THE JOURNAL OF GENE MEDICINE
J Gene Med 2004; **6**: 616–623.
Published online 25 February 2004 in Wiley InterScience (www.interscience.wiley.com). **DOI:** 10.1002/jgm.548

RESEARCH ARTICLE

Decline of surface MHC I by adenoviral gene transfer of anti-MHC I intrabodies in human endothelial cells – new perspectives for the generation of universal donor cells for tissue transplantation

Florian Beyer[1]
Cornelia Doebis[1]
Annette Busch[1]
Thomas Ritter[1]
Abner Mhashilkar[2]
Wayne M. Marasco[2]
Horst Laube[3]
Hans-Dieter Volk[1]
Martina Seifert[1]*

[1]*Institute of Medical Immunology, Campus Charité-Mitte, Schumannstraße 20/21, 10098 Berlin, Germany*

[2]*Department of Cancer Immunology & AIDS, Dana-Farber Cancer Institute, Harvard Medical School, 44 Binney Street, Boston, MA 02115, USA*

[3]*German Heart Institute Berlin, Augustenburger Platz 1, 13353 Berlin, Germany*

*Correspondence to:
Martina Seifert, Institut of Medical Immunology, Campus Charité-Mitte, Humboldt University Berlin, Schumannstraße 20/21, 10117 Berlin, Germany.
E-mail: martina.seifert@charite.de

Received: 24 July 2003
Revised: 20 October 2003
Accepted: 13 November 2003

Abstract

Background The seeding of small-calibre vascular polytetrafluoroethylene (PTFE) grafts with endothelial cells provides an increase in biocompatibility of the graft surface. The harvest and *ex vivo* culture of autologous endothelial cells is highly delicate. Allogeneic human umbilical vein endothelial cells (HUVEC) could be a potential cell source – however, rejection might occur due to major histocompatibility complex (MHC) I mismatches. Lowering cell surface MHC I expression on endothelial cells by gene transfer of an anti-MHC I intrabody might reduce graft failure. The intrabody consists of a single-chain variable fragment (sFv) of an anti-MHC I antibody, carrying a terminal KDEL sequence to retain the molecule together with the MHC I inside the endoplasmic reticulum.

Methods Adenoviral gene transfer was used to express the intrabody in HUVEC. The MHC I surface expression was measured 48 h after transduction by flow cytometry. Functional effects of the intrabody expression were analyzed in a calcein release cytotoxicity assay.

Results A transduction efficiency of more than 95% with EGFP-adenovirus indicates a sufficient gene transfer into HUVEC. Intrabody-adenovirus-transduced HUVEC show a massive reduction in MHC I surface expression creating almost a complete 'knockout' phenotype. Stimulation with inflammatory cytokines could not overcome this effect. The cell lysis of anti-MHC I intrabody-expressing HUVEC in a cytotoxicity assay is reduced when compared with the level of the MHC mismatched control.

Conclusions Our data indicate that HUVEC with reduced levels of MHC I might be used as universal donor cells for the seeding of vascular grafts.

Keywords intrabodies; MHC I; HUVEC; tissue engineering; adenoviral transduction; immunomodulation

Introduction

At the present time the grafting of autologous veins is the gold standard in vascular surgery. However, the vascular status of the patient often does not allow the transplantation of venous autografts. The usage of cadaver

allografts and the implantation of artificial vascular prostheses could help in this indication. Whereas allografts may be potentially infectious and show signs of rejection, small-diameter vascular prostheses made of polytetrafluoroethylene (PTFE) show low patency rates [1]. The luminal surface of PTFE lacks an endothelial layer that prevents thrombosis and the formation of a hyperplastic neointima. In the course of advances in cell culture and the concept of tissue engineering, *in vitro* endothelialized grafts and the development of fully tissue-engineered vessels consisting of a matrix seeded with different vascular cell types are now being used [2]. In the past, several clinical approaches using autologous endothelial cells for the seeding of small-calibre vascular prostheses to improve the long-term patency rate have been encouraging [3–6]. Preserved allogeneic endothelial cells, e.g. HUVEC, might facilitate the difficult generation of autologous seeded grafts, if rejection could be avoided.

In vascular allograft rejection several arms of the immune system are involved. Direct interaction of donor major histocompatibility complex class I molecules (MHC I) with host $CD8^+$ cells induces proliferation and cytotoxic differentiation [7–10]. $CD8^+$ cytotoxicity is mediated by the perforin/granzyme and by the Fas/FasL pathway following the target MHC I/TCR interaction, thus leading to target cell apoptosis [11]. Donor MHC II molecules are recognized by host $CD4^+$ and it is mainly host Th1 cells that are involved in rejection processes by inducing allo-antibody production and macrophage delayed type hypersensitivity as well as causing direct cytotoxicity via their FasL expression [12]. Indirect allorecognition by host antigen-presenting cells includes donor antigen processing and presentation on self-MHC II to host $CD4^+$ and $CD8^+$ cells being responsible for chronic rejection processes [13,14].

Ex vivo gene therapy with the goal of providing cytoprotection of allogeneic endothelial cells is a promising area of clinical investigation [15,16]. One method to accomplish this task is by modulating the MHC I surface expression of endothelial cells to reduce the immunogenicity of allogeneic cells. Because of the diversity and different loci of the MHC I genes a direct 'genetic' knockout is not possible. In the past several interferences of different steps of antigen processing have been described. These include disruption in antigen processing by the proteasome complex, peptide transport by the TAP transporter molecule and correct assembly of the synthesized MHC I-peptide complex with the $\beta 2$-microglobulin molecule as potential targets to reduce MHC I expression on cell surfaces [17–19].

