Expression-targeted gene therapy for the treatment of transitional cell carcinoma

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Targeted gene delivery for induced apoptosis of transitional cell carcinomas was carried out in vivo in mice via utilization of the murine cyclooxygenase type 2 (Cox-2) promoter (Tis10). MB49 cells, which constitutively overexpress Cox-2 like numerous other carcinomas, selectively expressed delivered genes that utilized this transcriptional control element. The products of the delivered genes were artificially inducible forms of caspases 3 and 9, which remained inactive until a chemical inducer of dimerization was later injected intraperitoneally. The genes were delivered intravesically as plasmids complexed with poly(ethylenimine). Significant improvements, in the form of reduced bladder mass, reduced tumor volume, anti-angiogenesis and inhibition of tumor growth were seen versus untreated or unactivated controls. In some instances, tumors were seen to go into complete remission. There were no apparent bystander effects associated with the treatments. This targeted gene therapy regimen could have wide applicability to numerous cancers due to constitutive overexpression of Cox-2.

Cancer Gene Therapy (2008), 1–10
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www.nature.com/cgt

Keywords: expression-targeting; gene delivery; bladder; cancer; Cox-2; caspase

Introduction

The practice of using promoters for targeted gene expression within specific cell types has been used in several laboratories for some time. Examples of promoters used and the intended cell targets include the cardiac troponin T promoter to target cardiac myocytes,1 the bovine opsin promoter for retinal targeting,2 and the SM22α promoter to direct transgene expression in smooth muscle cells.3 Enhancers have also been used both in the contexts of targeting specific cell types4 and raising the level of gene expression for plasmids that already employ a specific promoter for cell targeting.5 With such targeting technology in place, additional groups have targeted cancer cells with a similar strategy. This technique has been used for specific cancers such as ovarian (using the secretory leukoprotease inhibitor (SLPI) promoter)6 and pancreatic (using the promoter for Cox-2).7 Developing cancers have also been targeted, such as by using the enhancer for the Tie2 gene to target endothelial cells involved with angiogenesis.4

The cyclooxygenase enzymes participate in the metabolism of arachidonic acid, which is used by the body in the synthesis of prostaglandins. There are at least two types of cyclooxygenase in the body, with type 1 being constitutively expressed and type 2 being an inducible isomorf. Cyclooxygenase type 2 (Cox-2) mediates the production of prostaglandin-E2, which is associated with neoplasia through the promotion of cell survival, cell growth, migration, invasion and angiogenesis.8 A common avenue of research into cancer treatment has been to suppress the expression of Cox-2.9–11 This has been the rationale behind the use of the drug celecoxib (Celebrex™) for antitumor therapy.12–14 Although now thought to be an effective antitumor drug because of a Cox-2-independent mechanism,15,16 celecoxib was originally applied to cancer cells because of their elevated Cox-2 levels and the drug’s Cox-2-inhibitory actions. The mechanism of celecoxib is not at issue here, but rather the identification of elevated Cox-2 levels as a marker of carcinoma cells but not untransformed cells. It has been shown repeatedly that the Cox-2 gene is overexpressed in many types of cancers, including dozens of examples taken from esophagus,17 gastric,18 colorectal,19 and bladder20–22 tissues, but its expression is weak-to-undetectable in normal cells from the same tissues.17–21 We have also shown this to be the case in the murine system to be
described here, which uses transitional cell carcinoma MB49 cells as the model cell line. These facts are keys that will be utilized for expression-targeted gene delivery to Cox-2-overexpressing tumors. It was not our aim to halt this natural cellular process, but rather to use it to our advantage by creating a new downstream result of Cox-2 overexpression in cancer cells.

One desired effect of cancer treatment is tumor cell death. To induce this response, this investigation utilized mediators of cellular apoptosis. Cells undergoing apoptosis activate a cascade of suicide genes to effect their own self-destruction while packaging their contents so that neighboring cells remain unaffected. A lack of response in neighboring, healthy, untransformed cells was another goal of the described experiments. Because apoptosis is an intracellular event with vesiculized degradation products, it is a promising choice for singly instituting the two desired outcomes of cancer cell death with no bystander effect.

