Abstract. Background: Adeno-associated virus (AAV) vectors have significant potential as gene delivery vectors for cancer gene therapy. However, broad AAV2 tissue tropism results in nonspecific gene expression. Materials and Methods: We investigated use of the C-X-C chemokine receptor type 4 (CXCR4) promoter to restrict AAV expression to tumour cells, in subcutaneous MCF-7 xenograft mouse models of breast cancer and in patient samples, using bioluminescent imaging and flow cytometric analysis. Results: Higher transgene expression levels were observed in subcutaneous MCF-7 tumours relative to normal tissue (muscle) using the CXCR4 promoter, unlike a ubiquitously expressing Cytomegalovirus promoter construct, with preferential AAVCXCR4 expression in epithelial tumour and CXCR4-positive cells. Transgene expression following intravenously administered AAVCXCR4 in a model of liver metastasis was detected specifically in livers of tumour bearing mice. Ex vivo analysis using patient samples also demonstrated higher AAVCXCR4 expression in tumour compared with normal liver tissue. Conclusion: This study demonstrates for the first time, the potential for systemic administration of AAV2 vector for tumour-selective gene therapy.

Breast cancer is the leading cause of cancer-related mortality in middle-aged women despite significant advances in conventional therapies (1). The prevalence of locoregional recurrence and distant metastasis can be as high as 13% and 10% respectively despite improvements in adjuvant treatment e.g. chemoradiotherapy and hormone therapy. The main stumbling block with both chemotherapy and radiotherapy is the induction of dose-limiting normal tissue toxicity, which reduce their clinical effectiveness. Liver metastases are present in 15% of metastatic breast cancer patients and are the only site of distant disease in one-third of these patients. Therefore, targeted treatment of localized liver disease would have the potential to improve outcome in a significant proportion of these patients. Currently, surgical resection is the treatment of choice for isolated liver disease. The liver’s unique anatomy presents numerous challenges when evaluating for surgical resection, resulting in fewer than 20% of liver tumours being suitable for surgical resection, and up to 90% of unresected cases undergoing chemotherapy fail to maintain long-term remission and die from liver failure. These failings warrant new treatment options for this subset of patients.

Gene therapy could potentially offer a safe and effective treatment modality in such patients. An ideal delivery system in patients with liver disease should offer effective transduction in the liver with prolonged gene expression restricted to infiltrative tumour cells only. Furthermore, it requires the capacity to be delivered by both local and systemic routes for isolated deposits and disseminated disease respectively, minimal expression in non-target organs and an acceptable safety profile. The use of an adeno-associated virus (AAV) approach has a number of features that make AAV an ideal vector for gene therapy strategies: it offers long-term gene expression, there is little or no cell-mediated immune response to the virus, and it is not associated with any disease (2). While AAV-derived vectors have shown promise in many clinical trials to treat a number of non-malignant conditions (3-6), their use has not been examined widely in cancer settings (7-9). The suitability of AAV for tumour treatment lies in its ability to efficiently and stably transfect a wide range of cells, including dividing and non-dividing cells. It also has the ability to penetrate the stroma of solid tumours due to its small size and can offer an excellent safety profile combined with reduced potential for activation of inflammatory or cellular immune responses.
AAV2 serotype was the first parvovirus to be isolated from humans and the first to be used as a vector for gene therapy application (6). AAV2 has a broad tissue tropism due to the wide expression of AAV receptors in all tissues (10). This would enable the targeting of systemically disseminated disease and, furthermore, AAV vectors have been shown to direct stable gene transfer and expression in hepatocytes. This feature makes them an attractive tool for treatment of liver metastatic disease.

Currently, tissue targeting using AAV is mainly achieved by the localized delivery of treatments, and/or use of different, more recently identified serotypes with restricted tissue tropisms (11, 12). However, local delivery can only be achieved in limited situations such as for superficial deposits, skin and muscle, or may require invasive means for access. The most applicable treatment strategy for liver and distant metastasis is via systemic delivery. Yet the broad host spectrum of AAV may limit its systemic use due to transgene expression in normal tissue.