Intracellular antibodies, or intrabodies, provide a unique and powerful method to manipulate the expression and the cellular transport of proteins [20,21]. The intrabody itself usually consists of the sFv fragment, which is constructed from the variable regions of the antibody molecule (VH and VL) with a $(Gly_4\text{-}Ser)_3$ interchain linker (ICL) to create the VH-ICL-VL or VL-ICL-VH single-chain fragments (sFvs) [22]. The sFvs are then further engineered to become intrabodies by the addition of intracellular targeting signals. For example, by using an N-terminal immunoglobulin leader sequence to direct the expression of the sFv to the lumen of the endoplasmic reticulum (ER) and carboxy terminal KDEL sequence as a retention signal, the intrabody molecule remains inside the ER, where it can capture and retain its target molecule [21]. To date several intrabody targeting cell surface molecules, e.g. the IL-2 receptor, the EGF-receptor, the CD2 and the VLA-4 antigen, have been employed successfully in therapeutic settings [23–26]. Intrabodies recognizing a non-polymorphic MHC I epitope were shown to retain the MHC I molecule inside the ER and prevent MHC I surface expression. Our experiments with anti-MHC I intrabodies both transiently and stably expressed in different human and rodent epithelial cells and cell lines have demonstrated a dramatic reduction in MHC I cell surface expression [27].

The aim of this study is to evaluate the potency of anti-MHC I intrabodies to reduce the MHC I expression in HUVEC and to examine the functional consequences of this approach in immune recognition by *in vitro* cellular cytotoxicity studies.

Materials and methods

HUVEC isolation

Isolation of HUVEC was performed according to the method described by Jaffe *et al*. [28]. Briefly, umbilical cords obtained from the obstetric ward were stored and transported in sterile cold HEPES buffer. Umbilical veins were cannulated, rinsed with HEPES buffer, filled with 0.2% collagenase II (Biochrom KG, Berlin, Germany) in HBSS solution and incubated at room temperature for 15 min. Cells were flushed out with HEPES buffer, spun down at 200 *g*, taken up in 5 ml EGM-2 (Cell Systems, St. Katharinen, Germany) containing full supplements according to the manufacturer's instructions and seeded on T25 flasks (Falcon, Becton Dickinson, Heidelberg, Germany).

HUVEC culture

Multidonor HUVEC (Cascade Biologics, Portland, Oregon, USA) and single donor HUVEC were grown on plastic tissue culture ware (24-well plate, T25 flask, T75 flask; Falcon, Becton Dickinson) in fully supplemented EGM-2 medium at 37 °C and 5% CO_2-humidified atmosphere. For all experiments cells were used at passage level 5 to 10. Endothelial identity was verified by histological criteria as polygonal shape and "cobble stone" morphology, contact inhibited growth in monolayers, CD31 staining and uptake of DilAcLDL (Cell Systems).

Flow cytometry and antibodies

Flow cytometry was carried out on a FACSort (Becton Dickinson, Germany) using Cell Quest software (Becton Dickinson). Approximately 1×10^5 cells were stained in FACS tubes (ICN Biomedicals Inc., Ohio, USA) and from vital gated cells 1×10^4 events were collected per sample. Endothelial cells were stained with fluorescein isothiocyanate (FITC)-conjugated a-MHC I, a-MHC II (BD Biosciences, Heidelberg, Germany), a-CD71, a-CD54 (ImmunoTech, Beckman Coulter, Krefeld, Germany) and phycoerythrin (PE)-labelled a-CD31 (BD Biosciences) antibodies. The cytotoxic T lymphocyte (CTL) immunophenotype was determined by staining with FITC-labelled a-CD25 (ImmunoTech, Beckman Coulter), a-CD3, PERCP-conjugated a-CD8 or PE-labelled a-HLA DR antibodies (all from BD Biosciences). FITC- or PE-labelled mouse IgG antibodies (ImmunoTech, Beckman Coulter) served as isotypic control for all staining experiments.

Viral vectors and adenoviral transduction

Experiments were performed with the E1-deleted human Ad5 recombinant adenovirus containing the anti-human MHC I single-chain intrabody fragment (AdsFv, intrabody) as the therapeutic gene, the enhanced green fluorescent protein (AdEGFP, gift from A. Flügel, Munich, Germany) as reporter construct, and *E. coli* β-galactosidase (AdβGal) or human-α_1-antitrypsin (AdhAAT, gift from M. Kay, Stanford, USA) as negative control constructs. The construction of recombinant vectors, virus production, purification and storage are described elsewhere [29]. For gene transfer, HUVEC were grown to 80% confluence in 24-well plates or T25 flasks. Transduction of cells was performed with a multiplicity of infection (MOI) of 200 plaque-forming units (pfu) per cell in a total volume of 200 µl (24-well plates) or 2 ml (T25 flasks) in EGM-2 supplemented with 8 µg/ml hexadimethrine bromide (Polybrene; Sigma, Deisenhoven, Germany), respectively [30]. After 30 min incubation at room temperature, virus-containing solution was removed and fresh medium was added. In the case of cytokine stimulation the medium was supplemented either with 250 pg/ml human recombinant IFN-γ (R&D Systems, Wiesbaden, Germany) or 5 ng/ml TNF-α (R&D Systems). Forty-eight hours after the transduction the cells were analyzed by flow cytometry or used as target cells in cytotoxicity assays.