To achieve cellular apoptosis, sequences that code for members of the apoptotic cascade were used as the exons of delivered plasmids. The apoptosis cascade itself is made up primarily of proteins known as caspases. The higher numbered caspases, 8 (FLICE), 9 (Apaf-3) and 10 (Mch4) tend to be initiators, while the lower numbered caspases 1 (ICE), 2 (ICH-1) and 3 (CPP32, Yama, Apopain) are typically effectors. Both initiators and effectors have been used in transfection experiments in an attempt to induce apoptosis. Each of the caspase proteins has the potential for use as an anticancer agent.

Modified, inducible forms of caspases 1, 3 and 9 (iCasp1, iCasp 3 and iCasp9, respectively) also exist. The activators of these molecules are non-toxic, lipid-permeable, chemical inducers of dimerization (CID) that can be used both in vitro and in vivo. The CID acts to create homodimers of the modified caspases, with subsequent activation that allows them to commence the apoptotic process from their respective positions in the cascade. The molecule AP20187 (ARIAD, Cambridge, MA, USA) is an effective inducer of iCasp1, 3 and 9. The modified forms of caspases 3 and -9 (iCasp3 and iCasp9) are experimentally valuable because of the definitive controls they present in the absence of an activator. This inducible protein motif has been used in other situations to help demonstrate mechanisms behind degenerative diseases such as heart and liver failure.

### Materials and methods

All animal procedures were reviewed by the Tulane University Institutional Animal Care & Use Committee (project number 0273-UT-D).

#### Creation of tumor detects

The instillation of the bladder tumor model was performed as described in detail by Gunther et al. Following anesthesia with a combination of Ketamine (18.2 mg kg⁻¹), Xylasine (20 mg kg⁻¹) and acepromazine maleate (0.2 mg kg⁻¹), the backs of C57-B16 mice were shaved and coated with electroconduction gel. Prolonged anesthesia was maintained by inhalation administration of isoflurane. Each mouse was placed on its back onto a metal grounding plate and catheterized with a lubricated 24-gauge iv catheter to drain the bladder. A burn injury was delivered to the bladder by inserting a platinum wire into the catheter in such a way that there was exposed wire on each end of the catheter (the catheter serves as an insulator for the middle portion of the wire, which extended the entire length of the urethra). A 2.5 W current was delivered to the bladder by touching the exterior exposed portion of the wire with a Bovie electrocautery unit (Bovie Medical Corporation, St Petersburg, FL, USA), set for coagulation, for less than 1 s. The catheter and wire were then moved to a different portion of the bladder and the electrocautery was repeated one time. The wire was then removed, the mouse back was cleaned with water and the animal was placed on its back on a heating pad to help maintain body temperature during the procedure. A suspension of 1 × 10⁶ MB49 murine bladder tumor cells, with a concentration of 1 × 10⁶ cells ml⁻¹ (100 µl total volume), was instilled into the bladder under separate catheterization. The catheter was blocked to allow the cell suspension to remain inside the bladder for 90 min to allow for cellular attachment to the (dorsal) burn sites. After 90 min the catheter was removed and the bladder allowed to drain naturally.

#### Cancer treatment procedure

The transfection complexes were constructed prior to administration in a biological safety cabinet to preserve sterility. The base amount of transfecting DNA, hereafter referred to as ‘1 ×’, was 3.6 µg total/dose, split evenly between the inducible initiator and effector caspases already mentioned (1.8 µg of Cox-2-driven iCasp3 and 1.8 µg of Cox-2-driven iCasp9). The DNA was complexed with poly(ethyleneimine) (PEI) as previously described using a 7.5:1 PEI nitrogen to DNA phosphate ratio, in 100 µl total volume with 0.9% NaCl. For example, to create exactly 10 doses of 1 × transfection solution, a solution containing 18 µg of Cox-2-driven iCasp3 plasmids in 250 µl total volume was formulated in a poly(styrene) tube, and a solution containing 18 µg of Cox-2-driven iCasp9 plasmids in 250 µl total volume was formulated in a separate tube. Stock solutions of 0.22 µm-filtered PEI (1.75 mM amine) were used to add 250 µl (~438 nmoles amine) of PEI to each DNA-containing tube. The two tubes contained 500 µl of iCasp3 and iCasp9 transfection complexes, respectively. The contents of the two tubes were combined prior to transport to the animal procedure room to create 10 doses (1000 µl) of iCasp3 + iCasp9 transfection complexes.
transfection solution, followed by lubrication of the catheter for ease of insertion. The mice were catheterized transurethrally and the transfection complexes were delivered via the syringe. The transfection solutions remained in the anesthetized mice for 2 h before removal of the catheters, after which the bladders drained immediately. The mice were then permitted to recover from the anesthetic on their own.

