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Communication

Retroviral targeting of proliferating endothelial cells*

Alexander Gornikiewicz, Anna Zommer, Raimund Jakesz, Michael Gnant and Christine Brostjan✉

Department of Surgery, Vienna General Hospital, Medical University of Vienna, Vienna, Austria;
✉e-mail: Christine.Brostjan@meduniwien.ac.at

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Tumor growth requires the formation of new blood vessels by endothelial cells. Thus, surface molecules – such as angiogenin receptors – that are selectively expressed on growing endothelium represent an attractive target for directed delivery of compounds to tumor tissue. We attempted to obtain genetically engineered retroviral vectors targeted to the endothelium by inserting the human angiogenin sequence into Moloney murine leukemia virus envelope glycoprotein. Abundant expression of the chimeric protein could be verified. However, while being selective for proliferating human endothelial cells, the recombinant retroviral particles displayed low transduction efficiencies and thus have to be further improved.

Keywords: retrovirus, targeting, envelope glycoprotein, angiogenin, endothelial cell

Research on tumor angiogenesis has been greatly expanding ever since Judah Folkman hypothesized in 1971 that growth and dissemination of cancer cells is dependent on the formation of new blood vessels and might therefore be controllable by inhibiting neovascularization (Folkman, 1971). Numerous approaches have been taken to interfere with tumor angiogenesis at various steps of the process and have found their way to clinical trials by now. Another treatment approach involving tumor vasculature is based on targeted delivery of therapeutic compounds to tumor endothelium, which may subsequently act on endothelial as well as surrounding cancer cells. Surface molecules specifically expressed on proliferating (angiogenic) endothelial cells and up-regulated in tumor vessels represent a stable feature common to most solid tumor types and have thus been chosen for the targeting approaches (Wickham *et al.*, 1997). The techniques applied involve non-viral (compound) as well as viral (gene transfer) vectors. Therapeutic or toxic agents have been coupled to protein ligands or to monoclonal antibodies directed against EC surface markers (Thorpe & Burrows, 1995). Adenoviral vectors carrying effector genes have been combined with bispecific antibodies to enhance virus specificity for tumor endothelium (Wickham *et al.*, 1996). However, adenoviruses have a very broad intrinsic host range and confer transient expression of a trans-gene. Retroviral MLV-based vectors, in contrast, stably integrate into the host genome of dividing target cells and provide long-term gene expression with little immunogenicity. The tumor endothelium could thus be stably supplied with a "therapeutic gene" when packaged in a retroviral vector, which is modified to specifically target proliferating endothelial cells. Gene transfer of a locally produced and active anti-tumor agent would abolish the need for systemic and thus more toxic administration of therapeutic compounds. Directing retroviral infection towards a specific host range, i.e. limiting transduction to a given target cell type, has been attempted by various strategies, the most promising of which seems to be modification of the viral envelope glycoprotein env (Karavanas *et al.*, 1998). Interaction of env with target cell receptors mediates viral entry *via* fusion of the cellular membrane and the viral envelope, and thereby determines host specificity. The most commonly applied envelope proteins are derived from Moloney MLV

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Abbreviations: ang, angiogenin; AngBP, angiogenin-binding protein; CMV, cytomegalovirus; EC, endothelial cell; EGFP, enhanced green fluorescent protein; env, envelope protein; FCS, fetal calf serum; MFI, mean fluorescence intensity; MLV, murine leukemia virus; PD-ECGF, platelet derived endothelial cell growth factor; scFv, single chain variable antibody fragment; SU, surface protein; TM, transmembrane protein.

exhibiting an ecotropic host range restricted to rodent target cells, as well as from amphotropic MLV (MLV-A, 4070A) permitting infection of most mammalian (including human) cells (Wang *et al.*, 1991; Miller *et al.*, 1994). MLV env is generally expressed as 85 kDa precursor molecule that is subsequently processed to a glycosylated surface (SU, gp70) and a transmembrane (TM, p15(E)) unit, which remain associated and form homotrimers. While SU is considered to primarily confer receptor interaction, the fusiogenic capacity is mainly attributed to TM.

