Tissue-Specific Promoters Active in CD44⁺CD24⁻/low Breast Cancer Cells

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Abstract
It has been proposed that human tumors contain stem cells that have a central role in tumor initiation and posttreatment relapse. Putative breast cancer stem cells may reside in the CD44⁺CD24⁻/low population. Oncolytic adenoviruses are attractive for killing of these cells because they enter through infection and are therefore not susceptible to active and passive mechanisms that render stem cells resistant to many drugs. Although adenoviruses have been quite safe in cancer trials, preclinical work suggests that toxicity may ultimately be possible with more active agents. Therefore, restriction of virus replication to target tissues with tissue-specific promoters is appealing for improving safety and can be achieved without loss of efficacy. We extracted CD44⁺CD24⁻/low cells from pleural effusions of breast cancer patients and found that modification of adenovirus type 5 tropism with the serotype 3 knob increased gene delivery to CD44⁺CD24⁻/low cells. α-Lactalbumin, cyclo-oxygenase 2, telomerase, and multidrug resistance protein promoters were studied for activity in CD44⁺CD24⁻/low cells, and a panel of oncolytic viruses was subsequently constructed. Each virus featured 5/3 chimerism of the fiber and a promoter controlling expression of E1A, which was also deleted in the Rb binding domain for additional tumor selectivity. Cell killing assays identified Ad5/3-cox2L-d24 and Ad5/3-mdr-d24 as the most active agents, and these viruses were able to completely eradicate CD44⁺CD24⁻/low cells in vitro. In vivo, these viruses had significant antitumor activity in CD44⁺CD24⁻/low-derived tumors. These findings may have relevance for elimination of cancer stem cells in humans. [Cancer Res 2008;68(14):5533–9]

Introduction
Decades ago, it was proposed that aberrant stem cells might underlie the heterogeneous nature of many advanced tumors. However, it is only recently that this theory has gained support from molecular evidence. Nonetheless, because cancer stem cells have not yet been fully defined, it cannot conclusively be proven that they exist, and there are also competitive hypotheses (reversible epidermal to mesenchymal transition, mutant phenotypes, etc.), which might explain some of the puzzling features of certain tumor cell populations. With regard to breast cancer, the cancer stem cell hypothesis is supported by the finding that CD44⁺CD24⁻/low cells, frequently found in primary tumors and metastases, exhibit stem cell characteristics, including self-renewal and differentiation along various mammary epithelial lineages (1–3). A low number of CD44⁺CD24⁻/low cells, as opposed to unsorted cells, is sufficient for initiation of tumors in mice (4).

Because of slow turnover and the ability for expelling antitumor drugs, putative cancer stem cells are resistant to many conventional cancer therapies (5). Therefore, they may have an important role in relapse after treatment and might therefore be causative of the incurable nature of many advanced solid tumors including metastatic breast cancer. Because most antitumor agents have been approved based on tumor response, agents preferentially active on cancer stem cells (which form a minority of the bulk of the tumor) may have been missed.

Viruses capable of selectively killing tumor cells, including oncolytic adenoviruses, enter cells through infection and kill both proliferating and quiescent cells. Such viruses are rendered replication deficient in normal cells by engineered genetic changes that are transcomplemented in tumor cells. One useful approach in this regard is utilization of tissue or tumor-specific promoters (TSP), which are activated in target cells, whereas nontumor cells are spared (6).

As there are no previous reports on which promoters might be useful in the context of cancer stem cells, we constructed viruses featuring the α-lactalbumin (ala; ref. 7), cyclo-oxygenase 2 (Cox-2; ref. 8), telomerase (hTERT; ref. 9), and multidrug resistance (mdr; ref. 10) promoters. Given that many stem cell types express low levels of the rate-limiting coxsackie-adenovirus receptor (11–14), we studied the utility of viral capsid modification for enhanced delivery. Then, we constructed the respective capsid modified, promoter-controlled oncolytic adenoviruses, and investigated their utility for killing CD44⁺CD24⁺/low breast cancer stem cells in vitro and in vivo.