B lymphoblastoid cell line (BLC) generation

BLC were generated from umbilical cord blood taken from each donor of HUVEC. Cord blood mononuclear cells were isolated by density gradient centrifugation using Ficoll Paque (Pharmacia Biotech, Upsalla, Sweden). Mononuclear cells were transformed with the supernatant of the Epstein-Barr virus (EBV) secreting cell line B95/8, described elsewhere [8,9,31]. BLC were cultivated as bulk cultures in vertical T25 flasks at 37 °C and 5% CO_2-humidified atmosphere in RPMI 1640 medium (Biochrom KG, Berlin, Germany) supplemented with 10% fetal calf serum (FCS; Biochrom KG), 2 mM L-glutamine (Life Technologies, Karlsruhe, Germany), 100 U/ml penicillin (Life Technologies) and 100 µg/ml streptomycin (Life Technologies). HLA-typing by PCR was kindly performed by M. Nagy (Institute of Forensic Medicine, Campus Charité-Mitte, Berlin, Germany).

Isolation of $CD8^+$ cells

PBMC were isolated from tissue-typed healthy donors by density gradient centrifugation. $CD8^+$ lymphocytes were positively selected using a-CD8 antibody coated magnetic beads (Dynal, Hamburg, Germany) according to the manufacturer's instructions. Briefly, PBMC were suspended at 1×10^7 cells/ml and incubated with 1.2×10^7 Dynabeads/ml for 20 min at 4 °C while permanently rolling and shaking to prevent bead sedimentation. Dynabeads with attached $CD8^+$ cells were positively separated and washed. Dynabeads were removed by incubation with Detachabead solution (Detachabead CD4/CD8; Dynal) for 45 min at room temperature under permanent rolling and shaking. $CD8^+$ cells were restored by negative magnetic isolation. Washed $CD8^+$ cells were diluted at 1×10^6/ml in RPMI 1640 medium supplemented with 10% human AB serum (Sigma), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. About 1×10^7 $CD8^+$ cells were recovered from 1×10^8 PBMC.

Generation of specific cytotoxic T lymphocytes (CTL)

We used BLC of haplotype HLA-A 3 and 23 and HLA-B 8 and 44 (stimulator cells) to sensitize $CD8^+$ cells of haplotype HLA-A 1 and 29 and HLA-B 8 and 44 (responder cells). Cells of the haplotype HLA-A 1 and HLA-B 51 and 40 served as MHC mismatched controls. 1×10^5 $CD8^+$ cells were co-cultured with 2×10^4 allogeneic γ-irradiated (100 Gy) BLC in 200 µl full supplemented RPMI 1640 with 10% human AB serum per well in 96-well round-bottomed plates (Nunc, Roskilde, Denmark) [16,32]. On days 3 and 10 of co-culture, 5 ng/ml human recombinant IL-2 (Proleukin; Cetus Corp., Emeryville, CA, USA) were supplemented. At day 7 fresh medium supplemented with 5 ng/ml IL-2 and γ-irradiated BLC at a ratio of one stimulator cell per five responder cells was given to the proliferating CTL. On day 12 the cells were harvested and used as effector cells in cytotoxicity assays. FACS analysis of CTL was performed on days 3, 10 and 12 of co-culture.

Cytotoxicity assay

CTL cytotoxicity was quantified by a calcein release assay as described elsewhere [16,33,34]. Briefly, the harvested target cells were incubated in serum-free culture medium containing 10 mM calcein-AM (Molecular Probes, Leiden, The Netherlands) for 30 min at 37 °C, washed four times in cold full supplemented RPMI 1640 with 10% FCS and plated with 2×10^4 HUVEC or 4×10^4 BLC in 100 µl full supplemented RPMI 1640 with 10% human AB serum in 96-well round-bottomed plates (Nunc). Effector cells were harvested, adjusted to 8×10^6 cells/ml and diluted in four steps down to 1×10^6 cells/ml in full supplemented RPMI 1640 with 10% human AB serum. Then, 100 µl of effector cell dilution were added to 100 µl target cell solution and incubated for 3 h at 37 °C in a 5% CO_2-humidified atmosphere. Effector/target cell ratios ranged from 40 : 1 to 5 : 1 (for HUVEC as targets) or 20 : 1 to 2.5 : 1 (for BLC as targets) and all samples were plated. In parallel samples, the T cell receptor (TCR)-specific lysis was blocked by a 30 min pre-incubation of effector cell dilutions with 10 µg/ml OKT3 antibody (Jansen-Cilag, Neuss, Germany) at room temperature. For maximal calcein release, the target cells were lysed in 200 µl medium containing 0.9% Triton X (Serva, Heidelberg, Germany). For spontaneous calcein release, target cells were incubated in 200 µl medium alone. From each effector/target ratio, three replicates were analyzed. Maximal and spontaneous releases were determined 4-fold. After incubation the cells were pelleted by centrifugation at 200 rpm for 3 min and 100 µl of the supernatant were carefully transferred to 96-well flat-bottomed plates (Nunc). Fluorescence was measured with a fluorimeter (Tecan Spectrafluor; Tecan Deutschland GmbH, Crailsheim, Germany). Percent of specific killing was calculated as: [(experimental release – spontaneous release) × 100/(maximal release – spontaneous release)]. Blocking the CD3 molecule of the CTL with OKT3 antibodies, the TCR-dependent killing was inhibited and the real amount of unspecific killing was revealed. Subtracting this value from the 'specific killing' we termed the parameter 'TCR-specific killing'. For TCR-specific killing, specific killing values of OKT3-treated samples were subtracted from corresponding specific killing values.

