Replication-competent retrovirus vectors for cancer gene therapy

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1. ABSTRACT

Oncolytic virotherapy represents an emerging field with tremendous promise for harnessing the replicative capabilities of viruses against rapidly proliferating cancer cells. Among the different replicating virus technologies being tested, replication-competent retrovirus (RCR) vectors based on murine leukemia virus (MLV) exhibit unique characteristics. MLV exhibits intrinsic tumor selectivity due to its inability to infect quiescent cells, and can achieve highly selective and stable gene transfer throughout entire solid tumors in vivo at efficiencies of up to >99%, even after initial inoculation at MOIs as low as 0.01. RCR vectors with suicide genes mediate synchronized cell killing after prodrug administration, and due to their ability to undergo stable integration, residual cancer cells serve as a reservoir for long-term viral persistence even as they migrate to new sites, enabling multiple cycles of prodrug to achieve prolonged survival benefit. Further testing in various tumor models, new vector targeting and delivery strategies, and development of GMP manufacturing, are being pursued through a multi-national consortium, and preparations are now being undertaken for clinical trials using RCR vectors in glioblastoma.

2. INTRODUCTION: REPLICATION-COMPETENT VIRUSES AS ONCOLYTIC AGENTS

The use of replication-competent viruses represents an emerging technology with the potential to achieve highly efficient gene transfer to tumors, as the virus would multiply and spread after the initial infection event, and each infected tumor cell would, in effect, become a virus producer cell, sustaining further transduction events even after initial administration (1-3). It is now known that various types of viruses can replicate selectively in tumors by taking advantage of intrinsic defects in cellular defense mechanisms that normally guard against infection, but which are switched off or disabled in cancer cells (4-10). In fact, the idea of using replication-competent viruses as oncolytic agents actually dates back more than a century, to the first documented report in 1904 of a patient showing dramatic remission of leukemia after influenza infection (11), and during the 1950s-1970s, different viruses were tested in patients with advanced cancer (3, 12). However, these early attempts met with discouraging results, as initial tumor necrosis was typically followed by immune clearance of the virus and tumor recurrence. With the advent of modern chemotherapy, oncolytic virotherapy was largely abandoned.
Oncolytic viruses

- large, complex viruses: often difficult to manipulate
- basis for attenuation/selectivity: sometimes not well understood
- robust immune response: causes rapid clearance of virus
- lack of viral persistence: leads to recurrence of tumor

MLV retrovirus

- simple virus, well understood
- lack of active nuclear uptake: infects proliferating cells only
- not intrinsically cytolytic, but can achieve stable integration, less immunogenic
- can carry suicide genes, and thus achieve synchronous prodrug-induced killing
- availability of anti-retroviral drugs: can stop viral replication, added safety

Figure 1. Unique advantages of replicating MLV as a cancer therapeutic agent. Please note that the characteristics listed do not necessarily apply to all oncolytic viruses, but represents a generalized view based on the most commonly used oncolytic viruses.

Now, with advances over the past 25 years in elucidating and manipulating molecular mechanisms of viral infection and replication, and with the realization that conventional gene therapy approaches employing replication-defective vectors have failed to achieve significant therapeutic benefit, there has been a renewed interest in oncolytic therapy using many different replicating virus species, such as adenovirus (1, 9, 13, 14), herpesvirus (15-19), reovirus (20, 21), poliovirus (22), paramyxoviruses (12, 23-25), and VSV (4, 26, 27). Unique among replicating viruses being developed as oncolytic agents, murine leukemia virus (MLV)-based replication-competent retroviruses (RCR) replicate without immediate lysis of host cells and can maintain viral persistence through stable integration (Figure 1).

3. MLV-BASED RCR VECTORS FOR CANCER VIROTHERAPY: ADVANTAGES

3.1. Absolute requirement for cell division in order to propagate

MLV contains no nuclear localization signals in its capsid and cannot cross an intact nuclear membrane, hence can only infect cells during active mitosis; in fact, this was precisely the original rationale for the use of retrovirus vectors in cancer gene therapy (28). This absolute requirement for dividing cells enables tumor-selective replication of MLV-based RCR vectors.

