obtained from A.J. Kingsman, Oxford University, UK. Plasmid pHIT60 contained the viral *gag* and *pol* genes under the CMV promoter, and pHIT456 expressed the amphotropic *env* gene starting from the CMV promoter. Viral supernatant from transfected 293T cells was collected after 48 h, filtered through a 0.45 µm-pore membrane, and stored at -80°C. To determine viral titers, NIH-3T3 cells were transduced [34], followed by G418 (800 µg/ml) selection, resulting in titers of 1.5×10^4 cfu/ml for HSY3207 and 1×10^4 cfu/ml for HSY3212.

Viral infection of human skin fibroblasts

To infect primary human skin fibroblasts, cells were grown to a density of 50% confluency in a six-well tissue culture plate. 1.5 ml of viral supernatant was added to each well with 8 µg/ml polybrene (Sigma), followed by 30 min of centrifugation at 32°C to increase infection efficiency [29,39]. This procedure was repeated once after 6–8 h. Three days later, the cells were split, plated on 10 cm dishes, and selected for 2 weeks with 800 µg/ml G418.

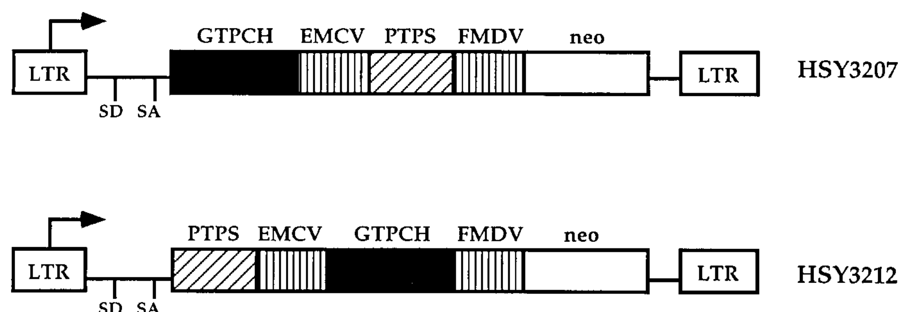

Figure 1. Schematic representation of retroviral, two IRES-containing vectors, pHSY3207 and pHSY3212, expressing the GTPCH, PTPS, and neomycin phosphotransferase (neo) genes. Arrows indicate approximate location of promoters and direction of transcription. EMCV and FMDV, two IRES elements, refer to the internal ribosomal entry site sequences from encephalomyocarditis virus and foot-and-mouth disease virus, respectively. LTR, Mo-MLV long terminal repeat; SD, splice donor; SA, splice acceptor

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Detection of GTPCH and PTPS activities in cell lysates and determination of biopterin in cell extract and in medium

Preparation of whole-cell lysates for enzyme activity measurements, and enzymatic assays for GTPCH and PTPS were carried out as described previously [6,32]. For stimulation of endogenous GTPCH expression, fibroblasts were incubated for 24 h in cDMEM in the presence of 250 U/ml IFN-γ (Sigma) and 100 U/ml TNF-α (Sigma) [3]. Determination of biopetrin in cell extracts and cell-free medium (supernatant) has been described previously [30]. Pterin analysis was performed by reversed-phase HPLC separation and subsequent fluorescent detection and quantification as described previously [40]. To determine the reduction state of biopterin, we applied the differential oxidation method described by Fukushima and Nixon [41]. Specific enzyme activities were given in μU/mg of soluble protein. For background activities, threshold values of 0.05 μU/mg for GTPCH or PTPS were defined. For statistical paired *t*-test analysis, the probability points (*p*-values) are relative to the activities of untreated fibroblasts ($*p<0.1$; $**p<0.05$; $***p<0.025$; $****p<0.005$).

Results

Generation of triple-cistronic vectors expressing the BH_4 enzymes GTPCH and PTPS, and the neomycin marker

The starting retroviral vector used in this report was MTIN, a derivative of MOI [42]. The MOI vector is a murine leukemia virus-based retroviral vector where the gene of interest is driven from the viral 5′-long terminal repeat (LTR) promoter and its mRNA is expressed efficiently as a splice product. A splice donor and a splice acceptor site were placed downstream of the 5′-LTR. The vector contains no viral coding sequences for *gag* and *env*. The MTIN retroviral vector carried two internal ribosome entry sites (IRES), an 'encephalomyocarditis virus' (EMCV)-IRES element and a 'foot-and-mouth disease virus' (FMDV)-IRES element, in front of the neomycin resistance gene (*neo*). Unique restriction sites for subcloning were present in front of each IRES element. More details about the construction of the *E. coli* based plasmid vector MTIN will be given elsewhere [S. H. Kim and S. Kim, in preparation].