In addition to the transfection procedure just described, the mice underwent daily injections of an activator solution following the first transfection day. The products of the delivered genes were inactive forms of caspases 3 and 9, which were made active following exposure of a chemical inducer of dimerization (AP20187, ARIAD, Cambridge, MA, USA). The activator was delivered intraperitoneally, in a 100 μl bolus consisting of 2 mg kg⁻¹ AP20187 suspended in poly(ethylene glycol) (PEG 400) and 2% Tween 20 (1.72% final). The dose volumes were approximately 5 ml kg⁻¹ (based upon 20 g mice), which dictated that a 0.4 mg ml⁻¹ dosing solution be formulated.

The entire treatment calendar consisted of instillation of the tumor cells on day 0, a 7-day incubation period to allow the establishment and development of tumors (which was verified by ultrasound), and gene therapy treatments beginning on day 7 and repeated every 3 days until day 22, for a total of five transfections/mouse. Activator solutions were administered daily beginning on day 8. The mice were euthanized on day 22 via pentobarbital overdose.

Ultrasound
Each mouse was anesthetized with isoflurane, followed by the shaving (clipping) of hair from the abdomen. Bladders were catheterized transurethrally and filled with sterile 0.9% NaCl for improved imaging quality. Ultrasound-conducting gel was then applied to the abdomen and 2-D ultrasound plus color and pulsed-Doppler readings were gathered primarily via a Sonosite 180 Plus (Sonosite, Bothell, WA, USA) portable ultrasound system, followed by catheter removal and weighing of the animals.

Real-time PCR
Bladders were dissected and stored in liquid nitrogen before use. Total RNA extraction was performed using the RNeasy mini kit (Qiagen Inc., Valencia, CA, USA), and the first strand cDNA synthesis was performed using the iScript™ Select cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Real-time PCR analyses were performed using an iCycler (Bio-Rad, Hercules, CA, USA) in a total volume of 25 μl that contained 12.5 μl qPCR mastermix (Eurogentec, San Diego, CA, USA), 200 nM forward and reverse primers, 300 nM Taqman probes and 1 μl template. (All primers and probes were supplied by Integrated DNA Technologies, Coralville, IA, USA.) Reactions were incubated at 95 °C for 3 min, amplified by 40 cycles of: melting at 95 °C for 10 s, annealing at 58 °C for 30 s and extension at 72 °C for 45 s. The housekeeping gene GAPDH (Genbank, locus: BC020407, accession: BC020407) was used as an internal reference.

The sequences of the primers and probe for the detection of murine Y chromosome (Locus: MMYORF Accession: X05260) were as follows:
- Forward primer: 5'-TTTTGCTCCCATAGTAGTAT TT CCT-3'
- Reverse primer: 5'-TGTA CCGCT CTGCC AACC A-3'
- Taqman probe: 5'-CY5™-AGGGATGCCCACCTCG CCAGA-BHQ2™-3'

Quantification was performed using the efficiency-corrected relative quantification method.33

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\text{Ratio} = \frac{(E_{\text{target}})^{\Delta \text{Ct}_{\text{target}}}(\text{control} - \text{sample})}{(E_{\text{reference}})^{\Delta \text{Ct}_{\text{reference}}}(\text{control} - \text{sample})}
\]

‘Ratio’ represents the relative Y chromosome level of the sample as compared to that of the control. ‘Target’ refers to murine Y chromosome expression. ‘Reference’ is GAPDH expression. ‘E’ is the amplification efficiency of each primer set. As the negative control (‘control’), we used the normal untouched bladder from C57-Bl6 mice, and ‘sample’ refers to the tumor bladders with or without treatment as indicated. Note that when a gene was not detected by PCR in negative control samples, the value 41 (total number of PCR cycles +1) was used to permit calculations with the above equation; when non-detection occurred elsewhere, the ratio was defined as zero.