3.2. Non-lytic replication, stable integration, and reduced immunogenicity

As an integrating virus, the life cycle of MLV is non-cytolytic, and with its selectivity for dividing cells, no acute toxicity to post-mitotic normal cells is incurred as a consequence of virus replication. While neutralizing antibody responses to MLV do occur, it has been shown that injection of wild type MLV or MLV-based retroviral vector producer cells causes only minor inflammation, with little effect on viral titer, and no associated pathology (29-31). Non-lytic replication, stable integration, and low immunogenicity enable prolonged persistence in tumors, allowing MLV-based RCR vectors to achieve high levels of intratumoral gene transfer.

Other replicating viruses also exhibit tumor selectivity, but generally are also capable of infection and lysis of normal cells at high doses, and provoke robust anti-viral immune responses. In fact, a major reason why many of these viruses have moved rapidly into clinical cancer trials is the availability of pre-existing data from prior trials in which they were originally developed as vaccine strains intended to elicit protective immunity in humans (24, 32, 33). While oncolytic adenoviruses were not derived from vaccine strains, it is well known that even conventional adenovirus vectors are rapidly eliminated by immune responses (34, 35), and can induce chronic persistent inflammation (36).

3.3. Regulatable cell killing through stable transfer of suicide genes

While not intrinsically cytolytic, MLV can be supplied with a suicide gene that will be seeded into tumor cells as the virus replicates. As suicide genes encode prodrug converting enzymes that catalyze intracellular production of toxic metabolites from a non-toxic substrate, simultaneous killing of tumor cells can be triggered by prodrug administration. Since intracellular prodrug conversion is confined to tumor cells, adverse side effects associated
RCR vectors for cancer gene therapy

Figure 2. Current design strategy for RCR vectors. These vectors consist of a full-length MLV genome into which is inserted an encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) sequence linked to a transgene cassette immediately after the env stop codon. The cassette is therefore present in both the spliced and unspliced viral transcripts and translation of the transgene is mediated by the IRES. Shown are some of the transgenes that have been tested in this vector system. The U3 region of the 5′ long terminal repeat (LTR) has been replaced with the cytomegalovirus (CMV) promoter for higher levels of initial virus production. Other abbreviations: ψ: viral packaging signal, gag/pol/env: virus structural gene loci, SD: splice donor, SA: splice acceptor, hGM-CSF: human granulocyte-macrophage colony stimulating factor.

with systemic administration of toxic chemotherapy agents can be avoided. As RCR vectors mediate permanent integration of the transgene, the non-toxic prodrug can be given at any appropriate time, e.g., upon recurrence of infected malignant cells that escaped initial treatment, and also serves as a mechanism to destroy inadvertently infected normal cells.

3.4. Efficient replication and gene transfer to cancer cells

While RCR vectors containing exogenous transgene inserts in the viral long terminal repeat (LTR) sequences have been described previously, these vectors generally exhibited genomic instability (37-41). We have recently devised a novel RCR design (Figure 2) that is highly stable over multiple serial passages in culture (42), and achieves extremely efficient replicative spread and gene transfer in human and murine tumor cells (43, 44), including gliomas (45-47). We and others (45, 48-54) have found that replicating retrovirus vectors can achieve a tremendous in situ amplification effect after initial intratumoral injection of even a small inoculum, and that as little as 10e4-5 total infectious units of RCR vector can transmit an inserted transgene throughout an entire solid tumor mass in vivo, achieving better therapeutic results than 1000-fold higher levels of replication-defective adenovirus (49), or those reported previously by others using 10,000-fold higher levels of highly concentrated conventional replication-defective retroviral vectors (55).