Modification of retroviral envelope proteins to incorporate "ligands" which may confer targeted transduction of a defined cell type has been tested in a variety of molecular engineering approaches summarized as follows: ligand sequences have either been inserted into (Cosset *et al.*, 1995; Schnierle *et al.*, 1996) or have been designed to replace part of the env gene (Kasahara *et al.*, 1994; Han *et al.*, 1995). Even though an impressive diversity of cloning strategies has been applied, no "ultimate" fusion modality could be defined which guarantees efficient viral incorporation and target cell transduction, since cloning site, spacer sequence, ligand identity and type of envelope protein give a joint effect which seems to be difficult to predict for each individual combination. With respect to retroviral targeting of tumor vessels, there has been a limited number of attempts primarily based on small peptide ligands, e.g. for integrin binding (Liu *et al.*, 2000; Wu *et al.*, 2000). However, efficient transduction could not be achieved unless amphotropic wildtype envelope was co-expressed, thereby losing target cell specificity. Furthermore, a van Willebrand factor-derived binding peptide to collagen (inserted at the N-terminus of amphotropic env) was applied to enhance retroviral accumulation at tumor sites where extracellular matrix is exposed (Hall *et al.*, 1997). Viral transduction, however, is mediated by the amphotropic receptor (Ram-1), i.e. also holding the potential risk of gene transfer into a wider spectrum of dividing cells throughout the body — especially when given systemically. In contrast, we have chosen an approach to directly incorporate the EC ligand angiogenin into Moloney MLV env to primarily target proliferating endothelial cells. Angiogenin is a potent EC mitogen expressed in tumor tissue (Montero *et al.*, 1998) and interacts with two surface receptors exposed on proliferating ECs. The 42 kDa AngBP has been identified as the dissociable smooth muscle α-actin (Hu *et al.*, 1993) and seems to mediate early steps in angiogenesis such as protease activation and EC migration. In contrast, expression of a distinct 170 kDa angiogenin receptor is mutually exclusive with AngBP and is likely to induce EC proliferation (Hu *et al.*, 1997). To date there is little evidence for expression of angiogenin receptors on the surface of cell types other than proliferating ECs or associated smooth muscle cells during angiogenesis. Furthermore, angiogenin uptake involves receptor-mediated endocytosis and lysosome-independent transport across the cytoplasm (Li *et al.*, 1997), a mechanism that resembles ecotropic viral entry and might therefore prove beneficial in mediating gene transduction. In this study, human angiogenin was inserted at the N-terminus of ecotropic env and chimeric constructs were analyzed for selective gene transfer into proliferating endothelial cells.

MATERIALS AND METHODS

Cloning of a chimeric env expression vector. The construct was based on the mammalian expression vector pIRES2-EGFP providing a strong constitutive promoter derived from the human CMV major immediate early promoter/enhancer (Clontech #6029-1). A plasmid encoding the complete Moloney MLV envelope protein sequence (pMLV-env) was generously provided by Prof. W.H. Guenzburg (Austrianova, Vienna, Austria). To introduce an *Xho*I cloning site at aa 6 of ecotropic env without altering the amino-acid sequence, two PCR fragments were amplified covering the sequences 5′ and 3′ of aa 6, respectively. PCR fragment A was generated by primers ATGe*Sal*I (ATCCTGTCGACCGCCATGGCGCGTTCAACGC) and 3′e*Xho*I (TGAGGACTCGAGCCGGGCGAAGCAGTAC), thereby introducing a *Sal*I restriction site 10 bp before the start codon as well as an *Xho*I recognition motif at aa 5/6 *via* primer mutagenesis. In the same manner, the primer pair 5′e*Xho*I (GCCCGGCTCGAGTCCTCATCAAGTCTATAA) and STOPe (CGTCCCGAATTCATGGCTCGTACTCTATAGGC) was applied to amplify PCR fragment B harboring a corresponding *Xho*I site at aa 5/6 and the *Eco*RI recognition motif overlapping the stop codon. Both env fragments were subsequently cloned into the *Xho*I/*Eco*RI restriction sites of pIRES2-EGFP, thereby destroying the vector *Xho*I site (*via* ligation of compatible *Xho*I / *Sal*I overhangs). The endothelial cell ligand was subsequently introduced *via* the generated *Xho*I site at aa 6 in conjunction with the endogenous *Bst*EII site at aa 17, i.e. a short stretch of 10 aa was replaced by the heterologous sequence without exchanging the endogenous retroviral receptor binding domain (Fig. 1). The coding sequence for angiogenin was amplified by PCR using an appropriate set of mutagenesis primers; cDNA templates were derived from HT-29 colon carcinoma RNA. The primers Ang5′*Xho*I (CCGACCTCGAGTCAGGATAACTCCAGGTAC) and Ang3′*Bst*EII (GCCCGCGGTTACCGGACGACGGAAAATTGACTG) were designed to allow subsequent restriction digestion by *Xho*I and *Bst*EII for in-frame fusion with the env expression construct, by omitting start and stop codons as

