An anti-major histocompatibility complex class I intrabody protects endothelial cells from an attack by immune mediators

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Abstract

Objective: In vitro endothelialization has significantly improved the overall outcome of artificial prostheses in cardiovascular bypass surgery. A drawback of this tissue-engineering method remains the limited availability of suitable autologous endothelial cells (EC), especially in aged patients. Allogeneic EC with high proliferative capacity represent a potentially valuable alternative to a patient-specific vascular transplant. However, such cells carry the risk of being rejected due to Major Histocompatibility Complex (MHC) mismatches.

Methods: We investigated the effects of a very potent, intracellularly expressed antibody directed against MHC class I molecules, referred to as α-rat MHC I single chain variable fragment (sFv) intrabody. The intrabody was stably expressed in rat aortic EC (RAEC) following lentiviral vector-mediated gene transfer. The functional consequence of the MHC I down-regulation was tested in an allogeneic setting in two different in vitro assays.

Results: Stable expression of the α-rat MHC I sFv intrabody resulted in a highly efficient depletion of surface MHC I. Thereby those RAEC which displayed low MHC I levels over extended periods of time were protected against killing by allo-specific, cytotoxic T cells (CTL) and by allo-antibody/complement-mediated lysis.

Conclusions: These results demonstrate that intrabody-mediated down-regulation of MHC I reduces the immunogenicity of RAEC which may provide a suitable alternative supply for the lining of vascular prostheses.

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Keywords: Endothelial cell; Gene therapy; Intrabody; Tissue engineering

1. Introduction

As we were able to show in our previous studies, the α-MHC I sFv intrabody is a potent modulator of MHC I surface expression leading to reduced cell immunogenicity [1,2]. The intrabody consists of a single chain variable fragment (sFv) of the OX-18 antibody and carries the endoplasmic reticulum (ER)-retention signal Lys-Asp-Glu-Leu (KDEL).

The intrabody binds and retains MHC I molecules inside the ER and hence reduces the surface MHC I expression [3].

In the present study, we investigated the effects of the α-rat MHC I sFv intrabody on the immunogenicity of primary RAEC with the aim to reduce the immunogenic features of these cells in the context of an allogeneic cell transplantation.

In the past years the development of small-diameter vascular grafts has been a field of intense investigations. In contrast to large synthetic vascular grafts that have been successfully used for aortic repair under high blood flow/low resistance conditions, artificial grafts have proven to be ill-suited for the reconstruction of smaller caliber arterial beds.

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such as the coronary circulation and peripheral vasculature. Since these grafts do not become spontaneously endothelialized in humans [4] a luminal seeding with endothelial cells prior to \textit{in vivo} implantation has to be used to prevent thrombus formation. A few groups have been able to demonstrate the efficacy of those \textit{in vitro} endothelialized femoropopliteal expanded Polytetrafluorethylene (ePTFE) grafts [5–8] and one study even showed a high patency rate in cardiac artery bypass grafting as well [9]. But the clinical advantage of any endothelialization has not been proven so far and more studies that compare native vein grafts with endothelialized artificial grafts are necessary. However, the availability and expansion of autologous EC remains the limiting step of this method as the restricted growth potential of EC from aged patients and the time required to obtain a sufficient number of these cells represent major drawbacks. Studies on other sources of immunologically compatible EC may provide novel insights in the development of non-thrombogenic artificial grafts [4].

Allogeneic EC that are easily isolated and expanded \textit{in vitro} could overcome these limitations. However, due to MHC incompatibilities, allogeneic cells carry the risk of being rejected by the host’s immune system. Recognition of the transplanted cells by antigen presenting cells (APC) of the recipient through uptake of shedded MHC structures activates processes of chronic rejection, which involve allospecific, cytotoxic T cells as well as allospecific antibodies and complement, and cause damage to the grafted allogeneic cells [10–14].

Additionally, recent data suggest that the EC itself can act as non-professional APC for direct allo-recognition by CD8+ T cells. [15,16]. For example, allogeneic human endothelial cells were described in the past to be capable of serving as competent albeit less efficient activators of CTL differentiation compared with normal professional APC like B-lymphoblastic cell lines [17].

