Oncolytic Viruses for Induction of Anti-Tumor Immunity

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Abstract: Oncolytic virotherapy is an evolving but, as yet, unrealized treatment option for cancer. This approach harnesses the cancer-restricted replicative activity of engineered viruses to achieve tumor cell kill. Tumors that are resistant to chemotherapy or radiotherapy can be susceptible to viral oncolysis because of distinct cell kill mechanisms. There is now compelling evidence that collateral induction of anti-tumor immune responses contributes substantially to viral anti-tumor activities. In addition to the expected anti-viral immune clearance, the "danger" signal created by virus-infected cells can generate immune co-stimulation known to override immune suppression and reverse tolerance within the tumor microenvironment. Our recent findings indicate that immune activation augments the clinical outcomes of oncolytic virotherapy. Strikingly similar and robust clinical response rates (>25%) were observed among advanced cancer patients following intratumoral treatments with adenoviral (AdD24) and herpes simplex (JS1/34.5-/47) constructs armed with an integrated granulocyte-macrophage colony-stimulating factor (GMCSF) payload. Both agents produced regressions in injected as well as distant, uninjected lesions, demonstrating systemic effectiveness. We discuss the innate and adaptive immune activating events that may contribute to these clinical outcomes, and examine systemic delivery strategies to tilt the immunological balance from viral clearance to tumor elimination.

INTRODUCTION

Oncolytic virotherapy is an evolving but, as yet, unrealized treatment option for cancer, especially for cancer patients who have failed other therapies. This approach, which harnesses cancer-selective viral replication to achieve tumor cell kill [1-10], has employed genetically-engineered constructs of adenovirus (ADVs), herpes simplex virus (HSV), Newcastle disease virus, reovirus, vesicular stomatitis virus, measles virus, and vaccinia virus [11]. Tumors that are resistant to chemotherapy or radiotherapy will likely be susceptible to destruction by oncolytic viruses because the mechanisms of cell killing are different [12-14]. Most oncolytic ADVs that have entered the clinic are derived from adenovirus serotype 5 (Ad5). Ad5 causes mild upper respiratory disease in infants, no disease in immunocompetent adults, is genetically stable, and can be produced to yield high titer stocks. Preclinical and clinical studies performed by us and others [6, 10, 11, 15-19] have demonstrated clinical safety of live ADVs administered by intratumoral, intra-arterial, or other systemic routes to cancer patients.

ONYX-015 is the best known oncolytic virus that has been evaluated in clinical trials. This vector is deleted of the E1B-55K protein that mediates export of ADV mRNA from the host cell nucleus to the cytoplasm. Presumably, cancer cells, particularly those with a defective p53 defective pathway would complement this defect in mRNA export, thereby facilitating viral replication [20]. Nonetheless, compared to wild type ADV, ONYX-015 replication is diminished even in cancer cells, most likely due to a decrease in downstream E4-100K mediated late viral RNA translation and export [21]. This construct has been evaluated in hundreds of cancer patients with head and neck, hepatocellular, pancreatic, ovarian, and metastatic colorectal carcinomas as well as malignant gliomas [15, 22-40]. ONYX-015 has proven to be safe, but its efficacy as a monotherapy was limited. ONYX-015 worked best in combination with chemotherapy, where a potentially synergistic effect was observed in patients with advanced head and neck cancer [29][39]. Viral replication and necrosis was observed within the injected cancer [24, 29, 30]. A similar construct named H101 (Oncorine^{R}) was approved in China in 2005 for treatment of head and neck squamous cell carcinoma [20, 41, 42] . H101 represents the first oncolytic virus vector of any kind approved for commercial use.

Late generation constructs, as exemplified by Ad5D24, utilize an alternative backbone deletion (E1A CR2). The CR2 region of the viral E1A protein activates E2F-transcription and forces cells into an S-phase-like state through binding and inactivation of pRb, in turn mitigating downstream pro-apototic tendencies of the infected host cell. The 24 bp E1A deletion eliminates the Rb-binding site for viral E1A, hence limiting Ad5D24 replication to cells with p16-Rb pathway defects which include most if not all human cancers [43]. In contrast, replication in normal tissues is minimized [44, 45]. Compared with the E1B55K delected constructs such as ONYX-015/H101, the 24 bp E1A deletion may be more advantageous because it does not attenuate viral replication in tumor cells [45]. The 24 bp constant region 2 dele-

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tion in *E1A* may actually increase the oncolytic potency of the virus [1]. In addition, Ad5D24 retains the adenoviral death protein (ADP), a viral gene expressed at very late stages of the infectious cycle that negates host cellular function of MAD2B/Rev7 at the spindle checkpoint [46], promoting host cell lysis and the release of virion progenies [47, 48]. Despite an improved efficacy in experimental models, the therapeutic effectiveness of systemic Ad5D24 remains poor [49] with clinical effectiveness largely restricted to locoregional applications.