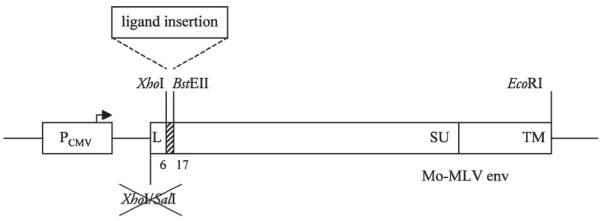

Figure 1. Schematic representation of the chimeric env expression construct.
The endothelial cell ligand sequence (angiogenin cDNA) was introduced at the N-terminal SU domain, thereby replacing amino acids 7–16 of Moloney MLV env. Restriction sites employed in the cloning process are indicated (and crossed out if destroyed during the cloning process). The construct includes the pertaining MLV env leader sequence (L) and C-terminal transmembrane domain (TM). Amino acid numbering refers to the first amino acid of processed SU as #1. Expression is driven by a constitutive CMV promoter (P_{CMV}).

well as leader sequences derived from the angiogenin gene. The plasmid was sequence-verified for the entire ligand and envelope coding region to exclude possible cloning artefacts.

Virus production. Retrovirus production was based on a transient transfection system of the Phoenix packaging cell lines established and generously supplied by Nolan and coworkers (Pear *et al.*, 1993). Phoenix-gp is derived from the human embryonic kidney cell line 293T by stably integrating an expression plasmid for MLV gag/pol genes. Phoenix-gp cells were grown in DMEM supplemented with 10% FCS. Calcium phosphate transfection of Phoenix-gp cells was performed with 8 μg of the retroviral vector pMSCV-EGFP carrying the reporter gene EGFP as well as 4 μg of the expression plasmid M13 for increased production of viral gag-pol [both vectors were generously provided by H. Klump, Hannover Med. School, Germany (Klump *et al.*, 2001)] and with 8 μg of wildtype or chimeric env expression plasmid. Two days later, Phoenix cell supernatants were harvested.

Target cell transduction. Human umbilical vein endothelial cells, HUVECs (Clonetics) were cultured in EGM2 medium (Clonetics) on fibronectin-coated dishes. Primary human fibroblasts (CCD-32Sk, ATCC) and murine fibroblast 3T3 cells were grown on gelatine-coated culture dishes in MEM or DMEM medium, respectively, and supplemented with 10% FCS. For viral transduction, cells were seeded at 1×10^5 (30 mm well) or 5×10^5 (10 cm dish) and exposed to 800 μl or 4 ml of virus supernatant, respectively (supplemented with polybrene to a final concentration of 4 μg/ml). Medium was replaced after 4–6 h of incubation, and 2 days later cells were analyzed by flow cytometry for retroviral transduction, i.e. for expression of the EGFP reporter gene.

Target receptor detection on endothelial cells. HUVECs were seeded at varying cell density and cultured for 24 h. Cells were then harvested with non-enzymatic cell dissociation solution (Sigma-Aldrich) for subsequent analysis of angiogenin receptor expression. To be able to simultaneously detect both angiogenin receptors, we used a special "sandwich" detection variant by applying human recombinant angiogenin (R&D Systems #265-AN) to endothelial cells, prior to labeling with polyclonal antiserum directed against angiogenin (Oncogene #PC317L) and detection with PE-labeled secondary antibody (Rockland #705-708-125). Negative controls were based on human recombinant PD-ECGF protein (R&D Systems #229-PE) tested with the same set of antibodies, as well as on combinations with the appropriate control serum (normal goat IgG, Oncogene #NI02). Receptor expression was ultimately detected by flow cytometry.

Analysis of chimeric envelope protein expression. To establish whether the env fusion protein was expressed in producer cells, we analyzed cell lysates of Phoenix-gp cells transfected with 8 μg of env expression plasmid, 8 μg of pMSCV-EGFP and 4 μg of M13 gag-pol expression plasmid. Protein extracts were generated on day 2 by a method previously described to be suitable for analysis of retroviral env proteins (Cosset *et al.*, 1995). Total protein (30 μg) were separated by SDS/PAGE and subsequently subjected to Western blot analysis with goat anti-SU antiserum (ViroMed #80S00019) or goat anti-angiogenin antiserum (Santa Cruz Inc. #sc1408). Secondary anti-goat IgG horseradish peroxidase conjugate (Dako) was applied for detection with a chemiluminescent substrate (Pierce).