Statistics
Groups of data were analyzed by single-factor analysis of variance (ANOVA). For pairwise comparisons, the F test was used to determine whether a given pair of population variances was equal (α = 0.05). This information was then used in designating the appropriate t-tests (typically heteroscedastic) to perform for comparing the means of population pairs. Significantly different pairs were defined as having \( P < 0.05 \)

Results
Selection of inducible caspases
In previous work, in vitro experiments were performed with various combinations of inducible caspases to determine the most effective caspase or caspase combination for obtaining apoptosis of the tumor cells to be used for in vivo investigations.33 Cell survival data indicated that using a combination of caspase genes is more effective than delivering the gene for a single caspase in bringing about cell death. Among the combinations of caspases, the use of an initiator caspase (caspase 9) with an executor caspase (caspase 1 or 3) tended to yield fewer surviving cells. The use of caspase 9 with caspase 3 was selected over its use with caspase 1 because of slightly (although not significantly) lower survival rates post-transfection, plus the fact that caspase 1 is associated with the production of pro-inflammatory cytokines,34 which could lead to unwanted side effects in vivo.

Validation and use of ultrasound
To reduce the number of mice required for the investigation, ultrasound was used over the course of the treatment
period to monitor tumor sizes within the bladders. Tumor volumes were calculated using \((\text{length} \times \text{width} \times \text{height}) \times 0.52\), which assumes an ellipsoid tumor shape. Figure 1 is a comparison of tumor volumes taken via ultrasound measurements or via necropsy. Comparison of panels (b) and (c) (ultrasound versus necropsy) yielded similar data between the two data collection methods, illustrating that the ultrasound method is a reliable means of obtaining general trends in tumor growth. It can be seen that even at 14 days after introduction of the carcinoma cells into the bladder (7 days after treatments have commenced), there are trends among the tumors that were receiving the gene constructs plus post-translational activation versus tumors that received no or incomplete treatments. These trends became more exaggerated at 21 days postinoculation (14 days after treatments commenced).

Ultrasound and Doppler data were collected throughout the investigation. Figure 2 illustrates typical tumor progressions at day 7, just before commencement of treatment regimens. Tumor volumes were generally on the order of 10\(^3\) mm\(^3\), and were subjectively scored by apparent size (0–3) to allow for equal distribution of tumor sizes for each treatment group before the first doses of drug were given. Figure 3 depicts typical data collected at day 14 (after 1 week of treatments). In the mice that were sham-treated, and the mice that received the \(1 \times \) formula without post-translational activation, growth of transitional cell carcinomas into the bladder lumens can be noted on 2-dimensional and color power Doppler. The color power Doppler was performed to demonstrate that the luminal masses were tumors by virtue of blood flow, and the flow detected via the color power Doppler was verified to be blood flow via further Doppler readings that revealed rhythmic pulsations to the flow patterns. There were few if any tumors visible via Doppler at the 14-day time point in the mice that received both \(1 \times \) treatments with subsequent post-translational activations. The only flow associated with these bladders, as detected by color power Doppler, was due to normal extraluminal bladder vasculature. These observations were supported by findings taken upon necropsy on day 22 (Figure 4). In selected instances, tumors seen via ultrasound at day 14 were not detected at day 21 in mice receiving the \(1 \times \) transfection dose concentration with subsequent post-translational activation.

**Direct observations**

One symptom of tumor progression was hematuria, presumably related to rapid angiogenesis within developing tumors and exacerbated by repeated catheterizations. It was consistently noted that the urine of untreated tumor-bearing mice contained blood by day 14, as did the urine of tumor-bearing mice that received transfections without subsequent activation. The concentration of red blood cells appeared to increase over the course of the experiment in these mice, and the volumes of urine collected diminished. Tumor-bearing mice having received transfections with subsequent dimerization of the delivered gene products displayed lower levels of blood in their urine, with those having received the \(1 \times \) dose concentration containing less blood than mice receiving...
the $2 \times$ dose concentration. It was occasionally observed that mice receiving the $1 \times$ transfections plus CID had hematuria on day 14 that eventually disappeared by day 21.