Materials and Methods
Cells. Pleural effusions were obtained with ethics committee–approved informed consent and washed with DMEM/F12 supplemented with 10ng/mL basic fibroblast growth factor, 20 ng/mL epidermal growth factor, 5 μg/mL insulin, and 0.4% bovine serum albumin (all from Sigma). All three patients (Fig. 2A–C corresponds to patients PL11-13) had metastatic ductal breast cancer. PL11: 58 y, grade 1, ER+, PR+, Her2– (CISH); PL12: 59 y,
grade 3, ER+, PR-, HER2-; PL13.85 y, grade 3, ER+, PR+, HER2--. JMT-1 cells were cultured as reported (15). Cells were sorted with FITC-labeled anti-CD44 and phycoerythrin-labeled anti-CD24 antibodies (BD Pharmingen), collected with magnetic beads (Miltenyi Biotech). Cells (911, 293, and A549) are from American Type Culture Collection (ATCC).

Viruses. Ad5/3/ala-d24, Ad5/3/mdr-E1, and Ad5/3/mdr-d24 were constructed by cutting promoter sequences from pGL3-ALA (7) and pMDmrd-p-hTFP (10) into pShuttle-CMV and pShuttle-d24 (16), from which the E1 promoter was removed. The viral backbone was rescued in BJ5183 cells using Pmel-digested pShuttle and Srlf-linearized pAdEasy1-5/3-d24 (serotype 3 knob, a 24-bp deletion in E1A) and a 965-bp 6.7K/gp19K deletion in E44) for transfection into 911 cells. Propagation of these viruses and of Ad5/3-d24 (14) and Ad5/3-CoxlL-D24 (12) was performed on A549 cells.

The same promoter sequences were cloned to replace cytomegalovirus (CMV) in pShuttle-CMV (AdEasy; QiBiogene) and luciferase was cut from pGL3-Basic (Promega) for homologous recombination with pAdEasy. Wild-type adenovirus (AdSw5) is from ATCC. Ad5/3a, Ad5/3/ala, and Ad5/3/mdr-1 have been reported (12, 13). E1-deleted viruses were propagated on 293 cells (Microbix). All viruses were purified on double CsCl gradients using standard methods. The E1 and E3 regions, fiber, and TSPs were checked with PCR followed by sequencing (11, 16). The viruses were titered for viral particles (VP) at 260 nm. Functional titer was determined with plaque assay with an initial overnight infection of 293 cells.

Luciferase assay. Cell lines were plated, infected the next day, and washed once. After 24 h, the medium was removed, cells were lysed with 200 μL of Reporter lysis buffer (Promega), and freeze thawed once followed by luciferase assay (Reporter Lysis Buffer; Promega). Standardization was accomplished by comparing to CMV (set as 100%).

Gene expression analysis. Messenger RNA from sorted CD44+/CD24low-- JMT-1 cells was isolated using the RNeasy Mini RNA extraction kit (Qiagen). Expression was analyzed with the OneStep reverse transcription-PCR kit (Qiagen). Primers were as follows: ala, 5′-GGGCAACGAGCATCACAATAAT-3′ (forward) and 5′-CACAAGCAGACGACACTA-3′ (reverse); cox2, 5′-CTCTGTGTCTGTCGTGA-3′ (forward) and 5′-GGGTCAATGGAGGCCTGTGAT-3′ (reverse); hTERT, 5′-AACCTGGCAGCAAAAGA-3′ (forward) and 5′-GAGAGCTCTGCTGTGAT-3′ (reverse); mdr, 5′-GACTGAACCTTGGAGTGG-3′ (forward) and 5′-CACCAGAGAGCTGAGTCG-3′ (reverse); and β-actin, 5′-AAATGCGAGCTGGAGTGG-3′ (forward) and 5′-TCAACCTGGAGCAAAAG-3′ (reverse).

Oncolytic assay. Cell lines were infected for 1 h and incubated in medium with 5% fetal bovine serum, half of which was changed every other day. Cells were checked daily and when the most oncolytic virus seemed to have killed most of the cells at 1 VP per cell, MTS assay (CellTiter96 Aqueous One Solution Reagent; Promega) was performed.