RESULTS

The cloning strategy that we chose to generate a chimeric protein composed of ecotropic env and human angiogenin, was based on previous reports of similar ligand/env fusion modalities which resulted in successful targeting of retroviral particles to a defined host cell type (Somia *et al.*, 1995; Konishi *et al.*, 1998; Khare *et al.*, 2001). In a similar manner, we inserted the angiogenin sequence at amino-acid position 6 of the envelope glycoprotein, flanked by *Xho*I and *Bst*EII restriction sites. Retrovirus production was then performed in a packaging system based on transient transfection of Phoenix-gp producer cells with the chimeric and/or wildtype envelope expression construct as well as a retroviral vector carrying the EGFP reporter gene for facilitated detection of gene transfer.

Expression of the chimeric envelope protein was initially verified in producer cell extracts. It should be noted that in previous reports of com-

parable chimeric env molecules, co-expression of wildtype ecotropic envelope protein was favorable to viral incorporation (Somia *et al.*, 1995). Therefore, we compared virus production with the chimeric angiogenin/env protein in the absence or presence of wildtype ecotropic env (at a 1:1 ratio). Since Moloney MLV interactions are restricted to rodent cells, co-expression should not interfere with targeting of human host cells. Producer cell extracts were analyzed by Western blotting with antiserum directed against the ecotropic SU domain or against human angiogenin (Fig. 2). Expression of wildtype (70 kDa) as well as chimeric (85 kDa) envelope protein was detectable with anti-SU antiserum, and was even more pronounced for the chimeric than the wildtype protein. Incorporation of the angiogenin ligand could further be confirmed for the chimeric construct by anti-angiogenin antiserum.

Before testing target cell transduction with the generated chimeric retroviral particles, the expression of the respective targeted angiogenin receptors was verified on human umbilical vein endothelial cells. It is of importance to note that expression of angiogenin receptors is greatly dependent on cell density, i.e. is restricted to proliferating, sparse cultures (Hu *et al.*, 1997). We thus seeded HUVECs at varying density (2×10^4 or 6×10^3 cells/cm^2). Flow cytometric analysis was performed on the following day (as is the case for retroviral infections). For concomitant detection of both angiogenin receptors, a particular immunostaining procedure was applied involving addition of human recombinant angiogenin and subsequent detection of bound ligand by anti-angiogenin antiserum (Fig. 3). Expression of angiogenin receptors was clearly detectable on HUVEC cultures and was further enhanced by 2.5-fold at the low seeding density.

Low-density HUVEC cultures were subsequently exposed to chimeric retroviral particles as harvested from Phoenix-gp producer cells transiently transfected with chimeric and/or wildtype env constructs and a retroviral vector carrying the EGFP reporter gene. Proliferating cultures of primary human skin fibroblasts and of murine fibroblast 3T3 cells (which do not express human angiogenin receptors) were similarly tested for retroviral transduction by flow cytometric analysis of EGFP expression (Table 1). Retroviral particles generated with wildtype amphotropic envelope protein and thus capable of infecting mammalian cells (including human cells) consistently gave a titer of 10^5 infectious particles/ml — thus demonstrating the functionality of our retroviral production and the HUVEC transduction system. As expected, virions carrying the ecotropic wildtype env protein could not transfer the EGFP gene to human endothelial cells, since the ecotropic host range is restricted to rodent cells. However, infection of murine 3T3 cells confirmed a viral titer of 10^6/ml. The chimeric angiogenin/env construct mediated a very low level of gene transfer to primary endothelial cells, which was not improved by concomitant expression of wildtype ecotropic envelope glycoprotein ($1–2 \times 10^1$/ml) but was directed to the targeted cell type, since infection of human fibroblasts could not be detected. When the chimeric env virions were tested on murine 3T3 cultures, an infectious titer of 10^3/ml was established – indicating that the insertion of angiogenin at the N-terminus of Moloney MLV env had led to an impairment of viral transduction *via* the remaining endogenous ecotropic receptor-binding domain. Co-expression of wildtype and chimeric env could restore the infectivity to normal titers of 10^6/ml, reflecting 3T3 target cell transduction *via* the interaction of wildtype MLV env and its murine Rec-1 receptor. Thus, while showing a target preference for proliferating endothelial cells, the generated chimeric retroviral envelope protein carrying human angiogenin did not result in efficient target cell transduction and gene transfer.

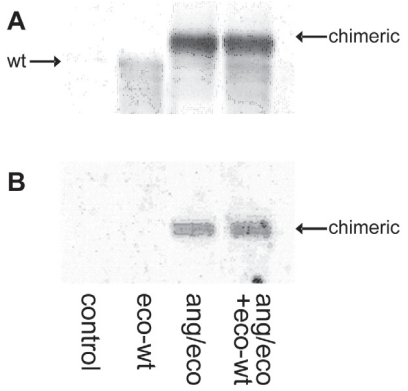

Figure 2. Western blot analysis of Phoenix-gp cell extracts following transfection with wildtype (eco-wt) and/or chimeric (ang/eco) env expression plasmids.
Extracts of non-transfected Phoenix-gp cells were included as a negative control. Immunoblotting was performed with anti-SU (A) and anti-angiogenin antiserum (B).