The MTIN-plasmid vector was modified to express BH_4-biosynthetic enzymes. To this end, the two retroviral constructs pHSY3207 and pHSY3212 were generated containing the human cDNAs for GTPCH and PTPS, and the neomycin phosphotransferase gene (Figure 1). Whereas pHSY3207 harbored the PTPS-cDNA downstream of the GTPCH-cDNA, in pHSY3212 this order was permutated. Expression of the second and third gene from these triple-cistronic vectors was achieved by translational coupling with the two different IRES elements in tandem. To determine the strength of GTPCH and PTPS transgene expression from the modified MTIN vectors, transient co-transfections of COS-1 cells were performed with the pRSVβgal control plasmid expressing β-galactosidase (see Materials and methods). Subsequently, specific enzyme activity was determined. For GTPCH, an activity of 0.6 and 1.2 μU/mg was measured, while for PTPS, an activity of 0.7 and 2.4 μU/mg was observed for pHSY3207 and pHSY3212, respectively. (The activity for the two enzymes in COS-1 cell background was <0.05 μU/mg.) Thus, although the specific enzyme activities in pHSY3212-transfected cells appeared to be slightly higher than for pHSY3207, both constructs turned out to express similar levels of GTPCH and PTPS, showing that at least the first two genes from these triple-cistronic retroviral plasmid-constructs were expressed in mammalian cells.

Transduction of PTPS-deficient patient fibroblasts with retroviral constructs HSY3207 and HSY3212

The MTIN-derivatives were transfected to the 293T-based packaging line [38] to generate amphotropic HSY3207 and HSY3212 recombinant retrovirus with reasonable titers. The cell-free viral supernatants were used to transduce primary skin fibroblasts from a control subject, KII, and two PTPS-deficient patients, HW and NG. After transduction, cells were selected for neomycin resistance, expanded, and enzyme activity was determined (see Figure 2). Whereas untransduced KII fibroblasts revealed normal PTPS activity of 2.7 μU/mg (mean value), the patients' fibroblasts showed no PTPS activity (<0.05 μU/mg). Furthermore, as expected, no GTPCH activity was observed in any of the untransduced fibroblasts (<0.05 μU/mg), as the corresponding gene is not expressed in these cells. Upon transduction with HSY3207, GTPCH activity significantly increased to mean values between 9.7 and 11.5 μU/mg, whereas PTPS activity was relatively low and reached levels of ~ 1 μU/mg for the two patients, and 3.8 μU/mg for the control. In contrast, cells transduced with HSY3212 virus had PTPS activity between 11.8 and 18.5 μU/mg, and low GTPCH activity (≤ 1 μU/mg). These results indicated that although coordinated translation of three genes was demonstrated from both triple-cistronic retroviral constructs, expression level appeared to be somewhat lower at least for the second gene, i.e. relatively low PTPS activity for the HSY3207 construct, and low GTPCH activity for HSY3212.

Detection of BH_4 in cell extracts and cell-free supernatant from transduced fibroblasts

Since the fibroblasts were transduced with BH_4-pathway enzymes for potential application in grafting experiments, the most important parameters were the BH_4 biosynthesis