Ultimately, clinical justification for oncolytic virotherapy hinges on the demonstration of systemic safety as well as efficacy. The vast body of clinical experience with ONYX-015, Ad5D24, and other conditionally replicative adenoviruses (CRADV) indicate that significant hurdles need to be overcome, including 1) the safe yet suboptimal infectious process attributable to the attenuated viral construct; 2) pharmacokinetic limitations due to rapid viral clearance, most likely from uptake by reticuloendothelial cells (heparin sulfate proteoglycan, Factor IX, X-mediated), platelets, and leukocytes (Fc receptors); 3) viral activation of the proinflammatory cascade through tumor necrosis factor-alpha (TNFα) and interferon-gamma (IFNγ) induction that reduces viral survival, and 4) activation of adaptive immune responses with increased viral neutralizing antibody titer, which further reduces viral circulating half life [6, 11, 50]. In this regard, late generation oncolytic viruses have been designed with alternative viral backbone modifications and anti-tumor transgene inserts to further improve oncolytic potency [11, 51]. Altered viral tropism, through masking or substitution of viral surface components has been widely considered to further tumor-selective targeting and safety [11, 52-56].

HARNESSING TUMOR HOST'S SYSTEMIC IMMUNE RESPONSE: CONVERTING A LIMITATION INTO THERAPEUTIC ADVANTAGE

The immunological sequelae following adenoviral exposure at the systemic or intratumoral (IT) level are well characterized [57-59]. Upregulation of proinflammatory cytokines and chemokines [11] is commonly observed, an innate immune response that is mediated by the ADV particle and does not require viral transcription [60]. Anti-viral response is commonly initiated by pattern recognition receptor-binding in epithelial cells, macrophages, and dendritic cells, exemplified by TLR9 recognition of unmethylated CpG viral DNA [61]. Cytokine induction and the activation of effector leukocytes lead to inflammation at the locale of infected tissues. The resulting clearance of >70% of viral load within 4-6 hours [60] severely limits the pharmacokinetics of viral administration.

By comparison, adaptive immune responses to viral gene therapy may both negatively and positively impact anti-tumor activity [62]. In animal models, preexisting anti-viral antibodies abbreviate systemic viral replication and dissemination [63-65], whereas cytokines generated throughout the infection process, including tumor necrosis factor-alpha (TNFα), interferon-gamma (INFγ), and monocyte chemoattractant protein-1 (MCP-1) [66, 67] can activate antigen presenting dendritic cells (DCs). Oncogenic changes alone can induce a pro-inflammatory microenvironment. However, this "smoldering" inflammatory state actually fosters tumor angiogenesis and metastasis [68]. Tumor infiltrating CD8+ T activation is often hampered by inadequate or inappropriate antigen presentation due to the lack of costimulatory signals and/or the presence of endogenous immune suppressive proteins [57]. The strong "danger" signal (viral induced DAMP, danger associated molecular patterns; calreticulin) produced during oncolytic viral replication provides the necessary co-stimulation to override immune suppression and reverse immune tolerance [62, 69, 70]. Coupled with the tumor cell oncolytic event and release of tumor-associated antigens, these events promote tumor antigen sensitization and natural killer (NK) and cytotoxic T lymphocyte (CTL)-mediated antitumor activities [71, 72]. The adjuvant properties of immunogenic viral components (e.g. hexon capsomere protein) further augment T helper and CTL mediated elimination of viral infected and uninfected, bystander tumor cells [73].

Nevertheless, this process may be inadequate or out of sync with the kinetics of localized antigen presenting cell (APC) maturation, as well as compromised by the competing intratumoral kinetic processes of immune mediated inactivation versus viral diffusivity. To this end, cytokine transgenes delivered and expressed by viruses has been beneficial for promoting T cell responses by the tumor bearing host. Localized, promoter-regulated release of TNFα generated highly significant clinical activity following delivery by a non-replicative adenovector (TNFerade) [74, 75]. Similarly, IFNγ coexpression by the oncolytic adenovirus CTV developed in the Fisher Laboratory significantly improved antitumor outcome through NK activation [76].