Recently we have also tested RCR delivery via loco-regional infusion in a hepatic metastasis model of colorectal cancer, followed by combined optical imaging, flow cytometry, and molecular analysis to monitor RCR vector spread (56, 57). Robust RCR replication was confirmed in both human WiDr and murine CT26 colorectal cancer cells in vitro, with transduction levels reaching >90% in less than 12 days after virus inoculation at multiplicities of infection (MOI) of 0.01 to 0.1 (56). In vivo, infusion of RCR supernatant into the portal circulation resulted in progressive and significant transduction of multi-focal intrahepatic CT26 tumors in syngeneic mice, averaging about 30% but with up to 60% transduction in some tumors within 4 weeks (56). However, immunohistochemistry and quantitative PCR analysis showed no evidence of RCR spread to adjacent normal liver, or any other normal tissues (56, 57). Thus, loco-regional infusion of RCR vectors can also be used to deliver therapeutic genes efficiently and selectively to tumor cells in the liver, resulting in effective tumor growth inhibition while sparing normal hepatocytes and without dissemination to extra-hepatic normal tissues (57).

4. RCR VECTORS FOR CANCER VIROTHERAPY: POTENTIAL HAZARDS AND SAFETY CONSIDERATIONS

4.1. Potential hazards

Of course, the possibility that insertional mutagenesis by integrating vectors might lead to carcinogenesis has been of foremost concern to the field of gene therapy, especially after a report of fatal lymphomas in 3 of 10 rhesus macaques after lethal irradiation and transplantation of bone marrow heavily contaminated with wild type revertant MLV (58). While similar experiments using less immunosuppression showed no evidence of pathology due to MLV (59, 60), the potential for adverse events now has clinical precedent, as evidenced by emergence of T cell leukemia in 3 of 11 immunodeficient children after retroviral gene transfer for IL-R γc deficiency (61). As the vectors used were replication-defective and
there was no evidence of RCR reversion, insertional activation of the LMO-2 proto-oncogene by the retrovirus vector, combined with a selective growth advantage of the IL-R corrected cells, are likely to have contributed to leukemogenesis.

4.2. Safety considerations

Nonetheless, in contrast to gene replacement therapy, we propose that several considerations mitigate concerns regarding potential genotoxicity and leukemogenesis in the case of RCR-mediated suicide gene therapy for cancer. Again, the initial rationale for using retroviral vectors in gene therapy still holds true for RCR vectors, i.e., MLV can only transduce cells that are actively dividing, and thus is selective for rapidly dividing tumor cells, as the majority of normal cells in vivo are quiescent (particularly hematopoietic stem cells). In contrast, for retroviral gene transfer in the X-SCID trial, hematopoietic stem cells were harvested and forced to divide in culture by cytokine stimulation.

In our own studies, after intratumoral administration, RCR vectors showed no detectable spread to normal tissues by PCR analysis in short term studies in either immunodeficient or immunocompetent tumor models; in longer term studies some spread to bone marrow and spleen was observed in immunodeficient nude mouse models by more sensitive quantitative real-time PCR methods, but this was not seen in immunocompetent rat syngeneic tumor models (45, 48, 50). A recent study by Klatzmann et al. (49) also reported wild type MLV spread in bone marrow and spleen at levels quantitated as 0.0037-0.21 copies per cell by real-time PCR, but this was only after intravenous injection, and again only in immunodeficient nude mice. Significantly, no such transduction was observed in any tissue after intravenous injection in immunocompetent adult mice (49).

Also as noted, incorporation of a suicide gene would itself constitute a self-destruct mechanism and provides a built-in safeguard, as normal cells inadvertently infected by RCR vectors would also be eliminated, and spread would be inherently self-limited. Furthermore, anti-retroviral drugs such as 3'-azido-3'-deoxthymidine (AZT) can readily terminate replication of wild type MLV (62) as well as MLV-based RCR vectors (45, 49). In fact, the low level contamination of bone marrow and spleen by wild type MLV after intravenous injection in nude mice as reported above, was shown to be completely suppressed by AZT (49).