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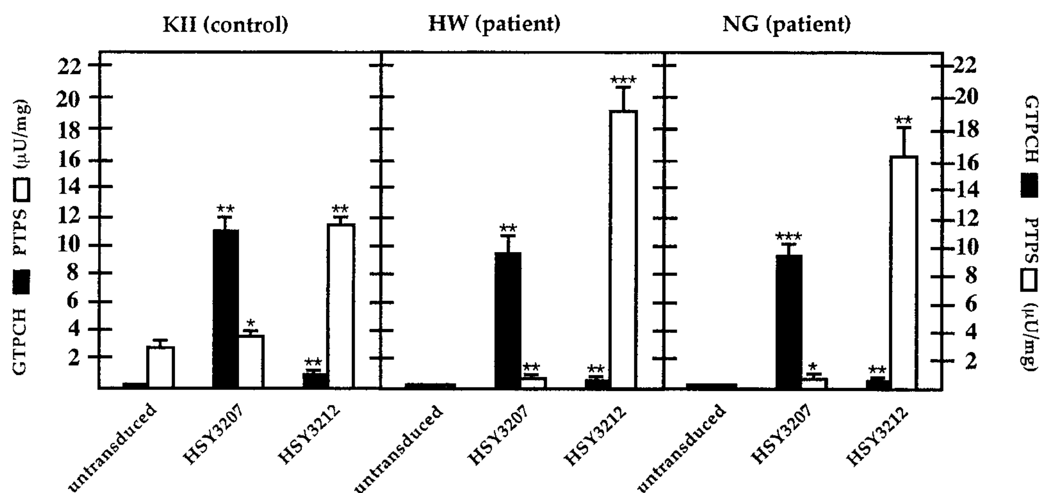

Figure 2. Specific enzyme activities of fibroblasts transduced with the polycistronic vectors containing two BH_4-biosynthetic genes. Fibroblasts from a control subject (KII) and two PTPS-deficient patients (HW and NG) were either untreated or transduced with the retroviral constructs pHSY3207 or pHSY3212. Subsequently, GTPCH and PTPS activity was measured from soluble cell extracts. The graph shows mean values collected from two to four independent experiments. Specific enzyme activity is given in μU/mg of soluble protein

and release from the cells. We thus determined BH_4 concentrations in the cytosol and supernatant from the same cells as those shown in Figure 2. Upon determining the reduction state, we found for intracellular biopterin that at least 80% is in the biological active tetrahydro form. Due to relatively high oxygen exposure of cultured cells, only 10–20% of the exported BH_4 is found as the tetrahydro form in the medium. A summary of the results from total biopterin, i.e. oxidized plus reduced biopterin, in cell extracts and in medium is depicted in Figure 3. Untransduced cells do not synthesize detectable levels of BH_4. After transduction with HSY3207, fibroblast cell extracts contained between 20 and 90 pmol of BH_4 per mg protein. In contrast, cells transduced with HSY3212 accumulated between 220 and 390 pmol of BH_4 per mg protein, suggesting that transduced HSY3212 virus might be more efficient in BH_4 biosynthesis than HSY3207 due to potentially limiting PTPS activity in the latter (compare also to Figure 2). However, determination of BH_4 concentration in the medium revealed the opposite of such a premature assumption. Here, the HSY3207-transduced fibroblasts released between 1700 and 1900 pmol biopterin per 10^6 cells, whereas fibroblasts transduced with HSY3212 virus had only 60–200 pmol biopterin per 10^6 cells (Figure 3, middle panel). Thus, HSY3207-transduced cells released significantly more BH_4 into the medium than cells infected with HSY3212 retrovirus. This phenomenon was further investigated in subsequent studies (see below). The *sum* of biopterin given in absolute picomols that is found inside the cells ('cell extract') plus cofactor that is released ('medium') was also calculated. As shown in Figure 3, lower panel, transduction with the HSY3207 vector leads to three to fivefold more BH_4 production than transduction with the HSY3212 vector.

BH_4-cofactor release into the medium, as presented in Figure 3, was determined for growing fibroblasts in culture plates during a 4 day incubation. In a series of experiments with the same cells, we determined the rate of BH_4 release in quiescent, i.e. confluent, non-dividing cells (data not shown). There, we observed a reduced BH_4 release of approximately factor 5, independent of whether HSY3207 or HSY3212-virus were transduced to the cells. A similar observation was previously made for fibroblasts double-transduced with individual GTPCH- and PTPS-expressing retroviral vectors [30].

Biosynthesis and release of BH_4-cofactor in cytokine treated fibroblasts that express endogenous GTPCH

So far, we demonstrated that retroviral transduction of two biosynthetic enzymes, GTPCH and PTPS, lead to BH_4 cofactor biosynthesis in originally PTPS-deficient primary skin fibroblasts that lack endogenous GTPCH activity. From the results shown in Figure 3, production of BH_4 up to a concentration of at least 400 pmol/mg protein in the HSY3212-transduced cells resulted in relatively inefficient release of cofactor into the medium. One interpretation is that GTPCH enzyme-levels, and, as a consequence a certain intracellular accumulation of BH_4 may be required to deliver cofactor. In order to investigate this phenomenon in more detail, we sought to increase the specific enzyme activity of GTPCH, which might be the limiting enzyme for cofactor biosynthesis specifically in HSY3212-transduced cells. Since cytokines are known to induce endogenous GTPCH expression in fibroblasts, we stimulated *de novo* biosynthesis of GTPCH in cells untransduced or transduced with HSY3212 by the addition of IFN-γ and TNF-α to the culture medium (see Materials and methods). As expected, this resulted in stimulation of specific GTPCH enzyme activity (Figure 4). Subsequently, we measured intracellular accumulation of