CRADV exposure is not commonly associated with increased GMCSF levels, although ectopic expression of this cytokine within the tumor environment has been highly effective at inducing systemic antitumoral immunity in murine tumor models [77]. Co-administration of GMCSF is limited by its very short half life. However, cells constitutively secreting GMCSF can sustain local cytokine concentrations for upwards of 21 days [78-81], serving to attract increased numbers of antigen-presenting DCs (a subset of APC) locally and to the drainage lymph nodes *in vivo,* and stimulate DC activation marker expression (e.g. CD40, CD80, CD86, and MHC II) [82]. This process promotes tumor antigen processing, presentation, and activates host CD8+ effector T-cells [83, 84]. Conversely, CD8+ effector T lymphocytes can induce robust DC maturation during priming by secreting "licensing factor(s)", which was recently identified to be GMCSF [85]. In comparative analyses with irradiated B16 melanoma cancer cells transfected with various retrovirally-delivered cytokine transgenes including IFNγ and TNFα, GMCSF transfected cells were the most potent cytokine inducer of anti-tumor immunity [77, 86]. We and others have confirmed that GMCSF expressing autologous or allogeneic cancer cell vaccines effectively induced tumor immune activation in clinical trials [87-91]. Approximately 75% of the roughly 120 patients with melanoma, non-small cell lung, renal and prostate carcinomas who received GMCSF-expressing cancer vaccines [89] have demonstrated no significant toxicities despite repeated vaccinations, with their antigen presenting DCs displaying maturational and activational features [92].

SUSTAINED ONCOLYTIC ACTIVITY IN THE GMCSF-INTEGRATED AD5D24

Based on the premise that adenovirus replication *per se* is usually inadequate for eliciting robust clinical responses, the Hemminki laboratory has armed the well characterized Ad5D24 [43, 45, 93] with the GMCSF transgene in order to override tumor immune suppressive mechanisms and to activate APCs [18]. GMCSF is cloned into the *gp19k and 6.7k*-deleted region under the control of the E3 promoter, resulting in replication associated transgene expression at high levels starting at about 8h after infection [18]. The virus replicates in a tumor selective manner and GMCSF is produced only locally, which might be important from the point of view of avoiding stimulation of myeloid derived suppressor cells (MDSCs). Although not all reports agree, there is some emerging evidence suggesting that this recently identified class of suppressors may be stimulated by high systemic GMCSF concentrations [94, 95]. Biologically active GMCSF secretion was seen when the virus replicated but not when UV inactivated virus was used *in vitro*. Further, Ad5D24GMCSF retained its oncolytic potency in all tested cell lines, indicating that replacement of *gp19k and 6.7k* with GMCSF did not impact viral oncolytic activity. Conversely, deletion of gp19k may serve to enhance tumor selectivity. Loss of gp19 likely attenuates viral replication in normal cells by removing the blockade of MHC I-restricted viral antigen presentation [96].

In immunocompetent hamster models where human GMCSF is active biologically [97], Ad5D24GMCSF demonstrated an augmented anti-tumor efficacy against the syngeneic HapT1 pancreatic tumor [18]. Treated animals acquired the ability to reject a subsequent HapT1 challenge though not against an irrelevant tumor. By comparison, treatment with parental Ad5D24 delayed tumor growth but did not reject tumor challenge. As expected, Ad5D24-GMCSF did not produce enhanced antitumor activities as compared with parental Ad5D24-E3 against A549 human tumor xenografts in SCID mice that lack T or B cell immune functions. Human GMCSF also lacks cross reactivity in mice. These findings support an immunostimulatory function by the GMCSF transgene in context of Ad5D24 virotherapy Fig. (**1**). Confirmatory analysis, however, is needed to determine the transferability of antitumor immunity by cellular or humoral effectors.

