Retroviral targeting of proliferating endothelial cells

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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
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 fusogenic 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 ecotropic env and chimeric constructs) 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 pIRE52-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 XhoI 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 ATGeSalI (ATCCTGTGAGCCCAT- GCCCGCTCAACGC) and 3’eXhol (TGAGGACTCGAGCGGCGTAC), thereby introducing a SalI restriction site 10 bp before the start codon as well as an Xhol recognition motif at aa 5/6 via primer mutagenesis. In the same manner, the primer pair 5’eXhol (GCCCGGTCTCCAGTCTCAT-CAGTCTATAA) and STOPe (CGCCGCGGCTCATCGAGGCGGGAAGACGTAC) was applied to amplify PCR fragment B harboring a corresponding Xhol site at aa 5/6 and the EcoRI recognition motif overlapping the stop codon. Both env fragments were subsequently cloned into the Xhol/EcoRI restriction sites of pIRE52-EGFP, thereby destroying the vector Xhol site (via ligation of compatible Xhol / SalI overhangs). The endothelial cell ligand was subsequently introduced via the generated Xhol site at aa 6 in conjunction with the endogenous BstEII 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 Ang5Xhol (CCGCACCCTCAGTCAAGGCACGTTA) and Ang3BstEII (CCCCGCGGGTACCGAGCAGCAGAAATTGACTG) were designed to allow subsequent restriction digestion by Xhol and BstEII for in-frame fusion with the env expression construct, by omitting start and stop codons as
**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 XhoI and BstEI 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 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).

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).

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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 ana-
lyzed by Western blotting with antisera 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 antisera, 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 recep-
tors was verified on human umbilical vein endothe-
lial 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 cul-
tures (Hu et al., 1997). We thus seeded HUVECs at
varying density (2 × 10^4 or 6 × 10^2 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 ap-
plied involving addition of human recombinant an-
giogenin and subsequent detection of bound ligand
by anti-angiogenin antiserum (Fig. 3). Expression of
angiogenin receptors was clearly detectable on HU-
VEC cultures and was further enhanced by 2.5-fold
at the low seeding density.

Low-density HUVEC cultures were subse-
quently exposed to chimeric retroviral particles as
harvested from Phoenix-gp producer cells transiently
transfected with chimeric and/or wildtype env con-
structs and a retroviral vector carrying the EGFP re-
porter 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 (Ta-
ble 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, in-
fected of murine 3T3 cells confirmed a viral titer of
10^4/ml. The chimeric angiogenin/env construct medi-
ated a very low level of gene transfer to primary
endothelial cells, which was not improved by con-
comitant expression of wildtype ecotropic envelope
glycoprotein (1–2 × 10^3/ml) but was directed to the
targeted cell type, since infection of human fibro-
lasts could not be detected. When the chimeric env
virions were tested on murine 3T3 cultures, an in-
fecious titer of 10^4/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 nor-
mal titers of 10^4/ml, reflecting 3T3 target cell trans-
duction 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 effi-
cient target cell transduction and gene transfer.

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.

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<th>Retroviral envelope protein</th>
<th>Transduction efficiency</th>
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<tr>
<td></td>
<td>HUVEC</td>
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<tr>
<td>eco-wt</td>
<td>ND</td>
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<tr>
<td>ang/eco</td>
<td>2 × 10^4/ml</td>
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<tr>
<td>ang/eco + eco-wt</td>
<td>1 × 10^4/ml</td>
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<tr>
<td>amphi-wt</td>
<td>2 × 10^5/ml</td>
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Extracts of non-transfected Phoenix-gp cells were
used as a negative control. Immunoblotting was performed
with anti-SU (A) and anti-angiogenin antiserum (B).

Figure 2. Western blot analysis of Phoenix-gp cell ex-
tracts following transfection with wildtype (eco-wt) and/
or chimeric (ang/eco) env expression plasmids.

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<td>amphi-wt</td>
<td>2 × 10^5/ml</td>
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Figure 3. Angiogenin receptor expression on HUVECs. Cells were either seeded at $2 \times 10^5$ (A) or at $6 \times 10^3$ (B) cells/cm² 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 (Somnia et al., 1995; Konishi et al., 1998; Khare et al., 2001). In those cases, titers of infectious viral particles ranged around $10^7$/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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**REFERENCES**