In vivo analyses. Sorted JMT-1 cells (2 × 10^6) were injected into the topmost mammary fat pads of nude mice (18). Mice were infected with 1 mg/kg Estradurin (Pfizer) every 3 wk as reported (1). Intratumoral injections were performed with 10^6 VP thrice weekly for 5 wk. Tumor volume = length × width^2 × 0.5. Animal experiments were approved by the Provincial Government of Southern Finland.

Flow cytometry. Expression of CD44 and CD24 in tumors (n = 7 per group) was measured by flow cytometry after 17 d of treatment. Briefly, tumors were dissociated with collagenase (Blend type F; Sigma) overnight in +4°C. Cells (2 × 10^6) from each tumor were labeled with antibodies as above and samples were analyzed with FACsAria (Becton Dickinson).

Statistical analysis. The F-test was performed to see if there were differences between the oncolytic potency of the viruses in vitro. If the E1 was unequal distribution of the results, a two-sided Student's t test was used to assess significance, defined as a two-sided value of P < 0.05. In vivo, a nonparametric change-point test was used to determine a systematic change in the pattern of observations as opposed to chance. Proc Mixed (SAS v6.12; SAS Institute) was used to examine the effects of group and time on tumor growth. Pairwise comparisons were performed.

Results

Breast cancer cells were sorted for CD44 and CD24 and infected by viruses featuring TSPs (Fig. 1A). Mdr showed the strongest activity with 17.8% or 6% of the highly active but nonselective CMV promoter, respectively, in JMT-1 and fresh patient cells. hTERT also gave high activity at 5.1% and 12.8% of CMV, whereas Cox-2 was also active at 5.2% and 7.9%, respectively. Expression levels of ala were 0.4% and 1.9% of CMV and seem too low for successful control of oncolytic adenoviruses. RT-PCR analysis confirmed expression of genes that showed high luciferase expression (Fig. 1A, insert). However, correlation was not perfect between the assays suggesting that factors other than promoter activity may play a role in mRNA expression (e.g., mRNA stability factors present in untranslated regions).

For comparison, CD24+ JMT-1 cells were analyzed for TSP expression (Fig. 1A, center). The main difference was that mdr was expressed to lower and ala to higher degree than in the CD44+/CD24low population.

Capsid modified luciferase expressing viruses were used to investigate if gene transfer to CD44+/CD24low or CD24+ cells could be improved (Fig. 1B). 5/3 modification seemed the optimal capsid configuration as it achieved 10- and 100-fold higher gene transfer to CD44+/CD24low and CD24+ cells, respectively. These results prompted us to construct the respective oncolytic adenoviruses featuring the respective TSPs and incorporating the 5/3 capsid (Fig. 1C). In many cases, an Rb binding site deletion was included as our previous data suggests that this can improve selectivity without loss of efficacy (12).

When CD44+/CD24low cells fresh from pleural effusions were infected, we found that 5 of 3 modified viruses were more oncolytic than the wild-type Ad5 control (Fig. 2). In 2 of 3 samples, Ad5/3-mdr-Δ24 was most oncolytic, with Ad5/3-hTERT-Δgp and Ad5/3-CoxlL-Δ24 closely following. Impressively, despite including a TSP, these viruses were sometimes even more potent than the highly active Ad5/3-Δ24.

Adding the Rb binding site deletion to Ad5/3-mdr-E1A to make Ad5/3-mdr-Δ24 did not reduce the activity of the virus but actually increased potency. As expected from the luciferase data, the ala promoter-driven oncolytic virus did not show oncolytic activity. CD44+/CD24low cells fresh from pleural effusions did not propagate actively in vitro, which may support their “stemness”. Nevertheless, this precluded analysis of all viruses on all samples. For experiments that required large numbers of cells, the JMT-1 explant was used (Figs. 3–4).

To look at the kinetics of cell killing between the different viruses, JMT-1 cells were infected and daily cell viability assays were performed (Fig. 3A). Ad5/3-mdr-Δ24 and Ad5/3-hTERT-Δgp were the most effective TSP viruses and similar in the efficacy with the highly active Ad5/3Δ24, included as a positive control. Because probably not all CD44+/CD24low cells are “stem” cells (progenitors may be included), it is important to note that viruses were able to kill 100% of cells, therefore including also the actual “cancer stem cells.” As an alternative method for analyzing cell killing, trypan blue assays were performed as a function of time (Fig. 3B), with results quite similar to the MTS assay.