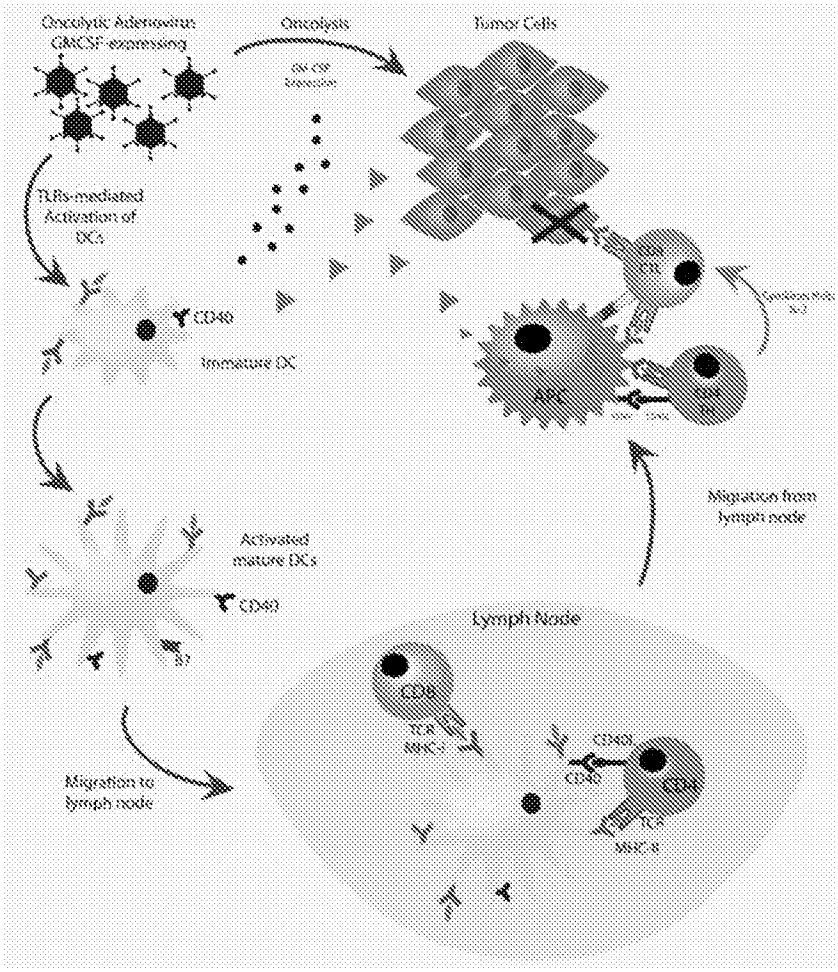

Fig. (1). Working hypothesis for an GMCSF-expressing oncolytic virus. Tumor infection by the oncolytic adenovirus leads to cell lysis. GMCSF transgene expression that is linked to the viral replicative cycle recruits antigen presenting dendritic cells (DCs). The "danger signal" produced by adenoviral infection serves as an intrinsic adjuvant effect for activating recruited DCs, which in turn loads tumor antigens released during viral oncolysis. Tumor antigen-loaded and activated DCs migrate to draining lymph nodes, where they activate CD4+ and CD8+ T cells to initiate a tumor-specific immune response.

CLINICAL SAFETY AND EFFICACY OF AD5D24-GMCSF

A European clinical study was recently completed with Ad5D24GMCSF (dose escalating cohorts from $8x10^9$ – $4x10^{11}$ vp) administered intratumorally, intraperitoneally, and/or intravenously in 20 patients with advanced refractory cancers. The starting dose of $8x10^9$ viral particles (vp) was chosen according to safety results [16, 23, 27, 70, 78, 98, 99]. Enrolled patients comprised breast, ovarian, cervical, colon, gastric, renal cell, nonsmall cell lung, medullary thyroid cancer, hepatocellular carcinoma, jejunum adenocarcinoma, mesothelioma, leiomyosarcoma, synovial sarcoma, choroidal melanoma, and melanoma. Virus was administered by ultrasound-guided single intratumoral injection, with one fifth of the dose given intravenously. Treatments were generally well tolerated at up to $4x10^{11}$vp. Most patients experienced grade 1-2 flu-like symptoms (19/19), fever (14/19), fatigue and injection site pain (8/19), with one patient exhibiting grade 3 toxicity after receiving an intermediate dose ($2x10^{11}$ vp) that was attributable to progressive disease. With respect to proinflammatory cytokine responses as a potential predictor of adenovirus toxicity [58, 100-103], only moderate elevations (<50%) of serum IL-6, IL-8, and/or TNFα elevations were observed in 20% of patients, suggesting that the viral doses used were well tolerated and below toxicity-inducing levels. Nine of 18 patients had detectable, circulating virus (<500–25787 vp equivalent) at 3-7 days post-treatment according to PCR amplification of the viral E1A region that flanks the 24 bp deletion region of AdD24 [18]. Virus remained detectable in 4 patients after 21 days. Since injected virus is rapidly cleared, the extended detection of circulating virus genomes in serum is suggestive of viral replication [16, 23, 104].

Sixteen patients were evaluable for radiological responses. Two patients (13%) had complete eradication of all measurable disease. One had a minor response and 5 additional patients (33%) had stabilization of their progressive disease. Therefore, the clinical benefit rate was 47% using RECIST criteria. Previous reports suggest that a decrease in tumor density may correlate with antitumor efficacy [105]. This was measurable in two patients, and in both cases the density decreased (by 4.6% and 27%). There was no difference with respect to clinical response in injected vs. non-injected tumors. Median overall survival of patients treated with Ad5D24GMCSF was 213 days. The 3 and 6 months survival was 89% and 53% respectively.

EVIDENCE OF IMMUNE ACTIVATION BY AD5D24-GMCSF

At 4-7 days post-treatment, low serum GMCSF levels (≥50% elevation) were detected in 5 of 19 patients, with no remarkable changes in total white blood cell levels. Thus GMCSF production was essentially restricted to the tumor. However, 10 of 11 patients demonstrated markedly elevated circulating CD8+ T cells (mean increase, 77%, n=11) following Ad5D24GMCSF treatment. ELISPOT analysis demonstrated markedly elevated CTL activities against both viral and, importantly, tumor target antigens in all of 4 patients tested when assayed with peptide pools for the human AdV5 penton [57] and the tumor-associated antigen survivin (expressed in essentially all human malignancies and low or no expression in differentiated normal tissues [106-108]). Confirmatory tetramer binding analysis demonstrated markedly increased frequency of CD8+/survivin-reactive T cells in 3 of 4 patients with an HLA-A02+ haplotype, illustrating the elevated T cell response following Ad5D24GMCSF treatment.