5. TUMOR-SPECIFIC TARGETING OF RCR VECTORS

The above considerations notwithstanding, it would still be advantageous to incorporate additional mechanisms so that RCR vectors would selectively target tumor cells. While we are continuing to pursue the use of untargeted RCR vectors for specific indications where tropism modification is less essential and the risk is well justified in poor prognosis malignancies (e.g. glioblastoma; see below), based on FDA recommendations we have now renewed our efforts to develop effective targeting strategies to enhance RCR transduction efficiency in tumors while further minimizing the risk to normal tissues. We are therefore testing strategies to design RCR vectors with additional mechanisms for tumor-selectivity and safety, by (1) introducing transcriptional control elements that restrict RCR replication to tumor cells, (2) targeting physical binding of RCR vectors to tumor cells by use of hybrid vector systems, and (3) employing tumor-homing cells as delivery vehicles for RCR vectors.

5.1. Transcriptional targeting of RCR vectors by incorporation of tissue-specific promoter elements

Transcriptional activity of retrovirus vectors can be regulated through the replacement of sequences in the viral long terminal repeat (LTR) with cell-specific promoter elements. The LTR consists of 3 distinct regions, designated U3, R, and U5, which are repeated at each end of the provirus genome. Promoter elements that control transcription of the RNA genome, and therefore replication of the virus, reside in the U3 region. The R region contains the start site of transcription, and therefore the upstream U3 promoter sequences are not included in the genomic RNA transcript. However, the transcript reads through to the U3 sequence in the 3' LTR, which is subsequently re-duplicated at the terminus of the newly formed 5' LTR during viral reverse transcription. Thus, modifications of the LTR promoter must be incorporated into the 3' LTR U3 region to be retained over serial replication cycles.

Similar strategies have been employed previously to target transgene transcription in conventional replication-defective retrovirus vectors to specific cell types (63-66). Through previous work, we demonstrated proof-of-concept for application of this strategy to RCR vectors using prostate-specific promoters, the first reported example of redirecting the tropism of MLV replication at the transcriptional level (44). Recently, Walter Günzburg, Brian Salmons, and colleagues have also reported successful transcriptional targeting of RCR vectors to hepatocellular and colorectal cancer cells (54). Here we will briefly discuss some details of our work using prostate-specific promoters:

5.1.1. Probasin and ARR2PB, prostate-specific androgen-dependent promoters

One of the most well-characterized proteins uniquely produced by the prostate gland is the rat probasin protein. The probasin promoter from -426 to +28 in the 5' untranslated region contains androgen responsive elements and has been shown to stringently direct prostate-specific gene expression in vitro (67) and in transgenic mice (68), particularly for targeted overexpression of SV40 T antigen, resulting in the establishment of transgenic models of prostate cancer (TRAMP mice) (69).

More recently, a synthetic probasin promoter, ARR2PB, with tandem duplication of the androgen responsive regions, has been demonstrated to confer a high
Figure 3. Prostate-specific transcriptional targeting of RCR vectors. Sequences used for hybrid probasin-LTR constructs engineered into RCR vectors. The probasin promoter depicted is ARR2PB, a synthetic probasin promoter with tandem androgen response regions (ARE). Transcription start sites are indicated by the open arrows. MLV: murine leukemia virus, LTR: long terminal repeat containing U3, R, and U5 regions as indicated, TATA: TATA box, CCAAT: CCAAT box. ACE-Ar, ACE-At, ACE-Ac: Amphotropic RCR vectors containing hybrid LTRs, containing ARR2PB promoter/enhancer elements fused as indicated at transcripational start, TATA, or CCAAT sites of MLV, respectively. ND: not done.

level of transgene expression specifically in the prostatic luminal epithelium and is strongly regulated by androgens (70, 71). The ARR2PB promoter has been successfully used to drive androgen-dependent, prostate cell-specific expression of transgenes in vitro and in vivo (71, 72), particularly in transgenic mice (70, 73-76) as well as from adenoviral vectors (77-79).

