Generation of retroviruses for the overexpression of cytosolic and mitochondrial glutathione reductase in macrophages in vivo

Marta Kisgati · Reto Asmis

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Abstract Retroviral gene transfer and bone marrow transplantation has been used by many investigators to study the role of macrophage proteins in different mouse models of human disease. While this approach is faster and less expensive than generating transgenic mice with macrophage-specific promoters and applicable to a wider array of mouse models, it has been hampered by two major drawbacks: labor-intensive cloning procedures involved in generating retroviral vectors for each gene of interest and low viral titers. Here we describe the construction of a MSCV-based retroviral vector that can serve as an acceptor vector for commercially available Cre-lox-compatible donor vectors. Using this new retroviral vector in combination with a FACS approach to enhance viral titers, we generated high-titer retroviruses carrying either EGFP-tagged cytosolic or EGFP-tagged mitochondria-targeted glutathione reductase. We show that the introduction of these constructs via retroviral

gene transfer and bone marrow transplantation into atherosclerosis-prone LDL receptor-null mice results in the long-term increase in macrophage glutathione reductase activity.

Keywords Retrovirus · Gene Transfer · Glutathione Reductase · Macrophage · Atherosclerosis

Introduction

Macrophages play important roles throughout the formation and development of atherosclerotic lesions. Cell culture models do not adequately mimic the complex interactions involved in atherogenesis, making it necessary to study the role of individual macrophage proteins in whole animal models, in particular in atherosclerosis-prone mice. Two approaches have been used to achieve macrophage-specific gene expression in mouse models: (1) insertion of the gene of interest under the control of a macrophage-specific promoter (Horvai et al. 1995; Wilson et al. 2001; Daugherty et al. 2001; Ghesquiere et al. 2005; Lang et al. 2002) and (2) retroviral gene transfer in hematopoietic stem cells (Ishiguro et al. 2001; Yoshida et al. 2001; Hasty et al. 1999). The advantages of the first approach are that the gene of interest would be expressed in all monocytes/macrophages and exclusively in cells of the macrophage

M. Kisgati
Department of Laboratory Medicine, Kenezy Gyula
Hospital, Debrecen, Hungary

R. Asmis (✉)
Division of Nephrology, University of Texas Health
Science Center at San Antonio and South Texas
Veterans Health Care System, 7703 Floyd Curl Drive,
MSC 7882, San Antonio, TX 78229, USA
e-mail: asmis@uthscsa.edu

lineage. However, the production of transgenic mice is very labor intensive and expensive. Few groups have used this approach successfully due to the mixed results with different macrophage promoters. Alternatively, transgene expression in macrophages can be achieved by retroviral gene transfer in bone marrow-derived stem cells followed by bone marrow transplantation (BMT) into lethally irradiated mice. With this approach, transgene expression is not limited to the macrophage lineage but will affect other hematopoietic cells. However, in atherosclerotic lesions, macrophages are the predominant hematopoietic cell type and transgene expression will therefore essentially be limited to macrophages and macrophage-derived foam cells. The drawbacks of the latter approach have been the labor-intensive cloning procedures involved in generating retroviral vectors for each gene of interest and the low viral titers obtained with common methods used for the generation of retroviruses. Here we describe the construction of a Cre-lox compatible MSCV-based retroviral vector for the facilitated insertion of genes of interest using commercially available Cre-lox compatible donor vectors, and the subsequent generation of high-titer retroviruses carrying EGFP fusion proteins of either cytosolic or mitochondrial glutathione reductase.