Neutralizing antibody titers were positive in 8/19 cases (42%) at baseline and increased within 2 weeks in most patients regardless of viral dose. However, there was no clear correlation between neutralizing antibody titers and viral dose, antitumor activity or toxicity as consistent with findings in other oncolytic virus trials [11, 16, 23, 34, 42, 50]. There did not appear to be a direct correlation of elevated CTL response to viral or surrogate tumor antigen response and clinical response or toxicity within the limited number of patients analyzed.

SYSTEMIC CLINICAL OUTCOME FOLLOWING INTRATUMORAL ONCOVEX^{GMCSF}

We have observed similarly positive systemic clinical outcomes following intratumoral treatment with a GMCSF-expressing oncolytic HSV [109]. Deletion of the well characterized 34.5γ gene from the highly lytic HSV confers tumor selectivity in a broad range of human tumor cells, regardless of their genetic aberration [110]. Following demonstration of safety and suggestions of clinical efficacy with the ICP34.5 deleted HSV, Coffin and coworkers have produced the OncoVEX^{GMCSF} from a recently isolated JS1 strain of HSV-1 that is more effective in tumor cell kill, and also incorporates the GMCSF transgene to stimulate an immune response under the control of the human cytomegalovirus immediate early promoter [110]. Further, the ICP47 HSV gene, which binds TAP1 and TAP2, is deleted to eliminate its attenuating function against MHC class I- and II-antigen presentation, thereby promoting an anti-tumour immune response. Loss of ICP47 also increases and transposes expression of the HSV US11 gene to the immediate early (IE) α47 promoter. This modification enhances viral growth (and cytotoxicity) in tumor cells by binding to PKR, prevents eIF-2α phosphorylation thereby offsetting the attenuated virulence resulting from ICP34.5 deletion. Thus the additional modifications in JS1 results in improved tumor-selective virulence without sacrificing safety [111]. Finally, the encoded human GMCSF transgene (in place of the deleted 34.5γ) is expected to promote DC maturation, proliferation, and differentiation, thereby amplifying the afferent process of tumor antigen sensitization derived from apoptotic tumor cells [112].

In murine syngeneic colorectal and melanoma tumor models, Toda and coworkers [113] demonstrated that intratumoral injection with HSV-1 G207 (34.5γ deleted/ICP6$^-$ [lacZ insertion]) produced cancer cell death and growth inhibition. Significantly, unilateral intratumoral inoculation produced growth reductions in both the inoculated and contralateral noninoculated tumors, an outcome that required viral infection of the tumor as intradermal inoculation of G207 did not generate any antitumor effects. Tumor growth inhibition correlated with the induction of tumor-specific CD8+ T cell response against a dominant "tumor-specific" MHC class I-restricted epitope (AH1) in the CT26 animal model. Thus

G207 achieved tumor growth inhibition by local cytotoxic viral replication as well as by its systemic immune activating function, i.e. as an *in situ* tumor vaccine.

These findings served as basis for a Phase I study with OncoVEX^{GMCSF}. Single and multidose intratumoral OncoVEX^{GMCSF} was well-tolerated at up to 10^8 pfu/ml in 30 patients with malignant melanoma or cutaneous/ subcutaneous breast, head and neck, and gastrointestinal cancers [110]. Biologic activity included evidence of viral replication, local reactions, GMCSF expression, and HSV antigen-associated tumor necrosis or apoptosis. Three patients had stable disease after treatment, six had tumors flattened in injected and/or uninjected lesions, and four patients showed inflammation of uninjected as well as the injected tumor, which, in nearly all cases, became inflamed.

In the subsequent Phase II study of OncoVEX^{GMCSF} led by our group (the Mary Crowley Cancer Research Center), IT injection of up to 4 mL of 10^6 pfu/mL of OncoVEX^{GMCSF} was given to 50 patients with advanced, metastatic melanoma (10 stage IIIc, 40 stage IV), followed three weeks later by IT injection of up to 4mL of 10^8 pfu/mL to accommodate seroconversion of seronegative patients. OncoVEX^{GMCSF} was administered every two-weeks for up to 24 treatments thereafter [109]. These patients received a median of 6 injection sets (mean, 9) and achieved an initial overall response rate of 26% by RECIST (8 complete remissions/CR; 5 partial remissions/PR). Regressions were observed in both injected and distant un-injected (including visceral) lesions Fig. (**2**). The 1-year survival for all patients was 58%, 58% for the stage IV subset, and 40% for the stage IVM1c subset. Seventy-seven percent of the responses have been maintained for >6 to 26 months, with two further responses ongoing at <6 months. There were significantly increased melanoma-specific (MART-1-reactive) T cells in regressed lesions, and reduced regulatory T cells (Treg), suppressor T cells (Ts), and MDSCs in virus-injected lesions [114]. Melanoma tumor infiltrating lymphocytes (TIL) from matched OncoVEX^{GMCSF} injected and uninjected sites in the same patients also recognized MART-1. Thus therapeutic responses to direct Oncovex^{GMCSF} injections were accompanied by local and systemic antigen-specific T cell responses and reduced Tregs, Ts, and MDSCs within established melanoma lesions (providing further evidence to support the contention that locally produced GMCSF without high systemic levels neither attracts nor stimulates MDSC).