5.1.2. Development of prostate cancer-selective RCR vectors

We have already tested a number of RCR vectors driven by both the wild type rat probasin (wt PB) promoter fragment used to generate TRAMP mice, as well as the synthetic probasin promoter construct ARR2PB (provided by Dr. Robert Matusik) (44), incorporated into the U3 region of the viral LTR. Three different chimeric promoter designs were tested for incorporation of the androgen-responsive probasin regulatory elements into the LTR, and a configuration that preserved the MLV TATA box but replaced all upstream elements with probasin sequences was found to be optimal (Figure 3). As noted above, this represents the first reported example of redirecting the tropism of MLV replication at the transcriptional level (44).

Replication of probasin-targeted RCR vectors was tested in both prostatic and non-prostatic cell lines, using FACS to monitor spread of the GFP transgene (44). In particular, ARR2PB-targeted RCR vectors exhibited stringent specificity for AR positive human prostate cancer in vitro and in vivo, with replication kinetics comparable to wild type virus. To assess the impact of transcriptional targeting on the potential dissemination and genotoxicity of RCR vectors, biodistribution and leukemogenicity of the immunocompetent and immunodeficient mice after systemic viral injection or bone marrow transplantation (BMT) from the donors transduced with the vectors was analyzed by real-time PCR. High copy number of integrated untargeted RCR vector was detected in the spleen and bone marrow, whereas the ARR2PB-targeted vector showed no detectable integration in normal tissues. No malignant changes were observed in any mice that received BMT. Targeted vectors did not mediate transgene expression in human PBMCs whereas untargeted vectors showed significant transduction. Our results thus indicate that transcriptional targeting may indeed improve the safety profile of RCR vectors (Kimura T, et al., manuscript submitted).

5.2. Physical targeting of RCR vectors to tumor cells

Retroviruses encode an envelope protein, which mediates binding and entry of the virion particle into the host cell through interaction with cognate receptors on the cell surface, and which is therefore an important determinant of viral tropism. Retroviral envelope proteins utilize a variety of cellular receptors, and it is well known
Figure 4. Targeted delivery strategies for RCR vectors. A high-capacity/helper-dependent adenovirus can serve as a first-stage carrier to deliver and transiently produce a second-stage retrovirus. Adenovirus vectors can be produced at high titer, can efficiently transduce even the non-dividing cell fraction in tumors \textit{in vivo}, and can be readily targeted by modification of fiber binding tropism, but generally do not undergo genomic integration and thus gene expression is only transient. Alternatively, liposomes or nano-particles can be used for non-viral delivery of plasmids that encode a retrovirus, and again, ligand- or antibody-targeted binding and transfection can be engineered into the lipoplex or polyplex. As a third option, tumor-homing cells can be used as motile carrier platforms to deliver RCR vectors to distant tumor sites. The secondary RCR vectors produced \textit{in situ} from the initially infected or transfected cells could then permanently integrate into adjacent cells as they undergo cell division over time, resulting in both stable transduction and a gradual amplification of the initial input titer. Furthermore, an additional level of selectivity can be achieved by regulating production and/or subsequent replication of the second-stage RCR with a tumor-specific promoter (transcriptional targeting).

that retrovirus particles have a broad capacity to incorporate envelope proteins from heterologous retroviral strains and genera, and even from widely disparate other species of viruses, a phenomenon known as “pseudotyping”. This property has been extensively exploited to broaden or redirect the tropism of conventional replication-defective retroviral vectors, as well as to achieve greater biophysical stability of the enveloped virion particle.

One commonly used pseudotype for conventional replication-defective MLV vectors is the envelope from gibbon ape leukemia virus (GALV), another gammaretrovirus (80). For entry into human cells, GALV uses the phosphate transporter PiT-1 (80), while amphotropic MLV utilizes another highly conserved phosphate transporter, PiT-2 (81). We recently reported the substitution of the MLV env gene in our RCR vectors with that of GALV (82). Unexpectedly, the env gene substitution initially rendered the chimeric RCR vector incapable of replication. However, extended passage of abortively infected cells resulted in selection for mutated forms of the chimeric RCR exhibiting rapid replication kinetics, and we found that different variants arose independently in different infections. Surprisingly, none of the revertants exhibited mutations in the GALV env gene itself, and all exhibited the expected tropism for PiT-1. In all cases, replication had been rescued by mutations in other parts of the viral genome. These second-site mutations were all found to functionally compensate for an imbalance in the viral mRNA splice isoform ratio that had been incurred by the env gene substitution (82). This study illustrates the power of natural selection, and the potential for molecular evolution strategies, in the generation of RCR vectors with novel tropism.