Glutathione reductase (GR) is a key enzyme of the glutathione-dependent antioxidant system, which protects cells against oxidative injury. GR is a homodimeric flavoprotein that maintains the cellular thiol redox state by catalyzing the reduction of glutathione disulfide (GSSG) to glutathione (GSH) with NADPH as the reducing cofactor (Tutic et al. 1990). With intracellular concentrations typically between 1 and 10 mM, GSH is essential for (a) the maintenance of protein thiols and antioxidants, (b) reduction of ribonucleotides to form the deoxyribonucleotide precursors of DNA, and (c) protection against oxidative damage, free radical damage, and other types of toxicity (Meister 1988). Mammalian GR is expressed in the cytosol and in the mitochondria. However, these isoforms are biochemically indistinguishable, suggesting that both isoforms are encoded by a single nuclear gene (George and Francke 1976). Several studies showed that both the human and the mouse GR

genes consist of 13 exons and both sequences code for an N-terminal mitochondrial targeting sequence (Iozef et al. 2000). Due to the fundamental role of GSH in the removal of deleterious reactive oxygen species (ROS), GR is crucial to the antioxidant defense mechanism of cells (Chaudiere and Ferrari-Iliou 1999). Oxidation of the catalytically essential Cys⁶³ into the corresponding sulfenic and sulfinic acid has been shown to inactivate GR (Savvides et al. 2002), a mechanism that is likely to contribute to the inhibition of cellular GR observed in cells exposed to intense or chronic oxidative stress (Asmis et al. 2005; Wang et al. 2006). Oxidative stress and ROS formation play important roles in endothelial dysfunction and the development of atherosclerotic lesions (Stocker and Kearney 2004).

One of the earliest events in atherosclerotic lesion formations is the recruitment of monocytes into the vessel wall (Ross 1999). Once in the subendothelial compartment, monocytes differentiate into resident macrophages and perform a wide array of functions (Glass and Witztum 2001). These macrophages scavenge extracellular modified lipoproteins, primarily OxLDL and lipoprotein aggregates, transforming the cells into lipid-laden foam cells, the hallmark of early atherosclerotic lesions. The ability of macrophages to generate large amounts of reactive oxygen species has led to the hypothesis that macrophages themselves are involved in the oxidative modification of LDL (Steinberg et al. 1989). However, local chronic oxidative stress within the vessel wall is likely to also affect macrophage function. We recently showed that inducing (thiol) oxidative stress in mouse macrophages *in vivo* impairs macrophage function and macrophage-dependent dermal wound healing (Asmis et al. 2006). Because the GSH-dependent antioxidant system plays a critical role in protecting macrophages from OxLDL-induced cell injury (Wang et al. 2006), we hypothesized that strengthening the GSH redox buffer of macrophages may prevent monocyte and macrophage dysfunction in early atherosclerotic lesions. However, we found that simply increasing intracellular GSH levels by supplementing cultured macrophages with GSH diethyl ester actually decreased the GSH/GSSG ratio, thus weakening the GSH redox buffer (Asmis et al. 2005). An

alternative strategy is to increase GR activity in macrophages, thereby increasing their GSH/GSSG ratio and the buffering capacity of the GSH/GSSG redox couple.

The two goals of this study were (a) to construct a retroviral vector for murine bone marrow-derived hematopoietic stem cells that can function as a general acceptor vector and (2) as a prove of principle, to generate recombinant retroviruses carrying either the mitochondrially targeted GR (MTS-GR-EGFP), cytosolic GR (GR-EGFP) or EGFP alone. Because the retroviral genome inserts into the host cell genome following infection, it can be utilized as a permanent gene delivery vehicle (Miller 1992). Up to 6.5 kb of foreign gene sequence can be packaged in a retroviral vector. Once a packaging cell line is transfected with retroviral expression vector that contains a packaging signal, the viral genomic transcript containing the target gene and selectable marker are packed into infectious virus within 48–72 h.