These survival findings compared favorably to recently published meta-analyses of 2,100 stage IV metastatic melanoma patients entered into 42 phase II trials from 1975 through 2005 [115], where the 1-year OS rate was 25.5%, with no trial providing a survival result that was statistically different from the mean (25% in 524 patients). In the same analysis, the 1-year OS for only those 1,024 patients with visceral disease (stage IV M1c) was 23.8%, as compared with 40% of Stage IV, OncoVEX^{GMCSF}-treated patients. The median survival time has not been reached at 16+ months for all patients treated with OncoVEX^{GMCSF} (58% currently remaining alive) as well as for the Stage IV subset (52.5% currently alive). The 26% response rate showed impressive durability of response in both injected and uninjected lesions including visceral sites and a 58% 1-year overall survival rate,and therefore, are compelling evidence of systemic effectiveness. Thus synergism between viral oncolysis and immune activation by the GMCSF producing virus can lead to durable, systemic complete and partial remissions in patients with advanced melanoma.

OVERCOMING CHALLENGES FOR SYSTEMIC VIROTHERAPY WITH A GMCSF PAYLOAD

Although these intratumoral clinical findings are based on a limited number of patients, overall safety and clinical response rates of >25% by the two GMCSF-armed oncolytic constructs are noteworthy. In particular, complete remissions and extended durations of survival were achieved at relatively conservative viral dosing among a heavily pretreated patient population having advanced disease. There have been similar demonstrations of safety and clinical activity of GMCSF-integrated poxvirus [98] and CRADV [116] therapy in recently completed Phase I trials.

Independent trials have confirmed the safety of prolonged administration of GMCSF. GMCSF (sargramostim; 125 ug/m^2 daily for 14 days followed by 14 days rest) has been administered over 3 years as a surgical adjuvant therapy

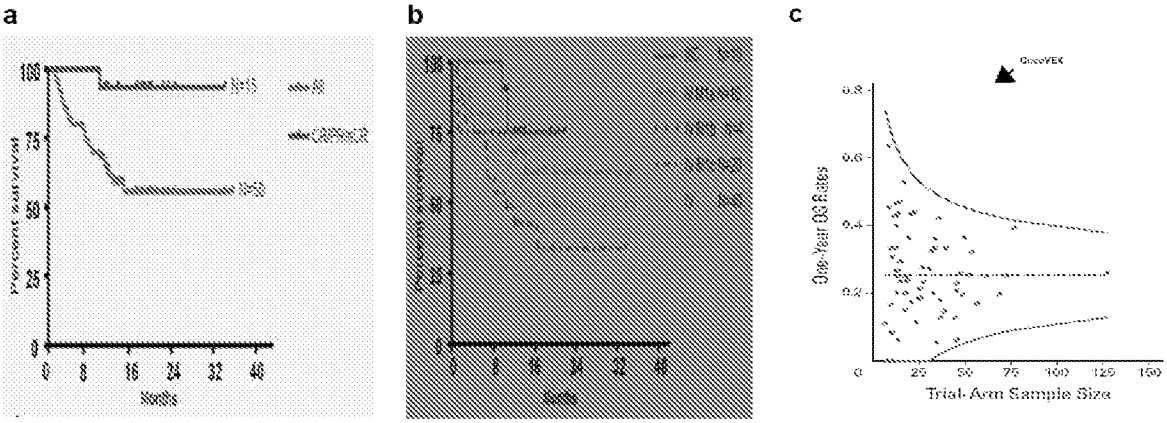

Fig. (2). Kaplan-Meier survival analysis for advanced melanoma patients following intratumoral OncoVEX^{GMCSF}. 3.a Survival curves for all patients enrolled, and those who achieved PR, CR or sCR; **3.b** Survival by disease stage. **3.c** Meta-analysis on one year overall survival of stage IV melanoma patients entered into trial (1975-2005). Survival for Oncovex-treated patient is shown in red.

in 98 evaluable patients with stages II (T4), III, or IV high recurrence risk melanoma patients [117]. This regimen was well tolerated. Grade 1 or 2 side effects occurred in 82% of the patients, with no grade 3 or 4 treatment-related side effects. A 5-year melanoma-specific survival rate of 60% was achieved. Median melanoma-specific survival has not yet been reached with a median follow-up of 5.3 years, hence supporting the relevance of GMCSF intervention for advanced melanoma. Two of 98 patients developed acute myelogenous leukemia after completion of 3 years of GMCSF administration. Preclinical studies showed that sustained, high dose GMCSF at the systemic level may induce tumor tolerizing myeloid suppressor cells [95]. However, these scenarios are unlikely with respect to viral-delivered GMCSF, given the relative brevity of viral pharmacodynamics and a corresponding duration of localized cytokine transgene expression that is expected to expire within 21 days post-treatment [16].