To analyze the effect of the viruses on CD44+/CD24low cells in vivo, tumors were allowed to establish, followed by intratumoral virus or mock injections every other day. In histologic analysis of mock-treated tumors, central necrosis was present in 20% of the tumor section, whereas the remainder seemed healthy. In Ad5/
3-mdr-Δ24-treated tumors, 50% of the section was necrotic and also non-necrotic regions displayed condensed nuclei, suggesting initiation of cell death (data not shown).

To assess antitumor efficacy, CD44+CD24-/C0-/low–derived tumors were established and injected with oncolytic viruses, all of which resulted in significantly smaller tumor size versus mock (all \( P < 0.001 \); Fig. 4A). Ad5/3cox2LΔ24 and Ad5/3-mdr-Δ24 showed the greatest antitumor efficacy and Ad5/3-mdr-Δ24 was superior even to Ad5/3-Δ24, a highly active positive control (\( P < 0.001 \)). Interestingly, despite promising activity in vitro, Ad5/3-hTERT-Δgp was less effective in vivo than Ad5/3-Δ24 (\( P < 0.001 \)).

Putative stem cells would be predicted to divide asymmetrically; each division producing one new stem cell and one progenitor. Progenitors would then divide to produce differentiated tumor cells. To assess this in a preliminary manner, tumors were induced by injection of 100% CD44+CD24-/C0-/low cells, and the proportion of CD44+CD24-/C0-/low cell was analyzed again when mice died or were killed. In mock-injected tumors, the proportion of CD44+CD24-/C0-/low cells had decreased from 100% to 6.2% (\( n = 2 \); SD, 6.8). In Ad5/3cox2L-Δ24–injected tumors, the proportion reduced to 1.2% (\( n = 1 \)), whereas in Ad5/3mdr-Δ24, Ad5/3-Δ24, and Ad5/3-hTERT-Δgp–injected tumors, the respective numbers were 3.8%
(n = 3; SD, 3.5), 1.2% (n = 3; SD, 0.4), and 1.6% (n = 1). The absolute number of CD44+/CD24−/low cells decreased from 2 million to a median of 669,000 (n = 8; SD, 1.2 million) in virus-injected tumors. However, because the number of tumors available for analysis was limited by practical aspects (duration of analysis and freshness of tumor), and the time points of analysis were not standardized in the efficacy experiment, we performed another set of experiments in more rigorous conditions (Fig. 4B and C). Tumors were established and treated with mock, Ad5/3mdr-Δ24 (able to kill CD44+/CD24− cells in vitro; Figs. 2–3), or Ad5/3-ala-Δ24 (not able to kill CD44+/CD24− cells in vitro) for 17 days, followed by fluorescence-activated cell sorting (FACS; Fig. 4B and C). Interestingly, the proportion of CD44+/CD24−/low cells was nearly identical [3.1% (SD, 1.35) versus 2.6% (SD, 1.17) in Ad5/3mdr-Δ24

Figure 2. Primary breast cancer pleural effusion samples from 3 different patients (A–C) were sorted for CD24-negative and CD44-positive cells and seeded at 20,000 cells per well on 96-well plates. After infection with promoter-driven oncolytic viruses, cells were checked daily by light microscopy, and MTS cell viability assay was performed when the most potent virus had lysed virtually all cells with the second lowest viral concentration. Results are presented as relative survival compared with mock-infected cells. Points, mean; bars, SE. *** P < 0.001 versus AdSwt.
versus mock-treated groups, and 3.3% (SD, 0.27) versus 3.4% (SD, 0.53) in Ad5/3-ala-Δ24 versus mock-treated groups, respectively; Fig. 4C).