The Cre-lox system is an attractive tool to facilitate the insertion of different transgenes into the same vector. The Cre-loxP DNA cloning and expression system uses Cre-loxP site-specific recombination to catalyze the transfer of target gene from donor vector—a plasmid containing the gene of interest—to an acceptor vector, a plasmid containing regulatory elements of the host expression system. Cre is a site-specific DNA recombinase means that the Cre protein can recombine DNA when it locates specific sites in a DNA molecule. These sites are known as loxP sequences, which are 34 base pairs long. The loxP sequence originally comes from the P1 bacteriophage, which is a bacterial virus that, contains DNA not found in animals. This 34 base pairs long consensus sequence consists of a 8 bp core sequence and two 13 bp palindromic flanking sequences. The asymmetric core sequence defines an orientation to the loxP site, which forces our insert to be transferred in a fixed orientation and reading frame. One of the advantages of the Cre-loxP recombination system is that there is no need for additional co-factor or sequence elements for efficient recombination (Nagy 2000).

Here we describe the construction of a Cre-lox compatible retroviral vector for murine bone

marrow-derived hematopoietic stem cells that can function as a general acceptor vector and a procedure for the generation of high-titer recombinant retroviruses, which, once introduced into mouse bone marrow, resulted in the increased expression of either mitochondria-targeted MTS-GR-EGFP, cytosolic GR-EGFP or EGFP in mouse macrophages.

Materials

- BD Creator DNA Cloning Kit, BD Science No K1670–1 (BD Science, Clontech, Palo Alto, CA, USA)
- Retroviral Gene Transfer and Expression Kit, BD Science No 634401 (BD Science, Clontech, Palo Alto, CA, USA)
- Creator acceptor Vector Construction Kit, BD Science No K1690–1 (BD Science, Clontech, Palo Alto, CA, USA)
- Qiaquick PCR Purification Kit, Qiagen No 28104 (Qiagen, Valencia, CA, USA)
- QIAprep Spin Miniprep Kit, Qiagen No 27106 (Qiagen, Valencia, CA, USA)
- polybrene, Sigma St.Louis, MO, USA. No H9268 (Sigma, St.Louis, MO, USA)
- G418, BD Science No 8056-1 (BD Science, Clontech, Palo Alto, CA, USA)
- Max Efficiency DH5 α Competent cells, Invitrogen No 18258-012 (Invitrogen, Carlsbad CA, USA)
- Lipofectamine Reagent, Invitrogen. No 18324111 (Invitrogen, Carlsbad CA, USA)
- C57BL/6J mice, Jackson Laboratories No 000664 (Jackson Laboratories, Bar Harbor, ME, USA)
- NIH 3T3 cell line, American Type Culture Collection CRL–1658 (American Type Culture Collection Rockville, MD, USA)

- Phoenix Eco cell line established at Stanford University (University of Kentucky, Lexington, KY, USA)
- Dulbecco's Modified Eagle's Medium, Invitrogen. No 11960–077 (Invitrogen, Carlsbad CA, USA)
- GlutaMAX, Invitrogen. No 35050–061 (Invitrogen, Carlsbad CA, USA)
- PenicillinG/Streptomycin, Invitrogen. No 15140–163 (Invitrogen, Carlsbad CA, USA)
- MEM non-Essential Amino Acids, Invitrogen. No 11140–050 (Invitrogen, Carlsbad CA, USA)
- Heat-inactivated fetal bovine serum, Invitrogen. No 10082–139 (Invitrogen, Carlsbad CA, USA)
- cDNA/GR-EGFP, German Cancer Research Center (German Cancer Research Center, Heidelberg)
- cDNA/MTS-GR-EGFP, German Cancer Research Center (German Cancer Research Center, Heidelberg)
- Cre recombinase, BD Science No 8480–1 (BD Science, Clontech, Palo Alto, CA, USA)
- Olympus C-71 fluorescence microscope, (Olympus America Inc., Center Valley, PA, USA)
- B6.129S7-Ldlr^{tm1Her}/J (stock no. 002207) mice, Jackson Laboratories (Jackson Laboratories, Bar Harbor, ME, USA)
- Retronectin-coated dishes, Takara. No T110A (Takara Bio INC, Otsu Shiga, Japan)
- mIL-3 (10 µg/mL in 1× PBS) Calbiochem No 407631 (Calbiochem, San Diego, CA, USA)
- mIL-6 (10 µg/mL in 1× PBS) Calbiochem No 407654 (Calbiochem, San Diego, CA, USA)