Thus scientific and clinical findings support the feasibility of synergism *via* viral oncolysis and immune activation by the GMCSF transgene. Systemic administration would likely 1) extend oncolytic effectiveness through concomitant infection of both primary and metastatic lesions, 2) accelerate onset of therapeutic activity, and 3) generate direct, multi-focal immune activation to primary and metastatic tumor antigens [118]. However, realization of these expectations is predicated on further optimization of systemic pharmacokinetics, bioavailability and viral gene expression. Within the context of the well characterized CRADV system, factors that negatively impact virus distribution, tumor targeting and tumor cellular viral uptake have to be overcome, which include the lack of the primary adenovirus CAR (coxsackie and adenovirus receptor) expression in human tumors, uptake of systemic circulating virus by liver Kűpffer cells and other non-target cells *via* secondary receptor (FX, heparin sulfate glycosaminoglycans)-binding, and immune mediated viral clearance.

To these ends, novel constructs that incorporate surface modifications and/or "stealth" techniques have been described [11, 119, 120]. Determinants of tissue tropism for ADV encompass the viral capsid proteins, hexon, penton base, and fiber [121]. The fiber contains the knob domain which interacts with CAR, its primary cellular receptor, and the penton base protein contains an RGD sequence motif that interacts with cell surface integrins essential for cell internalization [11]. Investigators have complexed bi-specific single-chain antibodies [45, 122] or ligands of tumor cell surface receptors [123] to the native fiber knob or penton [122, 124]. The most common modifications have been the insertion of an Arg-Gly-Asp (RGD-4C) integrin-targeting motif into the HI loop of fiber knob domain, expanding tropism toward the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, and the replacement of ADV serotype 5 knob with the Ad3 knob (Ad5/Ad3 chimera) to circumvent low CAR expression [125] . Others have considered replacements with non-CAR-interacting Ad35 fiber knob domains [126], the Ad9 fiber knob [126], or the nonhuman canine ADV type 2 (CAV2) knob [127]. The complexity of these novel constructs will likely require extensive manufacturing and quality assurance validation in scaled-up production needed for clinic study [128].

STEALTHED ADV FOR IMPROVED SYSTEMIC PHARMACOKINETICS AND BIODISTRIBUTION

A technically less challenging but equally effective attractive alternative is to encapsulate viral particles in liposomal complexes Fig. (**3**), in view of their biocompatibility, biodegradability, low toxicity, and low immunogenicity. "Stealthed" viral particles, typically with a diameter less than 400-500 nm, localize to the tumor microenvironment by the enhanced permeation and retention effect (EPR) in inflammatory sites and solid tumors, where the vasculature and, in particular, the larger endothelial junction, is "leaky" [129]. In addition to wider fenestrations, lack of a smooth muscle layer, and impaired angiotensin II receptors, vascular hyperpermeability is also attributed to overexpression of cytokines by the tumor or its stroma, such as vascular permeability factor (VPF), vascular endothelial growth factor (VEGF), as well as other factors such as the basic fibroblast growth factor (bFGF), bradykinin, nitric oxide and peroxynitrate [130]. EPR permits small particles to extravasate from the blood stream into the disease site [131] through the extracellular matrix, a highly hydrated, gel-like network which serves as scaffolding between the blood vessels and the tissue cells. The small size of the stealthed viruses facilitates their diffusion through the extracellular matrix to the cell surface [131]. Hama [132] showed recently that liposomal-delivered DNA exhibited a more efficient uptake by cells as compared with "naked" ADV, had a similar efficiency of intracellular endosomal escape, trafficked to the nucleus at a slower yet comparable rate, yet was 1,000 times less efficient at gene transfer. By utilizing liposomal stealth delivery for CRADV, enhanced systemic pharmacokinetics is integrated with the versatility of the evolutionarily-perfected viral transcriptional machinery that co-ops the host cell's manufacturing capacity to achieve the oncolytic process.