5.2.1. Targeting physical binding of virions by direct modification of the retroviral envelope

As an alternative to naturally occurring retroviral envelopes, it is also possible to engineer specific targeting ligand sequences into the env gene. However, while proof-of-concept for this approach has been demonstrated by several groups (83-89), significant difficulties have been encountered in achieving efficient levels of targeted transduction. Various approaches for direct modification of the retroviral envelope have been tested over the past decade, including the attachment (90, 91) or conjugation (92) of “adaptor” molecules, as well as direct incorporation of targeting ligands or single-chain antibody sequences (83-89), but have generally imparted targeted binding specificity to the virion at the cost of drastic reduction in overall infectious titers (2). We now understand this is because the process of virus-cell membrane fusion, normally triggered by binding of the wild type envelope protein to its natural receptor, instead fails to be activated upon targeted binding to heterologous receptors by the ligand-modified envelope (93). In addition, vectors targeted to alternative receptors may become sequestered and ultimately degraded after endocytosis into a non-productive pathway (85). Therefore, we have been seeking alternative methods for targeting physical delivery of RCR vectors to tumor sites \textit{in vivo} (Figure 4).
5.2.2. Adenovirus-retrovirus hybrid vectors

We are also exploring the use of helper-dependent ("gutted") adenovirus vectors, which are fully deleted of all adenoviral genes and thus have a cloning capacity of up to 36 kb which would allow the incorporation of complete replicative or non-replicative retrovirus systems, as well as offer the potential advantage of relatively low immunogenicity for Class I-mediated cellular responses. It has already been well-established that the target cell binding tropism of adenovirus vectors, including high capacity adenovirus vectors, can be altered by modifications to the fiber knob (94, 95). Thus, hybrid vectors based on helper-dependent adenoviruses directing the in situ production of RCR vectors could represent an ideal combination, with the potential for high titer production, low immunogenicity, and targeting of binding tropism via the first-stage adenovirus, with permanent transgene integration and amplification of initial input titer by propagation strictly restricted to actively dividing tumor cells via the second-stage RCR, whose production from the adenovirus genome in infected cells could further be regulated by the use of tissue-specific or inducible promoters.

We have already demonstrated that high-capacity/ helper-dependent adenovirus vectors (HDAd) can be employed as a first-stage carrier for expression of encoded secondary RCR vectors (96), which are thus produced in situ from initially adenovirus-transduced cells. We used a human adenovirus-derived HDAd vector to deliver an encoded RCR vector derived from ecotropic (i.e., murine species-specific) MLV (96). Human HDAd could infect both human and murine cells, but only transiently, and was incapable of efficient replication in murine cells. Conversely, the ecotropic RCR vector was incapable of infecting human cells, which lack the murine receptor required for binding of ecotropic envelope, but showed robust replication in murine cells. Hence this demonstrates proof-of-concept for the use of tropism-modified hybrid adenovirus-retrovirus hybrid vector systems targeting cancer cells, studies that are currently in progress (Kubo, S. et al, manuscript in preparation).

6. ADDITIONAL STRATEGIES FOR TUMOR-TARGETED DELIVERY OF RCR VECTORS

6.1. Transfection-initiated in situ RCR vector production

RCR vectors can be encoded by a single plasmid, and so viral production and replication can be initiated in situ directly in the tumor tissues by plasmid transfection via chemical or physical delivery methods. We have recently found that effective transfection and initiation of RCR replication can be achieved in the multifocal CT26 hepatic metastasis model after systemic administration of RCR-encoding plasmids via hydrodynamic transfection (Hiraoka, K. et al., unpublished data). This now opens the way to developing targeted nanoparticles for delivery of RCR vector-encoding plasmids, a strategy that represents a unique hybrid vector system combining non-viral delivery and replicating virotherapy. Additional mechanisms for selectivity could be incorporated by conjugating tumor-specific ligands with RCR-encoding plasmids to form cancer-targeted DNA-protein conjugate polyplexes (97).