Table 1. Infection of primary human endothelial cells or fibroblasts as well as of murine 3T3 cultures with chimeric retroviral particles carrying the EGFP reporter gene.

Transduction efficiency as detected by EGFP expression in target cells was calculated in infectious viral particles per ml of virus (producer cell) supernatant and varied strongly with the type of viral envelope protein applied: ecotropic wildtype (eco-wt), amphotropic wildtype (ampho-wt) or chimeric angiogenin/ecotropic env (ang/eco). ND, not detectable.

Retroviral envelope protein	Transduction efficiency		
	HUVEC	Human fibroblasts	Murine 3T3
eco-wt	ND	ND	2×10^6/ml
ang/eco	2×10^1/ml	ND	5×10^3/ml
ang/eco + eco-wt	1×10^1/ml	ND	1×10^6/ml
ampho-wt	2×10^5/ml	1×10^5/ml	3×10^5/ml

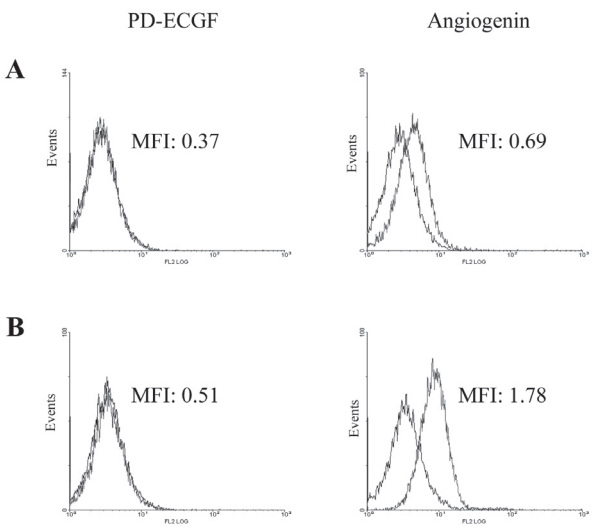

Figure 3. Angiogenin receptor expression on HUVECs. Cells were either seeded at 2×10^4 (A) or at 6×10^3 (B) cells/cm^2 and analyzed for angiogenin receptor expression on the following day. Binding of human recombinant angiogenin (or control PD-ECGF peptide) was followed by incubation with primary α-angiogenin antiserum (grey line) or control goat IgG (black baseline) as well as with secondary PE-labeled detection antibody. MFI reflecting angiogenin receptor expression is indicated.

DISCUSSION

Despite a high expression level of the angiogenin/env fusion protein by retroviral producer cells, the resulting virions were not capable of mediating substantial gene transfer to proliferating endothelial cells expressing the appropriate angiogenin receptors. The generation of the chimeric retroviral envelope protein was based on a fusion modality previously reported to permit efficient transduction of targeted cell types (Somia *et al.*, 1995; Konishi *et al.*, 1998; Khare *et al.*, 2001). In those cases, titers of infectious viral particles ranged around 10^4/ml when chimeric and ecotropic wildtype envelope proteins were co-expressed. In contrast to our approach, the authors introduced scFvs rather than actual ligands for the targeted surface receptors. Since efficient viral entry requires a conformational change of SU and TM domains which is triggered upon ligand/receptor binding, a comparable steric configuration may be provided upon scFv/receptor interaction but may not be the case for angiogenin/receptor binding. Thus, by substituting the angiogenin sequence with an scFv directed against the high-affinity angiogenin receptor, transduction efficiency of the chimeric retroviral particles might be enhanced. Further improvements of the chimeric construct may also support the required conformational change of retroviral env upon target receptor interaction: two point-mutations introduced at the C-terminal portion of ecotropic SU have recently been shown to greatly increase, i.e. "rescue", retroviral transduction by chimeric envelope proteins harboring a heterologous sequence at the N-terminus (amino acid 6) of SU (Zavorotinskaya & Albritton, 2001). Hence, we will further modify the present angiogenin/env construct by introducing the respective point-mutations (Q227R, D243Y) into the ecotropic SU sequence – to enhance targeted gene transfer into proliferating human endothelial cells, which is the prerequisite for possible *in vivo* applications in tumor angiogenesis settings.

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