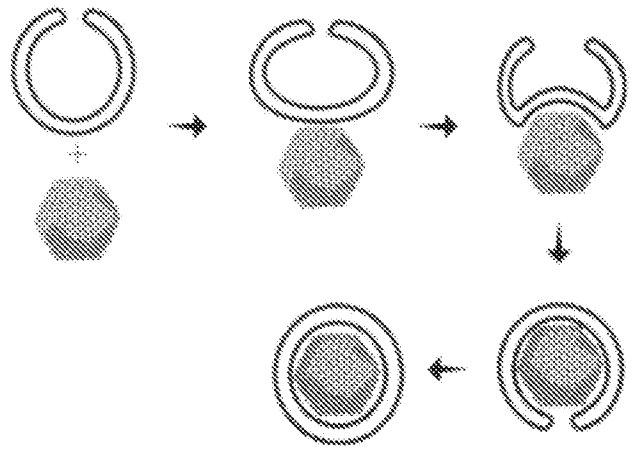

Fig. (3). BIV encapsulation of adenovirus. Graphic representation of ADV-BIV interaction-induced negative curvature and complexing with virus particle. Novel BIV-DNA [148] and BIV-ADV structures[55] have been confirmed by cryo-electron microscopy (cryo-EM).

ONYX-015/dl1520, when encapsulated by cationic DOTAP (dioleoyl 3-trimethylammonium-propane) and DOTAP:cholesterol as bi-lamellar vesicles (BIVs) efficiently infected CAR-negative tumor cells while displaying resistance to the neutralizing antiviral antibodies *in vivo* [56].

Antitumor efficacy was significantly improved as compared with naked dl1520 following IT or intravenous administration. dl1520/liposome was localized in the lung , with no detectable virus in the liver or other organs following iv injection of 10^9 pfu/mouse. Similarly, liposomal encapsulated ADVs, when administered intranasally, selectively transduced airway epithelial cells with minimal expression in alveolar cells in mice. IFNγ and inflammatory cellular infiltrates were greatly reduced as compared with mice that received the unencapsulated ADV [133]. Brunicardi and coworkers recently demonstrated the systemic applicability of BIV encapsulated delivery to human pancreatic cancer xenografts [134]. Thus tumor localizing advantages can be achieved without the potential issues of covalent viral capsid modification [11, 135], and can be self-assembled and scaled up effectively.

FUTURE STUDIES

Positive findings of clinical safety and efficacy through locoregional routes have paved the way towards considering GMCSF-armed oncolytic viruses as a systemic cancer therapeutic. Stealth delivery by bilamellar invaginated liposome (BIV) encapsulation represents a technical advance that can improve pharmacokinetics and tumor localization, as well as reduce sensitization to immunodominant viral antigens while in circulation. Recently reported enhancements have been shown to further minimize nonspecific uptake and promote tumor-targeted localization. The reversible masking strategy developed by the Templeton laboratory utilizes small, uncharged molecular weight lipids that loosely associate with the surface of BIV complexes to temporarily shield the positively charged lipoplex to bypass non-target organs. These small lipids are removed by sheer force in the bloodstream by the time they reach the target cell, re-exposing surface cationic charges at the time of target cell engagement, facilitating entry. Thus targeted delivery can be achieved *in vivo* without the use of polyethylene glycol (PEG). PEG is traditionally added to delivery vehicles including liposome formulations to extend circulatory half-life [136-139], but has been shown by us and others to induce steric hindrance in the BIV liposomal structures and thereby reducing the efficiency of DNA encapsulation resulting in substantially poorer gene expression. Furthermore, PEGylated complexes enter the cell predominantly through the endocytic pathway, resulting in degradation of the bulk of the nucleic acid in the lysosomes.

Reversible-masking has optimized the systemic delivery of a CAT-reporter-encoded expression plasmid to target organs [140]. However, further studies are needed to confirm its applicability for stealth viral delivery. Moreover, Templeton [140] and others [141, 142 129, 143] have shown that "decorated" BIVs (which are inherently fusogenic) that actively target tumor surface receptor/ligands can redirect intracellular trafficking to enhance uptake and minimize, if not bypass, lysosomal degradation, thereby achieving a prolonged intracellular half life and extends transgene expression. The approach by Shi and coworkers is particularly appealing, whereby small molecular bivalent beta-turn-mimics screened for tumor receptors binding can further facilitate CAR-independent entry [140] and viral/transgene expression. Thus the approach of stealthed, targeted tumor delivery of GMCSF-armed oncolytic virus strives to strike a balance by merging its "immunocentric" and "virocentric" features [59] within the tumor microenvironment, further enhancing the anti-tumor capabilities of such an experimental therapeutic agent.

The clinical implementation of GMCSF-armed oncolytic viruses will require an in-depth understanding of immune activating events that accompany treatment. To this end, the Syrian hamster model recently validated by the Wold laboratory for assessment of CRADV biodistribution, toxicity, and efficacy have already been established [144-146]. Hence the immunological outcome of GMCSF armed CRADV and its induction of effector and regulatory immunological subsets [59, 147] can be further characterized as hamster-specific immunological reagents become readily available.

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CONFLICT OF INTEREST

A.H. is founder and shareholder in Oncos Therapeutics, Inc.

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