Statistical analysis

The differences in MHC I surface expression of HUVEC were analyzed with a one-way ANOVA test followed by the Wilcoxon test. The different groups of the cytotoxicity assays were compared with the Wilcoxon test. Each group of the HUVEC passed a regression analysis using Friedman's test.

Results

Efficiency of adenoviral transduction

To assure a sufficient transfer and protein expression of therapeutic genes by adenoviral transduction we used an adenovirus expressing EGFP (AdEGFP) as a reporter gene. HUVEC were transduced with either AdEGFP or the control vector AdhAAT. In flow cytometric analysis AdEGFP-transduced cells displayed a strong signal in the green fluorescence (FL-1) due to their EGFP expression, whereas non-transduced or AdAAT-transduced cells did not (Figure 1). The transduction efficiency in our experimental system was 97.4% (median, data of two experiments, each performed in triplicate).

Inhibition of MHC I cell surface expression

Flow cytometric analysis of MHC I expression on AdsFv-transduced HUVEC was carried out in five independent experiments, each performed in triplicate. As a reference, the median level of the constitutive MHC I expression of HUVEC was set to 1. The MHC I expression of AdsFv-transduced HUVEC was significantly reduced to 0.08 ($p < 0.01$). In contrast, the MHC I expression of AdAAT- (negative control) transduced HUVEC achieved 1.19 without reaching a significant difference to the control (Figure 2). Stimulation of HUVEC for 48 h with 250 pg/ml of IFN-γ resulted in a 7-fold increase in surface MHC I expression for both non-transduced and AdhAAT-transduced cells in contrast to a 0.45-fold increase for AdsFv-transduced cells (Figure 3a). Stimulation with 5 ng/ml TNF-α over 48 h enhanced MHC I levels to 4.82 and 9.28 for non-transduced and control vector transduced HUVEC, respectively, versus 0.52 for intrabody-expressing cells

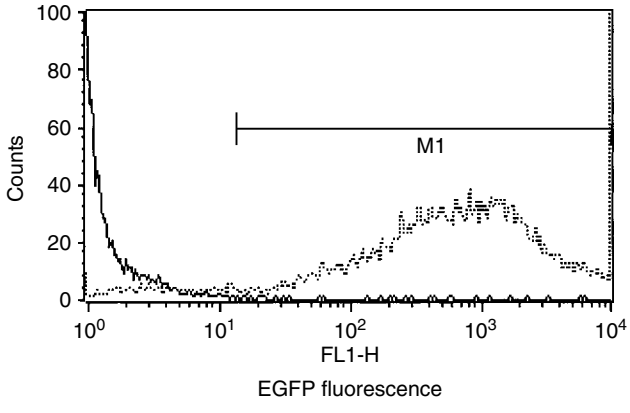

Figure 1. FACS histograms of AdEGFP- (reporter gene) and AdAAT- (control) transduced HUVEC 48 h after transduction. EGFP-expressing cells (dashed line) are gated by their enhanced signal in the FL1 channel. The overall rate of EGFP-expressing AdEGFP-transduced HUVEC was 97% in contrast to 0.5% of AdAAT-transduced cells (solid line)

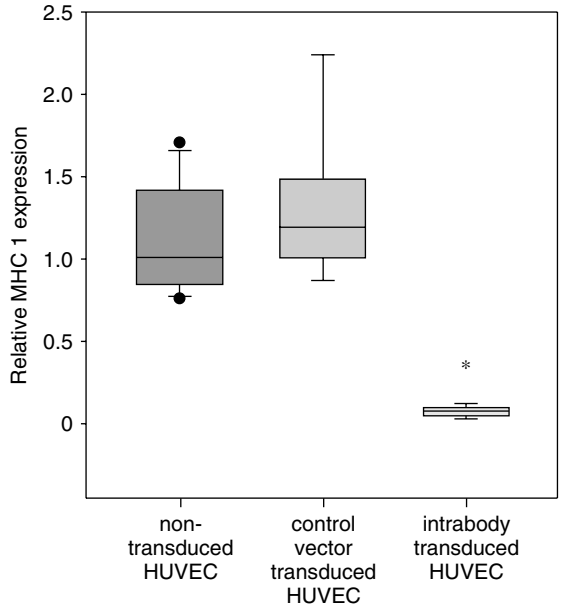

Figure 2. Box plots showing the MHC I surface expression of non-transduced HUVEC (dark grey boxes), AdβGal- (control vector, light grey boxes) and AdsFv- (intrabody, white boxes) transduced HUVEC measured by flow cytometry 48 h after transduction. Data from five experiments each performed in triplicate are included. Constitutive MHC I expression level was set to 1. For statistical analysis a one-way ANOVA was performed for each group (all three groups, $p < 0.01$), and single differences were determined by a Wilcoxon test. Stars over the boxes indicate significant differences. The solid line inside the boxes displays the median, the boxes themselves represent 25–75% of the values and the columns outside the boxes show 5–95% of variance. Fat dots represent outlier values. MHC I expression of HUVEC was analyzed 48 h after transduction. The relative MHC I expression of the intrabody-treated group was 0.075 (median) and significantly lower than the AdAAT-treated group (1.19, $p < 0.05$) and the non-transduced HUVEC (1, $p < 0.05$). The difference in MHC I expression of non-transduced and AdAAT-transduced cells was not significant

(Figure 3b). Taken together, unstimulated intrabody-expressing HUVEC almost completely lost MHC I surface expression. Remarkably, cytokine stimulation did not significantly overcome this inhibition (<10% of control transduced cells).