- mSCF (230 µg/mL in 1× PBS) Calbiochem No 569610 (Calbiochem, San Diego, CA, USA)

Procedures

Mice

Female LDL receptor-null (LDLR^{-/-}) recipient mice on a C57BL/6J background and C57BL/6J mice donor mice were obtained from The Jackson Laboratories. All mice were maintained in a barrier facility and fed normal mouse laboratory diet. All studies were performed with the approval of the University of Kentucky Institutional Animal Care and Use Committee.

Cell culture

The murine fibroblast NIH 3T3 cell line was obtained from the American Type Culture Collection. The Phoenix Eco ecotropic packaging cell line was obtained from Dr Gary van Zant (University of Kentucky, USA). Cells were cultured at 37 °C with 5% CO₂ 95% air in Dulbecco's Modified Eagle's Medium supplemented with 200 mM GlutaMAX, 10,000 U/mL PenicillinG/Streptomycin, 0.1 mM MEM non-Essential Amino Acids and 10% heat-inactivated fetal bovine serum. Cells were not used after passage 25. One day before transfection or infection, cells were seeded into 6-well plate or 60 mm tissue culture dish. On the day of transfection the cell line was 50–70% confluent.

Construction of retroviral vectors

The cDNAs for C-terminal EGFP fusion proteins of cytosolic glutathione reductase (GR-EGFP) and mitochondrial targeted GR (MTS-GR-EGFP) were kindly provided by Dr. Dieter Werner (German Cancer Research Center, Heidelberg). The sequences for enhanced green fluorescent protein (EGFP) or for C-terminal EGFP fusion proteins of cytosolic glutathione reductase (GR-EGFP) and mitochondrial targeted GR (MTS-GR-EGFP) were cloned into pDNR donor vectors (BD Biosciences Clontech). Both GR-EGFP and

MTS-GR-EGFP were excised from the pcDNA3 vector with HindIII and XbaI and cloned into the pDNR vector. The Murine Stem Cell Virus vector (pMSCVneo) was used as an acceptor vector (BD Biosciences Clontech). To generate a Cre-loxP compatible MSCV-based acceptor vector, a loxP cassette was inserted into the multi-cloning site of the vector (BD Creator DNA Cloning Kit). The genes for EGFP, GR-EGFP and MTS-GR-EGFP were inserted into this Cre-lox-compatible retroviral expression vector by linear recombination with Cre recombinase (BD Biosciences Clontech).

Generation of recombinant retroviruses

Infectious but replication-incompetent recombinant retroviruses expressing EGFP, GR-EGFP or MTS-GR-EGFP were generated in the Phoenix-Eco packaging cell line. Once high transfection efficiency was achieved, cell populations with high EGFP expression levels were selected by FACS, expanded, and viral titers of the supernatants from these stable virus-producing cell lines were determined in NIH 3T3 cells by flow cytometry.

Determination of retrovirus titers

NIH 3T3 cells were seeded at 2×10^5 cells per well in 6-well culture dishes and incubate in Dulbecco's Modified Eagle's Medium containing 0.4 mg/mL Polybrene. Retroviral supernatants were harvested 48 h after transfection and titered by limited dilution on NIH 3T3 cells using EGFP as a marker for infection (Webb et al. 2003).

Fluorescence microscopy

Cells were washed, fixed in 4% paraformaldehyde and mounted in Mowiol embedding medium (Heider et al. 2000). Cells were examined using an Olympus C-71 fluorescence microscope and digitally photographed using a Rotiga 1300 digital camera.