Mice were euthanized on day 22, and their bladders were photographed in situ, excised, bifurcated to allow for the drainage of any urine, photographed again, weighed and cryopreserved for future total RNA extraction for real-time PCR. The gross findings for the different bladder groups were consistent, with cancer-inoculated bladders from the groups having received transfections at the $1 \times$ dose concentration with subsequent activation having an appearance similar to that of normal controls, and cancer-containing bladders having received regular transfections at $1 \times$ or $2 \times$ dose concentrations without post-translational activation having an appearance similar to that of untreated, tumor-containing controls (Figure 4). Normal and fully treated bladders were translucent, had luminal capacities of approximately 150 μl and had discrete extraluminal vascularization. There was a noticeable white characteristic to the walls of $1 \times$ - and $2 \times$-treated tumor-inoculated bladders,
similar to sham-treated negative controls, that was presumably due to fibrous tissue resulting from repeated stretching of bladders during the transfection process and was not considered significant (sham data not shown). Small tumor masses were occasionally observed on the dorsal aspects of tumor-inoculated bladders that received the 2× dose concentration with subsequent CID administrations (Figure 4b). Bladders from tumor-inoculated mice that received either no treatment or transfections without subsequent post-translational activations were strikingly different from the fully treated bladders, with larger sizes, distinct rubor from tumor angiogenesis, greater mass and virtually no lumina. They did not lay flat following bifurcation as did their normal or fully treated counterparts due to the mass of the enclosed tumors, and often contained necrotic regions characteristic of rapid tumor growth.

Statistical analysis of bladder weights shown in Figure 5 revealed that there was no significant difference between the mean weights of tumor-inoculated bladders that received either no treatment, or transfections with the 1× or 2× transfection solutions without subsequent caspase activation (ANOVA, P = 0.703). Analysis of wild-type bladders and tumor-inoculated bladders that also received transfections with the 1× or 2× transfection solutions plus subsequent caspase activation also showed no significant difference between mean bladder weights, although the P-value approached significance due to the inclusion of suboptimal 2× dose concentration data (ANOVA, P = 0.055). F test analysis showed that

**Figure 4** Bladders as they appeared upon necropsy. (a) Bladders receiving the full treatment appeared similar to normal controls, while transfections without subsequent activation of the gene product were distinctively red from angiogenesis, and full of tumor or tumor by-products. The green arrow in panel (b) indicates tumor tissue on the dorsal side of the bladder (which was rotated 90° for the picture).

**Figure 5** Final bladder weights. Data were obtained on day 22 upon necropsy. *Significant difference between indicated groups (n ≥ 4, P = 0.044). See text for an in-depth statistical analyses. ‘Normal’, bladders without tumor inoculation; ‘1×’ (or ‘2×’), bladders that were treated with the 1× (or 2×) drug concentration (see text), with or without post-translational activation. Transfections without activation. Transfections with post-translational activation. ‘Untreated’, tumor-inoculated bladders, which did not receive treatment.

wildtype and 1×-treated (plus CID administration) groups had equal variances, and t-test analysis of these two groups showed no difference between the means (n ≥ 4, P = 0.420). The same analyses were run to compare wildtype and 2×-treated (plus CID administration) groups. The F test revealed unequal variances, and
although there was not a significant difference between the means of the two groups, this statement was made with less confidence than for wild-type versus the 1× (with CID) group \( n \geq 5, P = 0.064 \).

Comparison of the bladder weights of the 1×-treated tumor-inoculated bladders, with versus without post-translational activation of the inducible caspases, revealed a significant difference between the means \( n \geq 4, P = 0.044 \). The same manner of comparison for the 2×-treated tumor-inoculated bladders, with versus without product activation, did not reveal a significant difference between the means \( n \geq 4, P = 0.144 \), indicating that this dose concentration was less optimal than the 1× treatments. Concentrations of 4× treatments were also tested in initial trials, but such a high drug concentration proved to be toxic to normal tissue and the trials were immediately halted. The toxicity was presumably due to high PEI concentrations, and is addressed in the Discussion section.