Intrabody transduction protects HUVEC from CTL-mediated lysis

To analyze the functional effects of AdsFv gene transfer into HUVEC, we established a cell-mediated cytotoxicity model. It is well known that endothelial cells, which are non-professional antigen-presenting cells, trigger only weak CTL responses [9,10,34]. To generate potent anti-endothelial CTL we used autologous BLC as professional antigen-presenting B cells and IL-2 to sensitize allogeneic $CD8^+$ cells. PBMC isolated from umbilical cord blood were immortalized by EBV transformation and developed a BLC phenotype. The cytolytic potency of the generated CTL was quantified in a calcein release cytotoxic assay. First we investigated whether CTL sensitized against allogeneic BLC are able to lyse stimulator BLC in an allospecific fashion. For all effector/target ratios a significant difference between allogeneic and MHC-mismatched cell lysis could be detected (specific killing). This difference was also detected after calculation of T-cell-dependent killing using OKT3 (data not shown). The TCR-specific killing at a 20 : 1 effector/target ratio reached a median of 39 and 8% for allogeneic and MHC-mismatched BLC, respectively (Figure 4a). When HUVEC served as target cells the killing remained significantly allospecific although the total cell lysis decreased to a median of 18 and 3% at a 40 : 1 effector/target ratio for allogeneic and MHC-mismatched HUVEC, respectively (Figure 4b). Finally, when AdsFv- and AdβGal-transduced allogeneic HUVEC were used as targets, the killing of AdsFv-treated cells was significantly inhibited to a median of 6% vs.18% for AdβGal-transduced cells at a 40 : 1 ratio (Figure 4c). The synopsis of the cytotoxicity studies (Figure 5) shows that the killing of intrabody-transduced cells was comparable to the low levels of the MHC I-mismatched control cells whereas AdβGal-transduced cells were strongly lysed, similar to non-transduced allogeneic HUVEC.

Discussion

Allospecific T lymphocytes recognize transplanted cells/tissues via the MHC I presentation of allopeptides [9,11,12,35]. One way to prevent CTL-mediated lysis of those allogeneic cells is to down-regulate MHC I surface expression.

In our study we investigated an intracellularly expressed antibody against a non-polymorphic MHC I epitope. This intrabody binds MHC I molecules within the lumen of the endoplasmic reticulum, preventing their surface expression [27]. Adenoviral transduction of HUVEC generated a phenotypic knockout of MHC I surface expression that could be revealed in flow cytometric analysis. This effect was neither changed by stimulation of transduced HUVEC with inflammatory cytokines like IFN-γ nor TNF-α. The increase in MHC I expression of AdsFv-transduced and IFN-γ- or TNF-α-stimulated HUVEC seems to be a result of the raised MHC I expression of the small fraction of HUVEC that failed the adenoviral transduction. Other surface markers like MHC II, CD54, CD71 and CD31 were not altered by AdsFv transduction and showed comparable expression levels on control vector transduced cells (data not shown). However, the HUVEC surface expression of MHC I, and also of the other surface molecules, seems to be in general marginally increased by the adenoviral transduction procedure itself regardless of the construct used for gene transfer [35–37]. To prove functional effects of the MHC I down-regulation by this gene therapeutic approach we performed cytotoxicity assays.

Despite using sensitized allospecific CTL we found a dramatic decrease in the cytotoxicity, underlining the

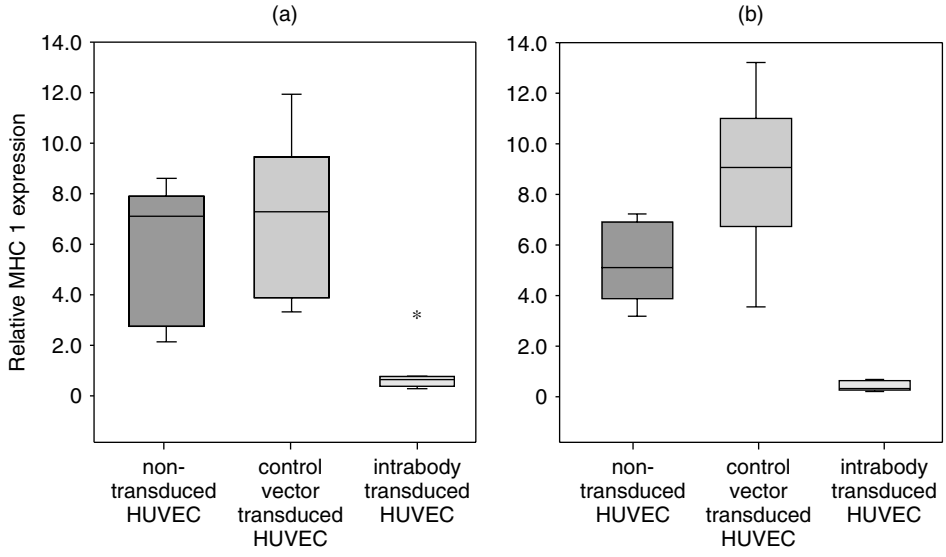

Figure 3. Box plots showing the MHC I surface expression of non-transduced HUVEC (dark grey boxes), AdβGal- (control vector, light grey boxes) and AdsFv- (intrabody, white boxes) transduced HUVEC measured by flow cytometry 48 h after transduction. Data from five experiments each performed in triplicate are included. Constitutive MHC I expression level was set to 1. (a) MHC I expression of HUVEC after 48 h stimulation with 250 pg/ml IFN-γ is displayed. Non-transduced and AdAAT-transduced HUVEC show increased relative MHC I levels of 7.04 and 7.22. The MHC I level of intrabody-transduced cells remained at 0.45 (the differences to non-transduced and control vector transduced cells were both significant with $p < 0.05$). (b) HUVEC following 48 h stimulation with 5 ng/ml TNF-α are depicted. Non-transduced and AdAAT-transduced cells show MHC I levels of 4.82 and 9.28 (the difference is not significant). AdsFv-transduced HUVEC express 0.52 of constitutive MHC I differs with $p < 0.05$ for non-transduced and control vector transduced counterparts