Infection of bone marrow cells

Donor mice (C57BL/6J) were pretreated with 5-fluorouracil (i.p. 150 mg/kg) three days prior to

harvesting bone marrow cells. Bone marrow cells were collected by flushing the femurs and tibias with PBS containing 2% FBS. Cells were pooled, washed and resuspended in DMEM with 15% FBS. Cells were cultured at 7×10^6 cells/mL in 60 mm culture dishes. Cells were incubated in DMEM supplemented with 15% FBS, mIL-3 (10 µg/mL), mIL-6 (10 µg/mL) and mSCF (230 µg/mL). Bone marrow cells transduction with EGFP, GR-EGFP and MTS-GR-EGFP was performed by transferring bone marrow cells into Retronectin-coated dishes containing 1.2 mL retrovirus supernatant mIL-3, mIL-6, mSCF and polybrene in 10 ng/mL, 20 ng/mL, 100 ng/mL and 5 µg/mL final concentration, respectively. Cells were incubated for 4 h, and the transduction procedure was repeated. The second incubation was 18 h and a 4 h incubation period followed the third transduction. Transduced bone marrow cells were washed with PBS prior to tail vein injection. A small amount of cells (500×10^5) was analyzed by flow cytometer to determine the percentage of EGFP expressing cells.

Bone marrow transplantation

Two weeks prior to irradiation and bone marrow transplantation recipient LDLR^{-/-} mice were put on acidified water containing sulfamethoxazole (160 ng/mL) and Trimetoprim (32 ng/mL). Mice were maintained on antibiotics for seven weeks. Prior to transplantation, recipient LDLR^{-/-} mice received two equal doses of 4.5 Gy three hours apart (9 Gy total, Mark I-68 Irradiator). Bone marrow cells transduced with either EGFP, GR-EGFP or MTS-GR-EGFP retrovirus were injected into the tail vein of the irradiated recipients ($n = 10$ per group).

Glutathione reductase activity assay

Resident peritoneal cells were harvested by lavage with 5 mL of ice-cold DMEM. Cell viability was determined by trypan blue extrusion and was >95% in all cell preparations. Cells were plated in culture medium supplemented with 10% FBS and cultured in a humidified atmosphere at 5% CO₂. After 3 h, non-adherent cells were removed through washing with culture medium.

Macrophage lysates were prepared with 50 mM KP_i (potassium phosphate), pH = 7.5, containing 1 mM EDTA and 1% TritonX-100 (Asmis et al. 2005). The assay was performed at 37 °C in 50 mM KP_i , pH = 7.5, containing 1 mM EDTA, BSA (1 mg/mL), 333 μ M NADPH. The enzymatic reaction was started by adding 1 mM GSSG and absorbance was monitored at 340 nm for 15 min on a VERSAmax plate reader (Molecular Devices, Sunnyvale, CA). GR from Baker's yeast served as a standard.

Results and discussion

For this study, we selected the commercially available pDNR donor vector to generate the three recombinant MSCV expression vectors. The pDNR vector contains two loxP sites, which flank the 5' end of the MCS and the 5' end of the open reading frame for the chloramphenicol resistance gene (Cm^r , Fig. 1). The vector also encodes for the ampicillin resistance gene (Amp^r) for its propagation and selection in *E. coli*, and the sucrose gene from *B. subtilis* (*SacB*) for selection of correct recombinants. Full-length cDNA for murine GR, which contains a mitochondrial targeting sequence (MTS-GR), and a truncated

form of the cDNA, which lacks only the mitochondrial targeting sequence (GR) (Iozeff et al. 2000), were cloned in-frame with the expression cassette for enhanced green fluorescent protein (EGFP). Both forms of GR are therefore expressed as C-terminal EGFP fusion proteins (GR-EGFP and MTS-GR-EGFP). Murine Stem Cell Virus (MSCV)-based MSCV_{neo} vector was chosen as an acceptor vector for this study because it achieves stable, high-level gene expression in hematopoietic stem cells. The MSCV_{neo} vector contains the extended retroviral packaging signal (Ψ), which promotes high-titer virus production. This vector also has a specifically designed long terminal repeat (LTR). The LTR provides enhanced transcriptional activation and decrease transcriptional suppression in stem cells (Hawley et al. 1994).