Importantly, because Ad5/3/mdr-Δ24 reduced tumor size, it also reduced the total number of CD44+/CD24- cells (Fig. 4A–C). In contrast, Ad5/3-ala-Δ24 did not affect tumor size nor the proportion of CD44+/CD24- cells (Fig. 4B and C). In mock versus Ad5/3/mdr-Δ24–treated groups, other cell populations had larger differences: 5.4% (SD, 4.72) versus 2.3% (SD, 0.97), 9.2% (SD, 6.75) versus 4.3% (SD, 1.37), 82.3% (SD, 11.3) versus 90.8% (SD, 3.35) for CD44+/lowCD24-, CD44+CD24+, and CD44−/lowCD24−/low, respectively. In mock versus Ad5/3-ala-Δ24–treated groups, the proportions of these cell populations were similar: 4.3% (SD, 1.93) versus 4.3% (SD, 1.07), 6.8% (SD, 1.07) versus 5.6% (SD, 1.44), 85.5% (SD, 2.88) versus 86.8% (SD, 2.54), respectively.

**Discussion**

Previous work suggests that cancer stem cells may underlie the relapsing nature of advanced cancer (1–4). Eradication of these cells seems daunting as they are resistant to chemotherapy and radiation (5). Nevertheless, oncolytic viruses may have potential in this regard, as they are not susceptible to ion pumps or lack of cell cycling. Although oncolytic adenoviruses have been quite safe in the dozens of trials, most of them have been performed with relatively attenuated early generation agents (19). A new generation of infectivity enhanced, armed, and highly effective viruses is rapidly emerging, and the first such viruses have already been tested in patients with good safety.9 Nevertheless, preclinical data suggests that enhanced potency can sometimes result in toxicity, and therefore, improved control of virus replication may become clinically relevant (20).

TSPs may be a useful way to prevent expression of adenoviral E1A in nontarget tissues (6, 12, 16). Dual control may be particularly useful in the context of nontumor cells positive for the TSP. For example, normal tissue stem cells might express hTERT or mdr. Nevertheless, they would be expected to be intact in the Rb-p16 pathway, and therefore, Δ24-type viruses should not replicate in them.

Given the paucity of TSPs known to be active in putative cancer stem cells, we focused on a handful of promoters theoretically useful. Luciferase assays indicated that all of these promoters, except ala, were active in CD44+/CD24- cells. 5/3 chimerism was identified as the optimal capsid configuration, and oncolytic viruses were subsequently constructed (Fig. 1). The respective viruses were effective in killing CD44+/CD24- cells (Figs. 2–3). However, in the most stringent assay (Fig. 4), Ad5/3/mdr-Δ24 and Ad5/3-Cox2L-Δ24 emerged as the most promising agents, with

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9 A. Hemminki, personal communication.
Ad5/3-mdr-D24 perhaps holding the advantage given slightly superior data on clinical specimens (Fig. 2). Nevertheless, Ad5/3-Cox2L-D24 also had activity in clinical samples, especially at lower doses, which may be promising with regard to in vivo application.

Importantly, in vivo data suggested that asymmetrical cell division does occur. Furthermore, sorting and FACS data from treated tumors supports in vivo killing of CD44+CD24-CD0/low cells by promoter controlled oncolytic viruses. Because virus-treated tumors were much smaller than mock-treated tumors, and had a similar or even slightly smaller proportion of CD44+CD24-CD0/low cells (Fig. 4C), it seems likely that viruses were able to kill CD44+CD24-CD0/low cells in vivo, similarly to what was seen in vitro. If viruses would only be able to kill differentiated tumor cells, the proportion of CD44+CD24-CD0/low cells would be higher, not lower, in virus versus mock-treated tumors. Nevertheless, neither tumors nor CD44+CD24-CD0/low cells were completely eradicated in vivo and, thus, work remains.

Finally, it was interesting that despite 100% CD44+CD24-CD0/low cells injected, the proportion found in tumors was between 2.6% and 3.8% with or without treatment and regardless of how long injections were performed. This suggests that the number of CD44+CD24-CD0/low cells may determine tumor size and each initiator cell is surrounded by a fixed number of differentiated cells. Taken together, these results suggest that mdr, hTERT, and Cox-2 promoters are active in CD44+CD24-CD0/low breast cancer cells. Also, oncolytic adenoviruses controlled by these promoters seem to be able to kill CD44+CD24-CD0/low cells. Viruses such as Ad5/3-mdr-D24 and Ad5/3-Cox2L-D24 may be appealing for testing in breast cancer patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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