In the present study we used the intrabody technology to modulate the immunogenenic feature of the primary RAEC by down-regulation of the surface molecule MHC I in order to circumvent such an immune response.

We were able to induce the efficient over-expression of the \(\alpha\)-rat MHC I sFv intrabody in RAEC by using lentiviral vector-mediated gene transfer. This resulted in strong down-regulation of surface MHC I expression which finally mediated the abrogation of cellular and humoral immune responses against these genetically modified RAEC. Those less immunogeneic, although allogeneic EC may represent a valuable alternative to autologous EC for the mentioned \textit{in vitro} endothelialization of vascular grafts.

Furthermore the same lentiviral \(\alpha\)-rat MHC I sFv intrabody gene transfer could be used to reduce the immunogenecity of EC and other cells of a solid organ in allogeneic transplantation. A promising application might be the \textit{ex vivo} manipulation of a donor heart in order to prevent cardiac allograft rejection and to achieve allograft tolerance as shown before by other groups using different immunomodulatory molecules [18,19].

2. Materials and methods

All animals studies for the isolation of the endothelial cells were performed in accordance to the guidelines described in the NIH Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996).

2.1. Cell culture

The isolation of RAEC was performed as described elsewhere [20]. RAEC were cultured in EC basal medium (EBM) supplemented with 10% FCS (both PAA, Pasching, A) and 50 \(\mu\)g/ml Gentamicin (Sigma, Steinheim, D). The medium was changed every 2–3 days. For lentiviral transduction, RAEC were seeded on 24-well plates and grown up to 80–90% confluence. For functional assays, RAEC were harvested by treatment with 0.05% Trypsin, 0.02% (w/v) EDTA (Life Technologies, Karlsruhe, D).

2.2. Lentiviral expression vector

The \(\alpha\)-rat MHC I sFv intrabody-containing vector NL-CEF-\(\alpha\)-ratMHCIsFv-hrGFP was generated based on the NL-CEF-EGFP vector [21] by replacing the EGFP reporter gene with an IRES-hrGFP cassette (pIRES-hrGFP-1a; Stratagene, La Jolla, USA). The \(\alpha\)-ratMHCIsFv insert was generated from the pAC-OX18sFv plasmid [1] by PCR using specific primers (For 5′-CCCTAGCTAGCATGGATTGGGTGTG-3′; Rev 5′-CCGCTCGAGCTATTACAGCTCGT-CCTTTTC-3′). The PCR fragment was inserted upstream of the IRES-hrGFP cassette. The generated vector constructs were tested for transgene insertion by conventional sequencing (MWG Biotech, Ebersberg, D). The NL-CEF-hrGFP vector was used as a control.

2.3. Production of lentiviral vector stocks

The production of lentiviral vector particles is described elsewhere [22]. Briefly, sub-confluent 293 cells were co-transfected using pNL-CEF-\(\alpha\)-ratMHCIsFv-hrGFP or pNL-CEF-hrGFP vector DNA, pCD/NL-BH*helper DNA [22] and pLTR-G envelope DNA [23] in the presence of Lipofectamin™2000 (Invitrogen, Karlsruhe, D). The virus were harvested 72 h after transfection and concentrated by using Vivaspin 20 columns (Vivascience/Sartorius, Göttingen, D).

2.4. Lentiviral transduction of target cells

RAEC were transduced with NL-CEF-\(\alpha\)-ratMHCIsFv-hrGFP or NL-CEF-hrGFP lentiviral vector stocks in complete EBM supplemented with 100 \(\mu\)g/ml Diethylaminoethy-Dextran (HCL) (DEAE-dextran; Sigma, Steinheim, D) using a multiplicity of infection (MOI) of 50. RAEC were incubated for 16 h at 37 °C. Then the viral solution was removed and cells were cultured in complete EBM for an
In order to enrich transduced cells expressing hrGFP, RAEC were sorted for hrGFP expression using a MoFlow-FACS-sorter (Dako Cytomation, Hamburg, D). In this way, the hrGFP-positive cell fraction was enriched up to 95%.