To make this MSCV-based retroviral acceptor vector, pMSCV_{neo}, Cre-lox-compatible, we inserted a loxP site into the multicloning site of the vector (Fig. 1). To this end, the loxP cassette was digested, purified and amplified through PCR with 5'-GATGCTGAATTCATAACTTCGTA-TAGCATACATTAT-3' as a forward primer and 5'-AGTCTCTCGAGACGTCAGGTGGCA-CTTTTCG-3' as the reverse primer (Integrated DNA Technologies). We amplified the loxP

Fig. 1 Restriction maps for pMSCV-GR-EGFP, pMSCV-MTS-GR-EGFP and pMSCV EGFP vectors: Relevant restriction sites are indicated

cassette with EcoRI and XhoI restriction site in frame. The pMSCV_{neo} plasmid contains unique EcoRI and XhoI restriction sites, allowing us to ligate the digested loxP sequence into the digested and purified pMSCV_{neo} expression vector. The Cre-lox-compatibility of this modified pMSCV_{neo} acceptor vector (ploxP/MSCV) and the availability of several different commercial Cre-lox-compatible cloning vectors will facilitate the cloning of any gene of interest into this retroviral vector, eliminating the need for time-consuming subcloning. Using Cre-mediated linear recombination, we transferred the genes for GR-EGFP, MTS-GR-EGFP and EGFP directly from the corresponding pDNR donor vector into our ploxP/MSCV acceptor vector. Cre recombinase binds to the loxP sites on both the donor vector and the acceptor vector, cleaves the DNA, and covalently attaches itself to the DNA. The Cre enzyme then catalyzes strand exchange and ligation of the DNA so that the insert is transferred from the donor vector into the acceptor vector.

The advantage of this system is that the two loxP sites in the donor vector flank the MCS and chloramphenicol open reading frame, so only this small sequence of the donor vector, gene of interest and chloramphenicol resistance gene with loxP in frame is transferred to the acceptor vector (Fig. 1). In other systems, there is only one loxP site in the donor vector causing both the donor and acceptor vectors to fuse into one large plasmid. The small expression vector is easier to use and ideal for variety of downstream applications, including retroviral expression.

After the recombination, we transformed DH5 α competent cells and isolated clones using Miniprep. Chloramphenicol plus sucrose selection allowed us to harvest only recombinant colonies containing a directionally correctly inserted GR-EGFP, MTS-GR-EGFP or EGFP gene. Clones containing the donor vector, without our gene insert expressed SacB, and therefore, did not grow on the sucrose-rich LB agar. The correct sequence of each construct was confirmed by sequencing analyses.

We generated recombinant retroviruses expressing EGFP, GR-EGFP or MTS-GR-EGFP in the Phoenix Eco packaging cell line. To generate stable, retrovirus-producing cell lines,

cDNA carrying EGFP, GR-EGFP or MTS-GR-EGFP gene was transfected into Phoenix Eco cells with Lipofectamine. Cells were grown in G418 to select for cells stably expressing the integrated vector. The transfection efficiency was 40–50% as determined by fluorescence microscope (Fig. 2). The population of cells displaying the brightest green fluorescence (10% of total cells) was single-cell selected by FACS and expanded. The application of EGFP as selectable marker of retroviral-mediated gene transfer has several advantages. EGFP has no requirement for secondary molecules, cofactors, substrates, antibodies or other reagents for visualization. Other emission channels can be used to further define the transducer cell populations on the basis of additional phenotypic markers.

Viral titers (infectious particles (ip) per milliliter) were determined in NIH 3T3 cells by flow cytometry. A total of 2×10^5 NIH 3T3 cells were transduced with serial dilutions of viral supernatant. Viral titers were calculated at limiting dilution according to the following formula:

$$\begin{aligned} \text{Titer (ip/ml)} &= (2 \times 10^5 \text{ target cells}) \\ &\times (\% \text{ EGFP - positive cells}) / \\ &\text{volume of supernatant (ml)} \end{aligned}$$

Viral titers were 3.6×10^6 ip/mL for EGFP, 1.1×10^6 ip/mL for GR-EGFP and 1.1×10^6 ip/mL for MTS-GR-EGFP-carrying retroviruses. Retroviral packaging cells routinely yield viral supernatants with titers in the range of 10^4 – 10^6 ip/mL, but our FACS approach allowed us to increase the titers significantly.