This paper studies the use of transcription targeting for AAV2 to restrict transgene expression to tumour cells, thereby sparing normal tissue, an outcome that conventional therapeutic approaches have failed to achieve. Transcription targeting using specific promoters is one of the oldest and most widely used strategies for targeting gene therapy (13, 14). Tumour-specific promoters (TSP) restrict the expression of genes of interest to tumour cells (15). Numerous TSPs have been applied in preclinical studies, including alphafetoprotein, carcinoembryonic antigen, survivin, C-X-C chemokine receptor type 4 (CXCR4) and osteocalcin (14). However, their use has not been investigated in AAV as widely as in adenoviruses. CXCR4 is a chemokine receptor that has been shown to be expressed at high levels in many types of cancer, including breast cancer, but is repressed in normal tissue (16, 17). CXCR4 regulates the growth of primary and metastatic tumours and tumour cells are believed to adopt the expression of chemokine receptors to facilitate metastatic spread through chemokine gradients (18, 19).

The aim of our study was to develop a strategy to increase efficiency and specificity of tumour gene delivery. This paper incorporates the use of the CXCR4 as a TSP in an AAV vector and investigates its tumour selective potential in models of both primary and metastatic breast cancer.

Materials and Methods

Cell culture. The human adenocarcinoma breast cancer cell line, MCF-7 (ATCC, Manassas, VA, USA) was maintained in Dulbecco’s minimal essential medium (DMEM) (GIBCO, Invitrogen Corp., Scotland) supplemented with 10% foetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and sodium pyruvate and grown at 37°C in a humidified atmosphere of 5% CO2.

Animals and tumour induction. All murine experiments were approved by the Ethics Committee of University College Cork. Mice were obtained from Harlan Laboratories (Oxfordshire, UK). They were kept at a constant room temperature (22°C) with a natural day/night light cycle in a conventional animal colony. Standard laboratory food and water were provided ad libitum. Before experiments, the mice were afforded an adaptation period of at least 7 days. Female mice in good condition, without fungal or other infections, weighing 16-22 g and of 6-8 weeks of age, were included in experiments. For routine tumour induction, 2×10⁶ tumour cells, suspended in 200 μl of serum-free DMEM were injected subcutaneously into the flank of female MF1nu/nu mice. The viability of the cells used for inoculation was greater than 95% as determined by Nucleocounter (ChemoMetec, Bioimages Ltd, Cavan, Ireland). Following tumour establishment, tumours were allowed to grow and develop and were monitored twice weekly. Tumour volume was calculated according to the formula $V = \frac{ab^2}{6}$, where $a$ is the longest diameter of the tumour and $b$ is the longest diameter perpendicular to diameter $a$. When tumours reached approximately 100 mm³ in volume, the mice were randomly divided into experimental groups.

Induction of hepatic metastases. Isolated MCF-7 liver tumours were induced in MF1nu/nu mice as described elsewhere (20, 21). Briefly, mice were anaesthetised using 100 μl intraperitoneal injection of phosphate-buffered saline (PBS) containing 1.5 mg ketamine hydrochloride (Vetoquinol, Dublin, Ireland) and 300 μg xylazine (Chanelle, Loughrea, Ireland) and placed on a heating pad. The abdominal skin was disinfected with 10% w/w iodinated povidone antiseptic skin cleaner (Videne, Ecolab, Leeds, UK). A 1-1.5 cm incision was made in the left subcostal margin. The abdominal muscles were divided, the peritoneum opened and the abdomen entered. The spleen was gently mobilised and the mid-body of the spleen divided between two 4/0 Vicryl ligatures (Ethicon, Johnson & Johnson, Berkshire, UK) placed between splenic vascular pedicles, such that each had its own vascular pedicle and was still in contact with the portal circulation. MCF-7 cells ($5\times10^5$) were injected into one hemic-spleen, which was removed 10 minutes later. The other hemic-spleen was returned intact, leaving the mouse with half an immunologically competent spleen. Mass closure of peritoneum and muscle was performed using 4/0 PDS (Ethicon) and the skin closed using 4/0 Prolene (Ethicon). Animals were fed a moistened diet for 24 hours. Carprofen (5 mg/kg, Norbrook laboratories, Newry, UK) was used for postoperative analgesia. Animals were observed for the development of disease and treated at day 7 post inoculation.

Creation of splenic port. The use of a splenic port for hepatic delivery has been described previously (22). Liver metastases were induced as described above. For this model, the intact hemic-spleen was transposed to a subcutaneous position while carefully preserving its vascular pedicle. The defect in the abdominal wall was reapproximated using 4/0 PDS to hold the spleen in position, over which the skin was closed. Postoperative recovery was as above. The subcutaneously placed hemic-spleen was easily visible and palpable for direct delivery into the portal circulation.