### 2.5. Detection of intrabody sequences at cDNA and genomic DNA level

Transduced RAEC and control RAEC were FACS-sorted and passaged over several times. The mRNA was obtained by the application of the “Absolutely RNA Miniprep Kit” (Stratagene, Heidelberg, D) and analysed for quality and quantity with a BioAnalyzer 2100 (Agilent, Palo Alto, CA). The cDNA was generated by reverse transcription as described in detail elsewhere [24]. Genomic DNA was obtained by using the Wizard® SV Genomic DNA Puri- fication System (Promega, Mannheim, D).

Detection of α-rat MHC I sFv sequence was processed by conventional PCR using specific primer (see below) followed by gel electrophoresis.

### 2.6. FACS analysis of surface MHC I

Detection of surface MHC I was performed by FACS analysis using the mouse-anti-rat RT1.A monoclonal antibody (mAb) OX-18 and an isotype control IgG1, mAb (MOPC-21), respectively (BD Biosciences Pharmingen, San Diego, USA). As a secondary antibody, a PE-labelled donkey-anti-mouse IgG (H+L) polyclonal antibody (Dianova, Hamburg, D) was used.

### 2.7. Cytotoxicity assay

RAEC were labelled with the fluorescence dye Calcein-AM (20 μM; Molecular Probes, Leiden, NL) in serum-free EBM for 30 min at 37 °C. Calcein-labelled RAEC were co-cultured at the given ratio with pre-activated cytotoxic T cells in complete VLE-RPMI (Biochrom AG, Berlin, D) including 10% FCS, 100 U/ml Penicillin, 100 μg/ml Streptomycin, 2 mM L-Glutamine, 5 mM HEPES, and 5 mM β-Mercaptoethanol for 3 h at 37 °C. Allo-specific, cytotoxic T cells were generated by the application of the “Absolutely RNA Miniprep Kit” (Stratagene, Heidelberg, D) and analysed for quality and quantity with a BioAnalyzer 2100 (Agilent, Palo Alto, CA). The cDNA was generated by reverse transcription as described in detail elsewhere [24]. Genomic DNA was obtained by using the Wizard® SV Genomic DNA Puri- fication System (Promega, Mannheim, D).

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using a day-5 mixed lymphocyte culture (MLC) using lymph nodes of Wistar Furth (WF; irradiated stimulator cells) or Lewis (Lew; responder cells) rats. T cell-mediated lysis of the RAEC was measured by the Calcein-release into the supernatant using a spectrofluorometer (TECAN, Crailsheim, D). Specific lysis was calculated in correlation to spontaneous (medium alone) and maximum (0.9% Triton X) Calcein-release by the RAEC.

2.8. Allo-antibody/complement-mediated lysis

Incubation of 2×10⁵ RAEC/sample with an allo-specific serum was performed in PBS/2% FCS at indicated dilutions for 30 min at RT. Allo-antibodies were washed off and cells were incubated with baby rabbit complement (Accurate Chemicals, Westbury, USA) at a dilution of 1:2 in GVB++ buffer (Sigma, Steinheim, D) for 1 h at 37 °C. After a final washing step, cells were stained with the vital dye 7-aminoactinomycin D (7-AAD; Sigma, Steinheim, D) for 15 min at RT. 7-AAD staining was measured by flow cytometry. A control serum from naive rats was used for the detection of non-specific lysis.

2.9. Statistical analysis

Results are presented as mean per group ±SEM. Statistical significance (⁎p<0.05; ⁎⁎p<0.01) was evaluated using the non-parametric Mann–Whitney U-Test for unpaired samples.

3. Results

3.1. Efficient down-regulation of surface MHC I

RAEC were transduced using the NL-CEF-α-rat MHC I sFv-hrGFP or NL-CEF-hrGFP lentiviral vectors. The FACS-enriched cell fraction was further expanded and analysed for the presence and expression of the intrabody sequences by conventional PCR (Fig. 1). α-rat MHC I sFv intrabody transgene sequences in genomic DNA and transcripts thereof were exclusively detected in the FACS-enriched α-rat MHC I sFv-hrGFP(+) cell fraction.
Detection of surface MHC I by flow cytometry showed that the intrabody-transduced RAEC expressed only low levels of MHC I compared to the constitutive MHC I levels of untransduced and hrGFP(+) transduced RAEC (Fig. 2A). After treatment of cell surface molecules using Papain, the specific intracellular staining for MHC I showed that intrabody expression resulted in enhanced accumulation of intra-cytoplasmic MHC I molecules as compared to untransduced and hrGFP(+) RAEC (Fig. 2B and C).