6.2. Cellular delivery of RCR vectors: the use of tumor-homing cells as carriers

Various groups are seeking to identify and exploit various cell types that exhibit preferential homing to tumor sites in vivo, including tumor infiltrating lymphocytes (98-101), mesenchymal stem cells (102-104), and endothelial progenitor cells (105-107), and it has already been demonstrated that these cells can also serve as carriers to chaperone viral vectors, including oncolytic viruses, to tumor cells in vitro and in vivo (107-111).

Particularly in the case of cytotoxic T lymphocytes (CTLs), there is unique potential for this approach to combine adoptive immunotherapy by tumor antigen-activated CTLs along with their use as vehicles for tumor-selective systemic delivery of targeted RCR vectors. We have therefore embarked on studies to engineer alloreactive cytotoxic T lymphocytes (alloCTLs) to function also as motile RCR delivery platforms particularly in the context of gliomas. AlloCTLs can be activated against host human leukocyte antigens (HLA) which are highly upregulated in gliomas but absent from normal CNS parenchyma, can traffic through tumors and themselves mediate a graft vs. malignancy response, an adoptive immunotherapy approach whose clinical feasibility has been confirmed in a Phase I trial (112, 113) by Kruse and colleagues. We hypothesize that vector-engineered alloCTLs may allow better RCR penetration by migration through the tumor mass and to tumor foci infiltrating normal brain tissue, compared to direct injection of virus supernatant preparations which initially achieve only limited diffusion away from the needle track. We further anticipate that this enhanced penetration will facilitate multi-focal dissemination of RCR vectors from each individual producer cell. The transduction efficiency, therapeutic efficacy, and safety of alloCTL-mediated RCR delivery, and how this delivery method might affect viral replicative kinetics and biodistribution in vivo are now being examined in various glioma models.

7. TOWARD CLINICAL APPLICATION OF RCR VECTORS FOR CANCER GENE THERAPY

RCR vectors could be optimally used to treat highly aggressive and rapidly progressive primary solid tumors with extremely poor prognosis arising from quiescent normal tissue. In this context, brain tumors such as glioblastoma multiforme (GBM) would be an ideal candidate disease for initial clinical trials of RCR-mediated virotherapy. Accordingly, we have been pursuing requisite preclinical studies in animal models of glioma to support the implementation of such clinical trials.

After intratumoral injection of cell-free RCR vector supernatant at MOIs as low as 0.05 into pre-established intracranial gliomas xenografted in nude mice, >98% transduction was achieved throughout the entire tumor mass over a period of several weeks, while notably, spread to normal tissues including peritumoral normal brain
was undetectable (45). In contrast, conventional replication-defective retroviral vectors exhibited gene transfer to ≤ 1% of the tumor. We further demonstrated that these enhanced tumor transduction levels can provide significant therapeutic benefit in two intracranial glioma models, human U-87 MG glioma xenografts in nude mice (45, 46) and syngeneic RG2 gliomas in Fischer rats (47). Stereotactic injection of RCR vectors carrying a suicide gene into intracranial human glioma xenografts, followed by a single cycle of systemic treatment with its non-toxic pro-drug, doubled the median survival time compared to control groups receiving vector alone or pro-drug alone. While relapse occurred due to residual viable tumor cells, the ability of this stably integrated RCR vector to persist in residual transduced cells also proved highly advantageous, enabling re-infection of tumor masses even as they resumed growth. Thus, additional cycles of pro-drug administration produced further therapeutic benefit, as evidenced by 100% survival over a >100 day follow-up period after a single intratumoral injection of the RCR-sucide gene vector and multiple cycles of pro-drug administration, compared to 0% survival of controls (p<0.0001) (45, 46). Again, MLV-derived RCR vector transduction was well-restricted to the tumor itself, with no evidence of systemic spread or toxicity in immunocompetent animals. Thus, our results demonstrated that RCR vectors can achieve significant therapeutic efficacy and survival benefit in malignant glioma models, while maintaining selectivity and safety.