To determine whether retroviral gene transfer of GR-EGFP and MTS-GR-EGFP results in long-term transgene expression and increased enzymatic activity in macrophages, bone marrow cells were infected with the transgene-carrying retroviruses and subsequently transplanted into lethally irradiated LDLR^{-/-} mice. Fifteen weeks after bone marrow transplantation, GR activity was measured in peritoneal macrophages isolated from these mice. Macrophages from mice that received GR-EGFP and MTS-GR-EGFP-expressing bone marrow cells showed a 57% and 70% increase in GR activity, respectively,

Fig. 2 Retrovirus-mediated expression of EGFP, GR-EGFP and MTS-GR-EGFP in Phoenix Eco cells: Cells grown on cover slips in 12-well plates were collected 48 h after transfection, fixed and mounted as described under

“Methods”. Cells expressing EGFP (**A, D**), GR-EGFP (**B, E**) and MTS-GR-EGFP (**C, F**) are shown. Panels (**A, B, C**) represent Hoffmann-contrast images of panels **D, E** and **F**, respectively (200×)

compared to cells isolated from mice reconstituted with EGFP-expressing bone marrow (Fig. 3).

Thus, using three different genes (cytosolic GR-EGFP, or mitochondrial MTS-GR-EGFP and EGFP) we, in each instant, successfully generated cell lines that produce high titer retrovirus, demonstrating the reproducibility and general applicability of our approach. We also demonstrated that retroviral gene transfer and subsequent bone marrow transplantation in mice results in long-term increased GR activity in macrophages isolated from mice that received GR-EGFP or MTS-GR-EGFP-expressing bone marrow cells. To our knowledge, this is the first report of retroviruses carrying glutathione reductase genes. Recombinant retroviral vectors are widely used in the laboratory, and in experimental clinical applications, to introduce new genetic material into hematopoietic cells in a stable form because of their potential to efficiently transduce replicating cells and integrate in the host genomic DNA (Nienhuis et al. 1991; Einerhand and Valerio 1992; Kerr and Mule 1994; Brenner et al. 1995;

Fig. 3 Glutathione reductase activity in peritoneal macrophages isolated from LDL-R^{-/-} mice reconstituted with EGFP, GR-EGFP or MTS-GR-EGFP-expressing bone marrow: Bone marrow cells were transduced with retroviral vectors carrying either EGFP, GR-EGFP or MTS-GR-EGFP and transplanted into lethally irradiated LDLR^{-/-} mice. Fifteen weeks after bone marrow transplantation, peritoneal macrophages were isolated from the recipient mice and cellular GR activity was determined as described under “Methods”

Vile and Russell 1995; Dunbar and Emmons 1994). Monocytes and macrophages are derived from bone marrow progenitor cells. Because they can migrate into the vessel wall and are involved in all stages of atherosclerosis, monocyte-derived macrophages are attractive targets for cell-mediated gene therapy of atherosclerosis (Ishiguro et al. 2001; Yoshida et al. 2001; Hasty et al. 1999). Using the retroviral vectors we described here, we recently showed that overexpression of GR and MTS-GR in macrophages of atherosclerosis-prone LDL-R^{-/-} mice reduces the severity of atherosclerosis (M. Kisgati et al., *Arterioscler. Thromb. Vasc. Biol.*, in revision), demonstrating the effectiveness of our retroviral vectors in vivo. These vectors are therefore useful tools to study, both in vitro and in vivo the role of the GSH redox state of hematopoietic cells, including monocytes and macrophages, in a wide array of pathologies and disease settings.

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