In a long time study aimed at surface MHC I expression on transduced RAEC, it could be shown that in comparison to hrGFP(+)RAEC, MHC I levels on α-rat MHC I sFv-hrGFP(+) RAEC were strongly diminished over time, possibly due to stable over-expression of the intrabody transgene. Reduced MHC I surface expression was detected in α-rat MHC I sFv-hrGFP(+) RAEC up to 10 weeks in culture whereas the expression levels in hrGFP(+) RAEC were not altered and stayed high within the same time frame (Fig. 3).

The transduction and sorting of the RAEC did not alter their typical cobble stone morphology and their proliferative behavior. Moreover the constitutively expressed adhesion molecules like ICAM-1, VCAM-1, P-Selectin and PECAM-1 were not changed and the activation marker MHC II was not up-regulated in comparison to wild type RAEC (data not shown).

Fig. 6. Increasing concentrations of IFN-γ can reverse the down-regulatory capacity of the intrabody on surface MHC I expression. RAEC were incubated with increasing concentrations of rat IFN-γ for 24 h and surface MHC I was detected by FACS analysis. Shown is the surface MHC I staining of untransduced, hrGFP(+) and α-rat MHC I sFv-hrGFP(+) RAEC after IFN-γ stimulation in comparison to untreated cells. Increasing amounts of IFN-γ led to an abrogation of the intrabody-mediated down-regulation of surface MHC I on a subpopulation of α-rat MHC I sFv-hrGFP(+) RAEC. This cell subpopulation expressed comparable levels of MHC I as detected for untransduced and hrGFP(+) RAEC after IFN-γ treatment whereas the other cell subpopulation showed only a moderate increase in surface MHC I upon stimulation. An isotype control is depicted. One representative experiment is shown.
3.2. Inhibition of surface MHC I results in reduced killing of RAEC by cytotoxic T cells

In a cytotoxicity assay, α-rat MHC I sFv-hrGFP(+) RAEC did not show detectable cytotoxicity over background levels up to a ratio of 50:1 of pre-stimulated allo-specific T effector cells per target RAEC. Even at high ratios (100:1), a significant reduction in cell killing in fewer than 20% of the cells was detected (Fig. 4). In contrast, co-incubation of untransduced and control-transduced hrGFP(+) RAEC with effector T cells induced substantial killing of over 40% of the target cells at a ratio of 100:1 effector to target cell. Non-specific killing was measured by using syngeneic RAEC which are only non-specifically recognized. RAEC from Dark Agouti (DA) rats, used as a third independent MHC I cell type, were also not killed using the same assay (data not shown).

3.3. MHC I down-regulation leads to a significant decrease of allo-antibody/complement-mediated lysis

Pre-incubation of RAEC with allo-specific serum and subsequent addition of baby rabbit complement resulted in robust cell lysis. Up to 50% of untransduced RAEC and hrGFP(+) RAEC were lysed in response to allo-specific serum in a dose-dependent manner (Fig. 5). Complement-mediated lysis was substantially reduced in α-rat MHC I sFv-hrGFP(+) RAEC. Less than 20% of the intrabody expressing cells underwent complement-mediated lysis. The use of lower serum dilutions revealed that cell lysis in these cells was significantly reduced reaching levels approaching those caused by non-specific lysis of RAEC after incubation with a control serum obtained from naive rats (Fig. 5).

3.4. Down-regulatory capacity of the α-rat MHC I sFv can be partially reverted by IFN-γ

In order to prove the capacity of the α-rat MHC I sFv intrabody under inflammatory conditions corresponding to the terms of a transplantation setting we performed MHC I expression studies following IFN-γ stimulation of RAEC. Untreated α-rat MHC I sFv intrabody expressing RAEC showed reduced levels of surface MHC I compared to control cells. In contrast, with increasing amounts of IFN-γ, the down-regulatory effect was partially reversed. A considerable fraction of these cells showed enhanced MHC I levels upon IFN-γ stimulation, whereas the α-rat MHC I sFv-hrGFP(+) RAEC population showed an IFN-γ-responsive as well as a non-responsive cell population (Fig. 6).