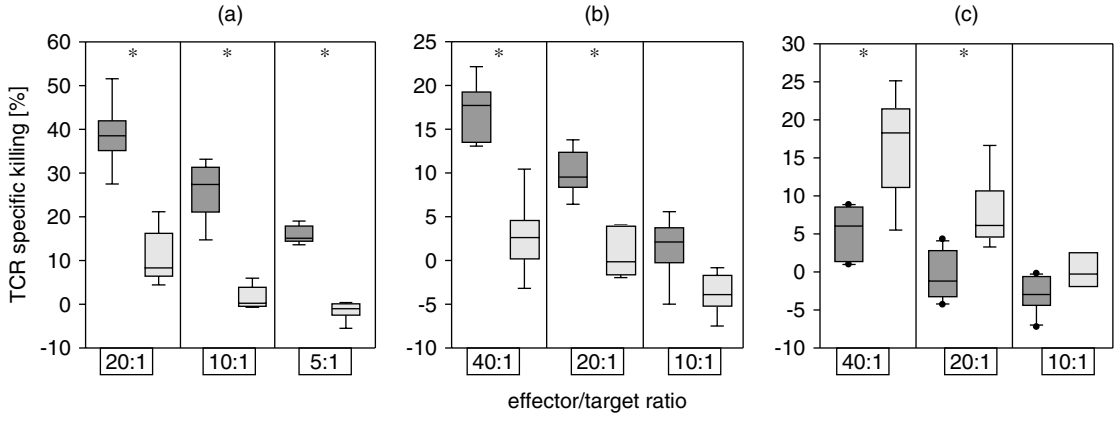

Figure 4. The results of the cytotoxicity assays as TCR-specific killing (five experiments, each performed in triplicate) are displayed as box plots. The solid line inside the boxes represents the median, the boxes themselves show the range from 25–75% of the values and 90% of all values are graphed by the columns outside the boxes. Fat dots mark outlier values. Stars above the boxes indicate significant differences between the two groups calculated with the Wilcoxon test. (a) Stimulator BLC served as allogeneic target cells (dark grey boxes) and MHC mismatched BLC as third party controls (light grey boxes). At all effector/target ratios significant differences between the groups exist with $p < 0.05$. (b) HUVEC of either allogeneic (dark grey boxes) or MHC mismatched (light grey boxes) haplotypes were used as target cells. With $p < 0.05$ the killing is significant in the two lower effector cell dilutions. The significance between the groups indicates the allospecificity of the killing. (c) AdsFv- or AdβGal-transduced allogeneic HUVEC served as targets in the cytotoxicity assay. Intrabody-transduced cells (dark grey boxes) are lysed to a lesser degree than control vector treated cells (light grey boxes). Differences are significant ($p < 0.05$) at the effector/target ratios 40 : 1 and 20 : 1

functional efficiency of our approach. Flow cytometric analyses of generated CTL showed a $CD3^+$- and $CD8^+$-dominant phenotype. During the co-culture activation markers like CD25, CD69 and HLA-DR were increased in contrast to non-BLC or IL-2 treated controls (data not shown). Intrabody-transduced HUVEC were protected from the cell lysis by allogeneic sensitized CTL, while non-transduced and control vector transduced HUVEC of the same donor were not. Cytoprotection due to MHC I down-regulation has previously been demonstrated [17,18,38]. Whereas other studies were based on viral immune escape strategies, the intrabody used in our experiments is the first engineered MHC I retaining molecule. Considering the application of those intrabody-treated cells in a transplantation model, some further aspects of the hosts' immune systems have to be discussed. First, an MHC

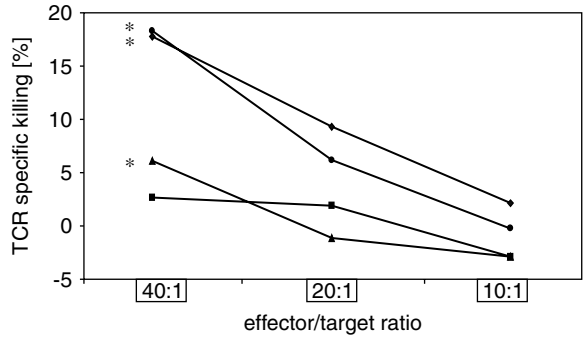

Figure 5. The results of the cytotoxicity assays with HUVEC as target cells in a synoptic view. TCR-specific killing is shown for AdsFv-transduced allogeneic HUVEC (▲), AdβGal-transduced allogeneic HUVEC (●), non-transduced MHC mismatched HUVEC (■), and non-transduced allogeneic HUVEC (♦). Stars represent significant regressions determined by the Friedman test. Allogeneic HUVEC and AdβGal-transduced HUVEC, on the one hand, and MHC-mismatched HUVEC and intrabody-transduced HUVEC, on the other, show comparable graphs

I down-regulation would neither protect grafts from unspecific, innate inflammatory responses nor inhibit an antigen presentation to the host and thereby initiate $CD4^+$-cell activation and humoral responses. Secondly, NK cells were shown to lyse cells with reduced protective MHC I expression [39–43]; however, they seem to be less efficient at killing tissues [27]. The data show promise to apply this immunomodulating principle *in vivo* (e.g. HUVEC-seeded grafts into SCID mice). In the case of a successful *in vivo* approach, we have to take in account alternative gene transfer methods to avoid the well-known problems of adenovirus of the first generation like immunogenicity. Furthermore, it could be that the murine origin of the anti-MHC I intrabody does induce undesirable immune responses. These problems might be avoided by the isolation of a complete human intrabody fragment with the same binding features, as recently described for a number of molecular targets [44–46]. Non-immunogeneic human intrabodies open up the way for their clinical use in a gene therapy setting.