**Real-time PCR**

The MB49 tumor cell line used to establish the tumor model originated from a male mouse. Since the mice used for the gene therapy experiments described herein were all female, the presence of tumor cells could be evaluated on a molecular level via real-time PCR using a primer/probe set specific for the Y chromosome.\(^{15}\) Figure 6 indicates the effectiveness of the treatments, with tumor-bearing bladders having received treatments with subsequent post-translational activation displaying Y chromosome levels that were not significantly different from normal mice (ANOVA, \( P = 0.45, n \geq 3 \) for each group). Direct comparison of wildtype and 1×-treated tumor-bearing bladders having received post-translational activation also displayed no significant differences between the two groups \( t\text{-test, } P = 0.36, n \geq 3 \). It should be noted that those bladders from mice receiving the 2× treatments plus activation tended to display a greater relative amount of Y chromosome present than both the 1×-treated and wild-type groups (186.8 versus 78.5 and 0.0, respectively). Untreated, tumor-inoculated bladders were found to contain significantly more relative Y chromosome (4292.2) than bladders having been treated with the 1× dosage with subsequent activation \( (t\text{-test, } P = 0.03, n \geq 3) \).

**Discussion**

For years the knowledge that Cox-2 overexpression is associated with tumor growth has driven research into the use of nonsteroidal anti-inflammatory drugs to slow tumor progression.\(^{36}\) The design of the gene therapy system described herein is not geared toward suppressing the overexpression of Cox-2 in developing transitional cell carcinomas, but rather celebrates it by capitalizing on the event to drive the transcription of delivered pro-apoptotic genes. The system has been used in in vitro settings for a variety of human cancers, with excellent effects seen for the induction of apoptosis in human bladder, prostate and breast cancer cell lines.\(^{32}\) The current investigations are an application of the system to an in vivo murine model, with modifications to the original plasmids incorporating the murine form of the Cox-2 transcriptional control sequence.

The reported data show that the growth of MB49 transitional cell carcinomas can be significantly inhibited by the described method of transfection and subsequent post-translational activation. This inhibition is due to the induction of apoptosis via expression of the delivered caspase genes followed by activation of the inducible gene products, as has been shown via expression of GFP-iCaspase fusion proteins plus positive TdT-mediated dUTP nick end labeling assays performed on cells transfected in culture.\(^{22}\) Although both the 1× and 2× treatment regimens, with subsequent activation of the translated gene products, showed marked reductions or reductions of tumor growth, bladder weight increase and angiogenesis versus untreated and unactivated controls, statistical analyses indicated that the 1× dose concentration was the optimum of the formulations tested.

There seems to be a trend to higher tumor volumes/weight in the group of mice receiving 2× dose concentrations. This was a phenomenon that was continued for mice receiving the 4× dose concentration as well. At the 4× concentration (and the 2× concentration, to a lesser extent), the gene delivery complexes were found to be cytotoxic without caspase activation, and this cytotoxicity was not targeted to only cancer cells. We believe that the levels of PEI in the transfection solutions were responsible for the non-specific cell death. We hypothesize that the cell death opens up space in the bladder luminal surface, which could be filled by normal or cancerous cells, and that the carcinoma cells out-compete the normal cells for the space. It has been our experience that MB49 cells detach easily in culture, and that the detached cells can be used to establish new colonies in vitro (data not shown). The 4× drug concentration yielded vast amounts of tumor

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**Figure 6** Detection of tumor cells via PCR. The relative amount of Y chromosome present in whole-bladder preparations of tumor-bearing mice that received treatments with subsequent post-translational activation versus normal mice was not significantly different (ANOVA, \( P = 0.45, n \geq 3 \) for each group) (—). Untreated, tumor-inoculated bladders were found to contain significantly more Y chromosome than bladders treated with the 1× dosage with subsequent activation \( (t\text{-test, } P < 0.05, n \geq 3, \text{labeled with } *\text{).} \text{ The } 1\times\text{-treated bladders were not found to be different from normal controls } (t\text{-test, } P = 0.36, n \geq 3)\).
progression, angiogenesis, and hematuria and experiments with this concentration were halted early because of animal suffering. The results of the limited $4 \times$ drug trials could explain why the $2 \times$ drug concentration produced poorer results than the $1 \times$ dosages. (A $0.5 \times$ concentration has also been investigated, but limited effectiveness was observed in in vitro trials.)

It should also be noted that in a few instances, tumors that were apparent via ultrasound in the middle of the time course were not found upon necropsy or real-time PCR performed on cDNA from total RNA of whole bladders. These encouraging instances of tumor regression occurred in mice receiving $1 \times$ dose concentrations with subsequent activation.