Preparations are now being undertaken for clinical trials using RCR vectors in glioblastoma. Further testing in tumor models, comparison of vector designs, and development of GMP manufacturing for use in clinical trials, are being pursued through a multi-national consortium that includes collaborating retrovirology and oncology groups in France (D. Klatzmann, C. Dalba), Austria (W. Günzburg, B. Salmons, M. Renner, D. Portsmouth), Denmark (F.S. Pedersen), the United Kingdom (F. Farzaneh, N. Habib), and various institutions in the United States, including the University of Southern California (USC; T. Chen, P. Roy-Burman, P. Cannon, W.F. Anderson, W. Wolf), Cedars-Sinai Medical Center (L. Medina-Kauwe, P. Lowenstein), Memorial Sloan Kettering Cancer Center (MSKCC; B. Bochner), Sidney Kimmel Cancer Center (SKCC; C. Kruse), Mayo Clinic (S.J. Russell), National Gene Vector Lab at Indiana University (NGVL; K. Cornetta), University of California San Francisco (UCSF; M. Berger, K. Bankiewicz, S. Chang) and University of California Los Angeles (UCLA; L. Liao, P. Mischel, T. Cloughesy, M. Wang, E. Srivatsan, N. Kasahara).

8. SUMMARY AND PERSPECTIVE

The inability of standard replication-defective retroviral vectors to achieve effective transduction of tumors in vivo has been a major obstacle to gene therapy for cancer. The use of replication-competent virus vectors for gene transfer would be more efficient, as each tumor cell that is successfully transduced would itself become a virus-producing cell, sustaining further transduction events even after initial administration. In contrast to various other replicating viruses now in development as cancer therapeutics, murine leukemia virus (MLV)-based replication-competent retrovirus (RCR) can replicate without immediate lysis of host cells and can spread via direct cell-to-cell budding, and are less likely to elicit robust immune responses that prematurely terminate virus propagation. Yet, until now, the use of such replication-competent vectors has rarely been contemplated due to the potential risks associated with uncontrolled virus spread. In fact, however, due to the intrinsic inability of MLV to infect quiescent normal cells, RCR-mediated gene transfer should be highly selective for rapidly dividing cancer cells. A variety of additional strategies for transcriptional and physical targeting of RCR vectors can also be envisioned and are being pursued; such strategies will further enhance the tumor-selectivity, therapeutic efficacy, and safety of this novel vector system.

The use of such RCR vectors could be well justified in clinical scenarios involving a highly aggressive and rapidly progressive primary solid tumor which arises from a normally very quiescent tissue and is associated with an extremely poor prognosis. In this context, brain tumors such as glioblastoma multiforme would be an ideal candidate disease for this purpose. Indeed, the selectivity of conventional MLV-based vectors for rapidly dividing glioma cells in the context of the quiescent adult brain was precisely the rationale underlying the original clinical trials of retrovirus-mediated cancer gene therapy (28, 114). Despite going to Phase III clinical trials, this approach ultimately failed because the conventional replication-defective retrovirus vectors employed could not achieve therapeutically adequate transduction levels (115). With the use of replicating retrovirus vectors, however, the original promise of this strategy might be fulfilled.

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**Abbreviations:** RCR: replication-competent retrovirus; MLV: Moloney murine leukemia virus; GALV: Gibbon ape leukemia virus; MOI: multiplicities of infection; LTR: long terminal repeat; wt PB: wild type rat probasin promoter; ARR2PB: synthetic probasin promoter; BMT: bone marrow transplantation; GBM: glioblastoma multiforme

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