4. Discussion

Our study demonstrates that the intrabody gene transfer approach can be used to successfully down-regulate MHC I expression in RAEC and markedly decrease the susceptibility of these cells to in vitro cellular and humoral rejection mechanisms. Thus, the genetically modified EC with reduced immunogenicity represent a potentially valuable new tool for the seeding of vascular grafts.

Peripheral arterial occlusive disease and coronary heart disease are major causes of morbidity and mortality in the Western world. Although the application of the saphenous vein or mammary artery is still the gold standard for vascular reconstruction, other sources of small-diameter grafts will be necessary in the near future due to unsuitable or unavailable autologous vascular material in a growing number of patients. Synthetic vascular grafts can be considered a valuable alternative only if seeded with EC which prevent the occlusive complications of thrombus formation [25]. In the past some groups proved the concept of in vitro endothelialization of peripheral vascular bypass grafts showing an improvement in the performance of grafts seeded with autologous cells. But several limitations, like the isolation of the cells from the veins by additional surgical intervention and the long culture period required for the expansion of EC prior the seeding, have significantly limited the routine use of this procedure. Therefore, a need has emerged for additional EC sources that could compensate for the lack of suitable autologous cell material [4]. This gap may be filled by endothelial progenitor cells (EPC) harvested from the blood, by EC isolated from fat tissue or by the application of immune modulated allogeneic EC.

The present study underscores the potential of genetically modified allogeneic RAEC to serve as an alternative cell source for artificial graft studies. For this study, we used RAEC that can be easily isolated and kept in culture. In order to circumvent an immune attack against RAEC, we pursued a proof of concept study involving a gene therapeutic approach that genetically modified the immunogenicity of these cells. In general the field of gene therapy has opened new opportunities to modulate the genetic features of EC by over-expression of therapeutic molecules. Others have also shown that EC are suitable target cells for gene therapeutic interventions. Sonnenday et al. described a strong reduction of ICAM-I expression in vascular EC in vitro following transfection with a chimeric ribozyme vector as well as in vivo after intraperitoneal delivery of the chimeric ribozyme in a rat hepatic Ischemia Reperfusion Injury model. Inhibition of ICAM-I resulted in decreased adherence of neutrophils to these cells in vitro [26]. Recently, the inhibition of VEGF-induced signalling in porcine aortic EC using an α-VEGFR2 intrabody was documented. These EC-targeted intrabodies co-localized with the VEGFR2 receptor and did not show any sign of degradation [27].

Since non-viral strategies showed only low efficiency, viral transfer methods seem to have a greater potential for a successful gene transfer. Using the lentiviral vector-based gene transfer system, we achieved stable and sustained expression of the highly potent α-rat MHC I sFv intrabody in primary RAEC. These RAEC showed a strong down-regulation of MHC I on their surface as early as 48 to 72 h after gene transfer. Co-expression of the hrGFP reporter gene allowed enrichment of transduced cell fraction by FACS.
This resulted in the enrichment of the cell subpopulation which expressed low levels of MHC I. Cell sorting by FACS did not harm the cells with regard to their activation state, proliferative capacity or morphology. Within the sorted RAEC, the stable association of MHC I sFv intrabody gene sequences with the host genome and the efficient expression of the α-rat MHC I sFv intrabody transgene were demonstrated. Down-regulation of surface MHC I was observed in α-rat MHC I sFv intrabody expressing RAEC for up to 10 weeks. In contrast, RAEC which expressed only the hrGFP reporter gene showed no difference in extra-cellular MHC I expression. In addition, both types of RAEC showed no change in the expression patterns of other surface markers, like different adhesion molecules and were negative for MHC II expression (data not shown). Hence, in accordance with other studies we were able to show that the intrabody strategy is a useful tool to modulate the expression pattern of an intracellular target gene [28,29]. For the past three years, siRNA-based strategies have made significant inroads [30]. However, due to the polymorphism of MHC molecules, this method is not suitable for our approach [31]. Furthermore, it is known that by using intrabody-mediated binding of intracellular target proteins higher levels of specificity can be attained compared with siRNA-based approaches [32]. The α-rat MHC I sFv intrabody provides a potent modulator of MHC I expression with efficient and sustained down-regulatory capacity. The intrabody binds the heavy chain of the RT1.A molecule and hence secures the inhibition of both MHC I haplotypes.