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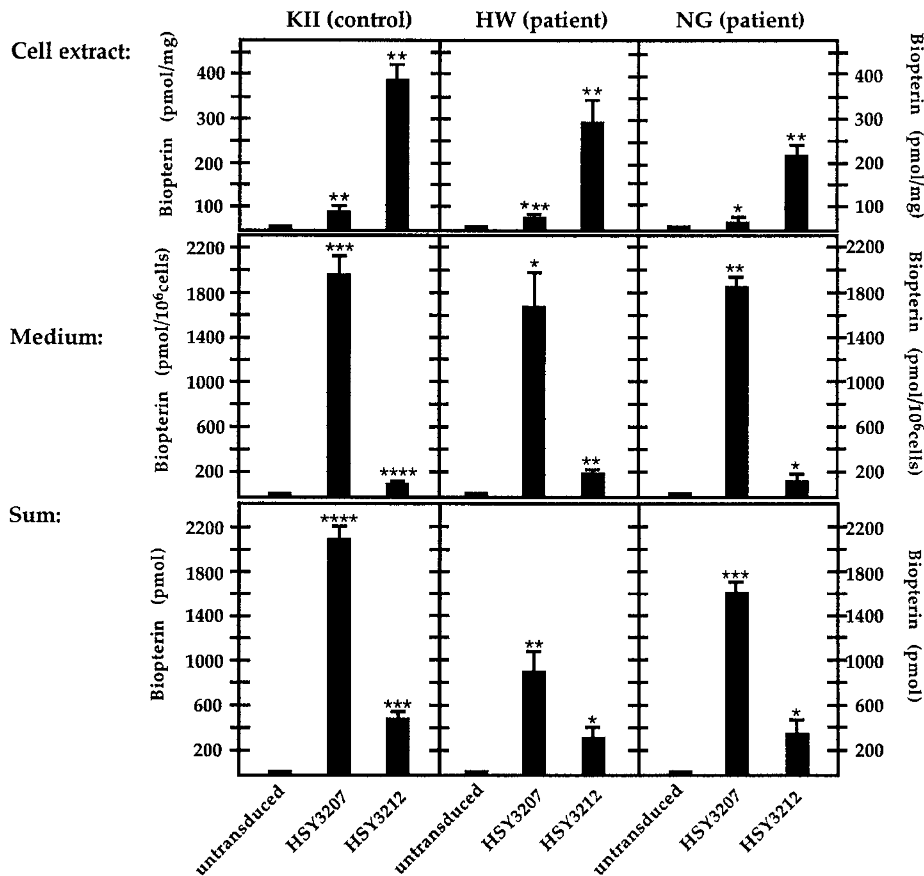

Figure 3. Intra- and extracellular biopterin content from the transduced fibroblasts shown in Figure 2. Cell extract (upper panel): mean biopterin content from two to four independent measurements in cells from the control, KII, and the two PTPS-deficient patients, HW and NG. Concentration is given in pmol biopterin per mg of soluble protein. Medium (middle panel): after 4 days of incubation, biopterin released into the medium was determined from the same cultures as those shown in the upper panel. Concentration in the medium is given in pmol biopterin per 10^6 cells. Sum (lower panel): the total amount of picomols of biopterin was calculated by adding the cofactor found in the cell extract (upper panel) to the cofactor that was released into the medium (middle panel)

BH_4, and found a mean value of around 225 pmol/mg protein for the cytokine-unstimulated, HSY3212-transduced cells from patients HW and NG (Figure 5).