While not the aim of these investigations, there was a positive side effect of a reduction in angiogenesis in transsected and activated bladders. It has been shown repeatedly that Cox-2 expression is responsible for the angiogenesis associated with numerous types of cancer, including colon, breast, gastric, uterine/cervical, Wilms’ Tumor and Hodgkin’s lymphoma. In the investigation presented here, the expression of Cox-2 was indirectly lowered by the selective death of cells that overexpressed Cox-2 in the bladder. This was also seen in cases where tumors were allowed to develop for extended periods before the gene therapy regimen commenced (data not shown). Although exposed to only a limited number of treatments, these bladders lost their red appearance, both inside and out, due to a reduction in blood within the bladders. Investigations into the phenomenon are currently underway.

A weakness of the gene therapy system presented lies in the fact that normal tissues will express Cox-2 as part of the inflammatory process. It could be argued that if the plasmids were delivered systemically to a person with inflammation somewhere in the body, then apoptosis of non-cancerous tissue could be induced in the inflamed area. While the described investigations did not involve systemic gene delivery, two counterpoints can be made to the argument based on existing data. First, in a previously published report, we investigated the effects of the gene therapy system in vitro on fibroblasts that were expressing Cox-2. It was found that the amount of Cox-2 expressed by the fibroblasts after exposure to temperature changes, pH changes, plus chemical and mechanical shocks was enough to detect via PCR but was not enough to spark therapeutic levels of transcription of the delivered Cox-2-directed genes for apoptosis. Second, in the current investigations, there was necessarily some trauma introduced via the repeated catheterizations and bladder distensions that are part of the treatment regime. However, the mice receiving the optimal treatment regimen behaved similarly to normal, untreated mice in terms of food and water consumption and activity levels. Necropsy did not show gross differences in $1 \times$-treated and activated versus wild-type bladders, other than some fibrotic tissue that is often associated with repeated bladder distensions. Further investigations into possible inflammatory effects of the treatment are underway in the corresponding author’s laboratory.

Other laboratories have investigated gene therapy methods for the treatment of transitional cell tumors in orthotopic and subcutaneously implanted models. One treatment employed the use of replication-deficient adenoviruses to deliver the murine CD40 ligand, which led to tumor regression in a subcutaneous model. However, when this treatment was applied to an orthotopic model, the infectivity of the adenovirus was reduced by the glycosaminoglycan layer within the bladder, so the enhancer of transduction Clorpactin was used to improve transduction efficiency. Liposomes containing DOTAP (N-[1-(2,3-DiOleoyloxy)propyl]-N,N,N,N-trimethylammonium methylsulfate) have been used to deliver genes coding for cytokines IFNα and GM-CSF (granulocyte macrophage colony-stimulating factor) to treat murine bladder cancer. Unlike the experiments described here, the investigation did not utilize a targeting method. Research closer to the method presented in this paper utilized a Cox-2 promoter-based replication-selective adenoviral vector to target subcutaneously implanted bladder cancer cells. The viral vectors were intratumorally injected, so possible interference to gene delivery by native uroplakins was not addressed. As already mentioned, membrane-associated glycosaminoglycans can negatively affect infectivity.

In conclusion, the experiments presented here have shown that delivery of genes coding for inducible caspas 3 and 9 under the control of the Cox-2-promoter, with post-translational activation of the gene products, has therapeutic benefits in an orthotopic murine model of transitional cell carcinoma. Benefits were seen grossly in terms of reduced bladder masses, reduced tumor volumes and the non-emergence of hematuria. Molecular analyses of the presence/absence of tumor cells via markers for the Y chromosome proved that there was a significant restriction of tumor growth. Anti-angiogenic effects were a positive side effect. The described treatment regimen has applicability to a wide array of cancers, since Cox-2 overexpression is a prevalent characteristic of many tumors.

Acknowledgements

We thank George Taylor, MD, and Patricia Dunning, RT(r), for assistance with ultrasound experiments, Carol Pilbeam, PhD for providing the TIS10 (murine Cox-2) promoter, ARIAD (www.ariad.com/regulationkits) for providing significant amounts of AP20187, and John C Prindle Jr, PhD for numerical discussions. This work was funded, in part, by the Louisiana Board of Regents (LEQSF(2004-07)-RD-A-28).

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