It is conceivable that inflammatory conditions might reverse the α-rat MHC I sFv intrabody-mediated effect on MHC I expression. Under the conditions of a transplantation setting, the pro-inflammatory cytokine IFN-γ seems to be a major player in the rejection process [33], although diverse and potentially contradictory effects were described for IFN-γ. Nevertheless, the arterial endothelium is regulated by IFN-γ in terms of both basal and induced MHC class I and class II expression [34]. Detection of surface MHC I after incubation with increasing amounts of IFN-γ showed that a partial reversion of MHC I down-regulation in intrabody over-expressing RAEC occurred. Nonetheless, a subpopulation of the RAEC was not affected and expressed low levels of MHC I molecules on the cell surface. This effect might be due to different efficiencies regarding the insertion association of intrabody sequences to the host genome on the single cell level, the different cell cycle of the single cell in a non-synchronized cell culture or diverse capacity of intrabody-binding to MHC I molecules within the ER. Notably, the high doses of IFN-γ used in our in vitro experiments are estimated to be non-physiological and may not occur in vivo. Further studies will show whether sorting of the IFN-γ unresponsive cell fraction within the intrabody-expressing RAEC might lead to the generation of cells that maintain low MHC I levels even in the presence of the pro-inflammatory cytokine IFN-γ. Preliminary studies in our laboratory indicate that indeed repeated sorting followed by IFN-γ stimulation of the IFN-γ unresponsive cells results in a cell population that constantly expresses reduced levels of MHC I (data not shown).

Beside the α-rat MHC I sFv intrabody described in this study, the MK3 protein of the γ-Herpes virus seems to have a strong capacity to modulate MHC I expression. Boname et al. could show that MK3 inhibited classical as well as non-classical MHC I molecules, even during IFN-γ stimulation, by using a retrovirally-transduced mouse cell line. This effect was due to a dual mechanism of MK3 which leads to the degradation of both the MHC I itself and the proteins of the transporters associated with antigen processing (TAP) [35]. Therefore, MK3 could be an attractive candidate as a “therapeutic molecule” in the context of genetically modified EC.

However, with regard to opportunistic infections, a remaining susceptibility to pro-inflammatory cytokines such as IFN-γ may be beneficial for those cells in order to handle the infection.

The functional analysis of the genetically modified RAEC showed that MHC I down-regulation mediated protective effects due to the inhibition of efficient antigen presentation by the RAEC. Allo-specific CTL were not longer able to recognize a considerable fraction of the anti-MHC I intrabody-positive RAEC and to mediate their killing. This is particularly impressive because CTL-mediated killing requires only low amounts of antigenic peptide presented by a target cell [36,37]. A similar effect was observed for the allo-antigen/complement-mediated lysis which was strongly reduced in RAEC over-expressing the α-rat MHC I sFv intrabody. Since the MHC I expression on the cell surface was drastically reduced, these effector components of the immune system presumably lost their ability to bind to the allogeneic cells which resulted in the strong reduction of cell destruction. We assume that the number of MHC I complexes declined towards a critical level that was insufficient to trigger T cell responses. As a result, α-rat MHC I sFv intrabody over-expressing RAEC were poorly recognized comparable to non-specific effects.

In summary, our gene therapeutic approach could serve as a general and potent strategy to prevent immune recognition of allogeneic EC thereby leading to a reduction of the chronic rejection of the transplanted donor RAEC. This might result in the sustained acceptance of the cells by the host immune system.

Moreover the immunomodulatory effect of the α-rat MHC I sFv intrabody might become an attractive alternative therapy for the inhibition of chronic rejection through the prevention of chronic allograft vasculopathy in cardiac allografts.

The outcome of the genetically modified RAEC will now be evaluated in vivo using an allogeneic aorta interdisposition transplantation model with seeded vascular grafts.

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