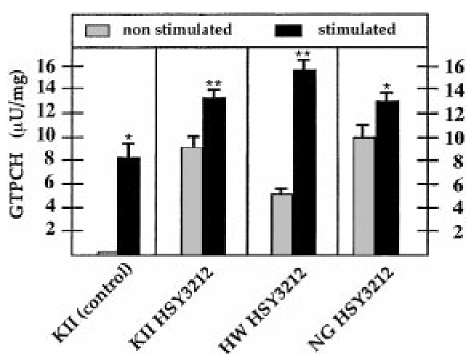

Figure 4. GTPCH activity in non-stimulated and in cytokine-stimulated fibroblasts transduced with HSY3212. Fibroblasts from KII, HW, and NG were treated with tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) for 24 h to induce GTPCH. Subsequently, GTPCH activity was measured and compared to GTPCH activity in non-stimulated cells. Enzyme activity is given in µU/mg soluble protein. In the graph, the average of two to four independent measurements is given

This amount of intracellular BH_4 was similar to what had been seen before (mean values between 236 and 305 pmol/mg; see Figure 3, upper panel). Upon addition of cytokines to fibroblasts from HW and NG, which enhanced GTPCH activity, biopterin content in cell extracts was apparently lowered to mean values between 25 and 49 pmol/mg protein. On the other hand, biopterin content in the medium was raised from a few hundred pmol per 10^6 cells for non-cytokine induced fibroblasts to as much as 7000 pmol/10^6 cells for cytokine-stimulated cells. In this series of measurements, the control cells KII without GTPCH stimulation appeared already to have reached an intracellular biopterin content to efficiently release BH_4. This is based on the observation that non-stimulated as well as cytokine-stimulated KII cells had no more than 50 pmol of intracellular BH_4 per mg of protein, with a medium biopterin content of 5500 pmol/10^6 cells and 8900 pmol/10^6 cells, respectively (see Figure 5). From these experiments we could not rule out a potential effect on cell death in culture and release of BH_4 into the medium. However, we did not observe any significant difference in cell numbers when HSY3212-transduced, cytokine-induced, and thus BH_4-producing fibroblasts

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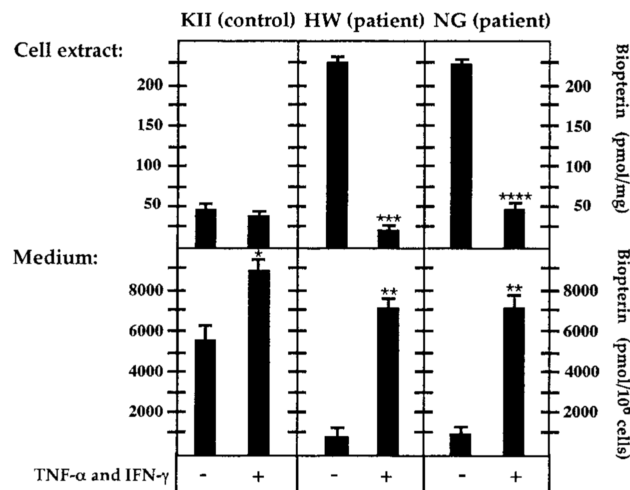

Figure 5. Comparison of biopterin production in cell extract and medium before and after cytokine-stimulation of fibroblasts transduced with HSY3212. Cell extract (upper panel): the same cells as shown in Figure 4 were used to determine the biopterin content in cell extracts. The cells were either non-stimulated or stimulated with tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) to induce GTPCH activity. The graph shows mean values, given in pmol/mg soluble protein, collected from two to four independent experiments. Medium (lower panel): determination of extracellular biopterin in the medium accumulated for 4 days. Concentration of biopterin is given in pmol/10^6 cells from the fibroblasts as shown in the upper panel

were cultured in parallel for several months and compared with the non-viral transduced, non-cytokine treated, hence non-BH_4 producing control fibroblasts (not shown). One possible explanation for these findings is that sufficient GTPCH enzyme expression and thus a critical intracellular cofactor concentration, probably above 400 pmol biopterin per mg of protein, may be required to efficiently release BH_4 in skin fibroblasts. The implications from these findings will be discussed below.