Acknowledgements

This work was partly supported by the BMBF (01 GN 0117). We thank Elke Effenberger and Heinz Tanzmann for skilful technical assistance.

References

1. Da Lio AL, Jones NF. New concepts and materials in microvascular grafting: prosthetic graft endothelial cell seeding and gene therapy. *Microsurgery* 1998; **18**: 263–266.
2. Teebken OE, Haverich A. Tissue engineering of small diameter vascular grafts. *Eur J Vasc Endovasc Surg* 2002; **23**: 475–485.
3. Rademacher A, Paulitschke M, Meyer R, Hetzer R. Endothelialization of PTFE vascular grafts under flow induces significant cell changes. *Int J Artif Organs* 2001; **24**: 235–242.
4. Zilla P, Deutsch M, Meinhart J, *et al*. Clinical in vitro endothelialization of femoropopliteal bypass grafts: an actuarial follow-up over three years. *J Vasc Surg* 1994; **19**: 540–548.
5. Laube HR, Duwe J, Rutsch W, Konertz W. Clinical experience with autologous endothelial cell-seeded polytetrafluoroethylene coronary artery bypass grafts. *J Thorac Cardiovasc Surg* 2000; **120**: 134–141.
6. Meinhart JG, Deutsch M, Fischlein T, Howanietz N, Froschl A, Zilla P. Clinical autologous in vitro endothelialization of 153 infrainguinal ePTFE grafts. *Ann Thorac Surg* 2001; **71**: S327–331.
7. Kreisel D, Krupnick AS, Gelman AE, *et al*. Non-hematopoietic allograft cells directly activate CD8+ T cells and trigger acute rejection: an alternative mechanism of allorecognition. *Nat Med* 2002; **8**: 233–239.
8. Biedermann BC, Pober JS. Human endothelial cells induce and regulate cytolytic T cell differentiation. *J Immunol* 1998; **161**: 4679–4687.
9. Biedermann BC, Pober JS. Human vascular endothelial cells favor clonal expansion of unusual alloreactive CTL. *J Immunol* 1999; **162**: 7022–7030.
10. Epperson DE, Pober JS. Antigen-presenting function of human endothelial cells. Direct activation of resting CD8 T cells. *J Immunol* 1994; **153**: 5402–5412.
11. Krupnick AS, Kreisel D, Popma SH, *et al*. Mechanism of T cell-mediated endothelial apoptosis. *Transplantation* 2002; **74**: 871–876.
12. Le Moine A, Goldman M, Abramowicz D. Multiple pathways to allograft rejection. *Transplantation* 2002; **73**: 1373–1381.
13. Valujskikh A, Lantz O, Celli S, Matzinger P, Heeger PS. Cross-primed CD8(+) T cells mediate graft rejection via a distinct effector pathway. *Nat Immunol* 2002; **3**: 844–851.
14. Vella JP, Magee C, Vos L, *et al*. Cellular and humoral mechanisms of vascularized allograft rejection induced by indirect recognition of donor MHC allopeptides. *Transplantation* 1999; **67**: 1523–1532.
15. Mann MJ, Whittemore AD, Donaldson MC, *et al*. Ex-vivo gene therapy of human vascular bypass grafts with E2F decoy: the PREVENT single-centre, randomised, controlled trial. *Lancet* 1999; **354**: 1493–1498.
16. Zheng L, Dengler TJ, Kluger MS, *et al*. Cytoprotection of human umbilical vein endothelial cells against apoptosis and CTL-mediated lysis provided by caspase-resistant Bcl-2 without alterations in growth or activation responses. *J Immunol* 2000; **164**: 4665–4671.
17. Furukawa L, Brevetti LS, Brady SE, *et al*. Adenoviral-mediated gene transfer of ICP47 inhibits major histocompatibility complex class I expression on vascular cells in vitro. *J Vasc Surg* 2000; **31**: 558–566.
18. Freland S, Chambers BJ, Andersson M, Van Kaer L, Ljunggren HG. Rejection of allogeneic and syngeneic but not MHC class I-deficient tumor grafts by MHC class I-deficient mice. *J Immunol* 1998; **160**: 572–579.
19. Hultman CS, Hunt JP, Yamamoto H, *et al*. Immunogenicity of cultured keratinocyte allografts deficient in major histocompatibility complex antigens. *J Trauma* 1998; **45**: 25–33; discussion 33–34.
20. Marasco WA, Dana Jones S. Antibodies for targeted gene therapy: extracellular gene targeting and intracellular expression. *Adv Drug Deliv Rev* 1998; **31**: 153–170.
21. Chen SY, Bagley J, Marasco WA. Intracellular antibodies as a new class of therapeutic molecules for gene therapy. *Hum Gene Ther* 1994; **5**: 595–601.
22. Huston JS, Levinson D, Mudgett-Hunter M, *et al*. Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *E. coli*. *Proc Natl Acad Sci U S A* 1988; **85**: 5879–5883.
23. Greenman J, Jones E, Wright MD, Barclay AN. The use of intracellular single-chain antibody fragments to inhibit specifically the expression of cell surface molecules. *J Immunol Methods* 1996; **194**: 169–180.
24. Richardson JH, Hofmann W, Sodroski JG, Marasco WA. Intrabody-mediated knockout of the high-affinity IL-2 receptor in primary human T cells using a bicistronic lentivirus vector. *Gene Ther* 1998; **5**: 635–644.
25. Jannot CB, Beerli RR, Mason S, Gullick WJ, Hynes NE. Intracellular expression of a single-chain antibody directed to the EGFR leads to growth inhibition of tumor cells. *Oncogene* 1996; **13**: 275–282.
26. Yuan Q, Strauch KL, Lobb RR, Hemler ME. Intracellular single-chain antibody inhibits integrin VLA-4 maturation and function. *Biochem J* 1996; **318**: 591–596.