Discussion

BH_4 is a redox cofactor that is produced from GTP by three biosynthetic enzymes, GTPCH, PTPS, and SR. The cofactor is required at least for aromatic amino acid hydroxylases to synthesize monoaminergic neurotransmitters, and for the nitric oxide synthase enzymes that produce the free radical NO. For patients with congenital defects in PTPS, oral doses of synthetic BH_4 are not sufficient for treating the neurotransmitter deficiency in the central nervous system (CNS). On the other hand, grafting of genetically modified depository cells such as autologous fibroblasts for drug delivery has been reported to be a potential alternative approach for CNS therapy (for reviews see Refs. [27,28,43]). However, fibroblasts from PTPS deficient patients do not exhibit functional PTPS and have no endogenous GTPCH activity, but express SR constitutively. Here we demonstrated the feasibility of retrovirus-mediated gene transfer to produce the BH_4 cofactor in primary skin fibroblasts with triple-cistronic vector constructs that express fully functional GTPCH, PTPS, and neomycin phosphotransferase (*neo*).

With the drug-selectable gene placed at the 3′-end, 5′-LTR promoter-dependent expression of the three-gene-containing mRNA was assured. The enzymes were synthesized independently from the single mRNA by translation of the first gene, which was cap-dependent, and the two others translated under the control of the IRES. Although reports on the use of di-cistronic retroviral constructs for gene therapy are well documented [31,42,44–46], the use of triple-cistronic vectors is still very rare, despite the fact that the feasibility of triple-cistronic two-IRES vectors is established [31]. To our knowledge, there is only one publication, besides this paper, on the application of such retroviral vector for transfer and expression in primary cells for gene therapy. In this earlier report, an MFG retroviral vector [47] was generated with two identical EMCV-IRES elements to express both subunits of IL-12 and a neomycin resistance-selectable marker gene from the same polycistronic message [48]. In an initial attempt to generate triple-cistronic recombinant retrovirus based on the pM48-vector plasmid with two identical IRES elements inserted in tandem, similar as reported by Zitvogel *et al.* [48], we failed to generate stable constructs and/or coordinate expression of more than two genes. (Plasmid pM48 was provided by J.M. Heard, Pasteur Institute [49]; EMC-IRES-element was obtained from C. Morgan, NIH [31]). This was presumably due to some recombination activity and thus instability of the viral constructs [50]; S. Laufs and B. Thöny, unpublished observation]. With the here presented MTIN vector harboring two different IRES sequences, the encephalomyocarditis virus (EMCV) and foot-and-mouth disease virus (FMDV) elements, we resolved such potential problems.

For both viral constructs, HSY3207 and HSY3212, the strength of GTPCH or PTPS expression, as revealed by specific enzyme activity in transduced fibroblasts, was always higher when a given cDNA was present adjacent to the promoter, i.e. when translation was cap-dependent. In contrast, translation under control of the first IRES resulted in a tenfold decrease of activity for GTPCH and a 20-fold decrease for PTPS (see also Figure 2). Such reduced expression levels of the gene downstream from the (first) IRES had been observed previously [48]. At least in case of cap-dependent translation, the levels of enzyme activities presented here were comparable to those of double transduced fibroblasts with individual GTPCH and PTPS expressing viruses that had two independent promoters, 5′-LTR and SV40, for mono-cistronic expression [30]. However, it turned out that a critical parameter to optimize BH_4 biosynthesis in fibroblasts transduced with the triple-cistronic vectors was the GTPCH activity. For instance, vector HSY3207 conferred higher GTPCH activity than HSY3212, which produced more BH_4, and resulted in relatively higher levels of BH_4 in the culture medium (Figures 2 and 3).

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Furthermore, when GPTCH activity was elevated by cytokine-treatment of the HSY3212-transduced fibroblasts, which stimulates expression of the endogenous gene, a significant boost of BH_4 production was observed (Figures 4 and 5). Extracelluar BH_4 increased to values five- to tenfold higher than those observed in unstimulated cells. However, the amount of cofactor released by the here-transduced but quiescent primary cells would be enough to use the fibroblasts as deposits for passive drug delivery in the CNS (see also below; for a more detailed discussion on the amount of BH_4 required empirically for CNS therapy see Ref. [30]).

By following the intra- and extracellular concentration of BH_4 in cultured cells, we did not observe in any of our experiments higher intracellular BH_4 levels than 400 pmol/mg protein. Furthermore, we ruled out excessive cell death in culture and thus release of BH_4 into the medium for cytokine-stimulated, GTPCH over-expressing fibroblasts. In this context it was reported that the physiological concentrations of BH_4 in at least catecholamine-producing rat adrenal pheochromocytoma and neuroblastoma cell lines and in human adrenergic neuroblastoma cell clones do not exceed 400 pmol/mg protein (between 120 and maximal 400 pmol/mg protein) [51,52]. Our finding that up to a concentration of at least 400 pmol/mg protein, almost no BH_4 appeared to be released, whereas with concentrations above this value large amounts of cofactor were found outside the cells, suggesting that an inducible transport system is involved. Although the biosynthesis pathway of BH_4 has been well studied, transport such as cellular import and export is only poorly understood. Hoshiga *et al.* demonstrated that BH_4 in different rat organs such as liver and kidney was not only supplied by intracellular *de novo* biosynthesis but rather by uptake of extracellular BH_4 [53]. Moreover, results by Anastasiadis *et al.* indicate that neurons and cultured rat pheochromocytoma (PC12) cells do not appear to have a specific membrane carrier for BH_4 and that BH_4 uptake into cells is due to passive diffusion [54]. Although it was not our aim to approach BH_4 transport studies, the experiments reported here provide new data on GTPCH levels or cofactor concentration, at least in fibroblasts, required for efficient release of BH_4. In line with these findings, we reported previously that BH_4 is more efficiently released from fibroblasts than its biosynthetic intermediates dihydroneopterin triphosphate and 6-pyruvoyl tetrahydropterin [30]. Further analysis will be required for investigating the existence and characterization of a potential transport system or membrane carrier that may be specific and/or inducible by intracellular BH_4.

The virus titers we obtained with the three-plasmid transfection protocol (around 10^4 cfu/ml) can be increased, for instance, by additional incubation of transfected producer cells with sodium butyrate for 12 h, as described by Soneoka and coworkers [38], or by further manipulating MTIN [S. Kim *et al.*, in preparation]. With such an increased virus titer, infection efficiency could be much higher and eventually render the selectable marker gene obsolete. Furthermore, by replacing the neomycin resistance gene with the sepiapterin reductase cDNA, the whole BH_4 biosynthesis pathway could be coordinately expressed from a single retroviral vector using one transcriptional unit. However, since we found that raising the levels of GTPCH and PTPS activities can enhance cofactor biosynthesis, we did not address the question of whether the endogenous, constitutively expressed SR was a potential limiting factor at all for BH_4 production. As an alternative, GTPCH and PTPS might be coordinately expressed from such triple-cistronic vectors together with, for instance, phenylalanine hydroxylase (see below) or nitric oxide synthase [55–60]. For example, in a gene therapeutic approach to treat hyperphenylalaninemia in a mouse PKU model, heterologous expression of phenylalanine hydroxylase in muscle to clear high serum levels of phenylalanine was only effective when the diet was supplemented with BH_4 [61]. In order to avoid continual cofactor administration, one could envisage transducing the phenylalanine hydroxylase, GTPCH, and PTPS genes to potentially treat PKU according to this model.

As mentioned in the *Introduction*, BH_4 replacement therapy is complex and not efficient in every case, as a stringent blood–brain barrier can limit BH_4 uptake [53] (for a more general review see Ref. [24]). Thus, gene therapy in the CNS seems to be a potential alternative for treating BH_4-dependent neurotransmitter deficiency. Besides using a strategy involving lentivirus vectors for stable infection of postmitotic neurons [62,63], intracerebral grafting has emerged as a feasible approach to CNS therapy. Although different cell types were exploited as grafts, skin fibroblasts have been most commonly used as vehicles for foreign gene expression in the CNS. It has been demonstrated, for example, that primary fibroblasts genetically altered to express tyrosine hydroxylase have the capacity to deliver L-DOPA locally to the striatum after implantation [64,65]. A major obstacle observed in studies conducted by these authors is the shut-off of LTR-driven gene expression after transplantation. A potential solution is the insertion of the mouse phosphoglycerate kinase (PGK) promoter element downstream of the 5′-LTR in the vector HSY3207, which may allow long-term expression also *in vivo* after transplantation in at least rodents [66]. Furthermore, such engineered depository cells may be used to correct the hepatic hyperphenylalaninemia in BH_4-deficient patients. Thus, the vectors generated and applied in the *in vitro* studies shown here provide the basic tool to proceed with *in vivo* studies.

In summary, we have demonstrated the successful generation of a functional triple-cistronic retroviral gene-transfer vector. The advantage of such vectors that coordinately express up to three genes from a polycistronic message using multiple IRES elements might be an efficient strategy to reconstitute metabolic pathways for drug delivery from depository cells, or to treat multigenetic disorders.

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