27. Mhashilkar AM, Doebis C, Seifert M, *et al*. Intrabody-mediated phenotypic knockout of major histocompatibility complex class I expression in human and monkey cell lines and in primary human keratinocytes. *Gene Ther* 2002; **9**: 307–319.
28. Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J Clin Invest* 1973; **52**: 2745–2756.
29. Kolls J, Peppel K, Silva M, Beutler B. Prolonged and effective blockade of tumor necrosis factor activity through adenovirus-mediated gene transfer. *Proc Natl Acad Sci U S A* 1994; **91**: 215–219.
30. Doebis C, Ritter T, Brandt C, Schonberger B, Volk HD, Seifert M. Efficient in vitro transduction of epithelial cells and keratinocytes with improved adenoviral gene transfer for the application in skin tissue engineering. *Transpl Immunol* 2002; **9**: 323–329.
31. Seifert M, Schoenherr G, Roggenbuck D, Marx U, von Baehr R. Generation and characterization of a human monoclonal IgM antibody that recognizes a conserved epitope shared by lipopolysaccharides of different gram-negative bacteria. *Hybridoma* 1996; **15**: 191–198.
32. Symington FW, Santos EB. Recognition of keratinocytes by cytotoxic T cells specific for conventional HLA class-I alloantigen. *J Invest Dermatol* 1990; **95**: 224–228.
33. Roden MM, Lee KH, Panelli MC, Marincola FM. A novel cytolysis assay using fluorescent labeling and quantitative fluorescent scanning technology. *J Immunol Methods* 1999; **226**: 29–41.
34. Dengler TJ, Johnson DR, Pober JS. Human vascular endothelial cells stimulate a lower frequency of alloreactive CD8+ pre-CTL and induce less clonal expansion than matching B lymphoblastoid cells: development of a novel limiting dilution analysis method based on CFSE labeling of lymphocytes. *J Immunol* 2001; **166**: 3846–3854.
35. Lorber MI, Wilson JH, Robert ME, *et al*. Human allogeneic vascular rejection after arterial transplantation and peripheral lymphoid reconstitution in severe combined immunodeficient mice. *Transplantation* 1999; **67**: 897–903.
36. Wedgwood JF, Hatam L, Bonagura VR. Effect of interferon-gamma and tumor necrosis factor on the expression of class I and class II major histocompatibility molecules by cultured human umbilical vein endothelial cells. *Cell Immunol* 1988; **111**: 1–9.
37. Qian HS, Channon K, Neplioueva V, *et al*. Improved adenoviral vector for vascular gene therapy: beneficial effects on vascular function and inflammation. *Circ Res* 2001; **88**: 911–917.
38. Goodman DJ, von Albertini MA, McShea A, Wrighton CJ, Bach FH. Adenoviral-mediated overexpression of I(kappa)B (alpha) in endothelial cells inhibits natural killer cell-mediated endothelial cell activation. *Transplantation* 1996; **62**: 967–972.
39. Efrat S, Fejer G, Brownlee M, Horwitz MS. Prolonged survival of pancreatic islet allografts mediated by adenovirus immunoregulatory transgenes. *Proc Natl Acad Sci U S A* 1995; **92**: 6947–6951.
40. Gumperz JE, Parham P. The enigma of the natural killer cell. *Nature* 1995; **378**: 245–248.
41. Itescu S, Artrip JH, Kwiatkowski PA, *et al*. Lysis of pig endothelium by IL-2 activated human natural killer cells is inhibited by swine and human major histocompatibility complex (MHC) class I gene products. *Ann Transplant* 1997; **2**: 14–20.
42. Correa I, Corral L, Raulet DH. Multiple natural killer cell-activating signals are inhibited by major histocompatibility complex class I expression in target cells. *Eur J Immunol* 1994; **24**: 1323–1331.
43. Zimmer J, Donato L, Hanau D, *et al*. Inefficient protection of human TAP-deficient fibroblasts from autologous NK cell-mediated lysis by cytokines inducing HLA class I expression. *Eur J Immunol* 1999; **29**: 1286–1291.
44. Bai J, Sui J. Zhu RY, *et al*. Inhibition of Tat-mediated transactivation and HIV-1 replication by human Anti-hCyclinT1 Intrabodies. *J Biol Chem* 2003; **278**: 1433–1442.
45. Heschhorn A, Admon A, Hizi A. Recombinant human antibodies against reverse transcriptase of human immunodeficiency virus type-1. *Biochem Biophys Acta* 2003; **1648**: 154–163.
46. Lobato MN, Rabbitts TH. Intracellular antibodies and challenges facing their use as therapeutic agents. *Trends Mol Med* 2003; **9**: 390–396.