Production of AAV vectors. A schematic representation of the relevant elements of the vector constructs utilised is shown in Figure 1. AAV-2 CMV expressing firefly luciferase was generated as
described in (7). Firefly luciferase-expressing CXCR4 AAV-2 vector was generated as follows: pDriveCXCR4LucZ plasmid was purchased from Invivogen (Cayla SAS, Toulouse, France). The CXCR4 promoter DNA sequence was PCR amplified using primers designed with MluI and EcoRI restriction overhangs, (forward: MluI5' CATACGACGGTGTTACAAATTCGCA1000GTCG3', reverse: EcoRI 5'CGCAAAGCTTGATTGTTCTCCAAGAT3') and cloned into pAS (AAV-luc). This CXCR4 promoter DNA sequence was inserted upstream of the firefly luciferase gene. Thus the levels of luciferase expression reflect the activity of the CXCR4 promoter. Clone sequence was validated by sequencing (MWG Biotech, Ebersberg, Germany) and restriction enzyme analysis. AAV particles were generated using the AAV Helper-Free System (Stratagene, Agilent, Dublin). AAV particles were purified using Virakit AAV Purification Kit (Virapur, San Diego, USA) as per manufacturer’s instructions.

Murine AAV treatments. All treatments were carried out under general anaesthesia in Class 2 containment hoods. Viral vector particles were administered in a volume of 50 μl for all delivery routes. In subcutaneous tumour and muscle delivery, 1x10⁸ infectious particles were injected slowly into the centre of the subcutaneous tumour or into the quadriceps femoris muscle of the lower limb. For direct intrahepatic delivery, a right subcostal incision was made and 1x10⁸ infectious particles were injected under direct vision into the liver capsule. For splenic port injections, 5x10⁸ infectious particles AAV vectors were injected into the subcutaneous hemi-spleen, allowing direct delivery of vectors into the portal circulation and therefore into the liver.

Human tissue samples. This study was approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals and informed consent was obtained from patients. Tissues were collected and cultured as described previously (23, 24). Briefly, breast tumour samples were obtained from patients undergoing wide local excision or mastectomy because of breast cancer. Tumour free liver tissue was obtained from three patients undergoing partial hepatectomy for metastatic liver disease. Fresh tumour material upon collection was placed in collection medium DMEM supplemented with penicillin (200 IU/l), streptomycin (200 μg/l) and fungizone (250 μg/ml) at 4°C. Slicing at 2000 μm was performed aseptically using a Leica Vibrotome (Laboratory Instruments and Supplies, Meath, Ireland). Slices were incubated in 6-well plates (1 slice/well) containing culture media at 37°C with 5% CO₂ in a humidified environment Slices were injected with AAV after 6 hours of incubation and maintained at culture conditions.

Bioluminescent imaging (BLI). For in vivo imaging, mice were anaesthetised and were injected intraperitoneally with 100 μl of an aqueous solution of luciferin (3 mg/ml) and placed under the chamber of the CCD camera system of the Xenogen IVIS 100 Imaging system (Caliper Life Sciences, Runcorn, Cheshire, UK) and photons were counted for 3 min. Regions of interest were identified and quantified using Living Image software (Caliper Life Sciences). For ex vivo imaging, tissues of interest were excised, placed in 6-well tissue plates with 150 μg/ml D-luciferin in PBS and imaged for 3 min. Luminescence is represented as expression per gene copy administered.

Flow cytometric analysis. Subcutaneous tumours were dissociated into a single cell suspension using Collagenase IV (1500 U per tumour) (Sigma-Aldrich), Dispase (4.8 mg per tumour) (Sigma-Aldrich) and DNAse (0.01 MU per tumour) (Sigma-Aldrich) for 45 min and then passed through a 70 μm cell strainer (BD Falcon, Dublin, Ireland). The cells were fixed using 70% ethanol and permeabilised using IFA-tx buffer (2 % FCS, 10 mM Heps, 0.1% Triton X-100, 0.1% Sodium Azide and 150 mM NaCl). To assess the transfection efficiency (luciferase positive cells), cells were stained sequentially for one hour on ice with primary luciferase antibody (10 μg) (clone Luci17) (Abcam, Cambridge, UK) and a secondary Cyanine 5 (Cy5) antibody (1.500) (Abcam). For CXCR4 cell analysis, cells were stained with anti-mouse CXCR4 (5 μg) antibody tagged with phycoerythrin (clone 2B11) (eBioscience, San Diego, CA, USA). For epithelial cell analysis, cells were stained with pan-cytokeratin (3 μg) antibody tagged with PE (clone C-11) (Abcam).

Statistical analysis. Calculation of means, standard deviation (SD), and standard error of the mean (SEM) was carried out in GraphPad Prism (V 3.0; GraphPad Prism Software Incorp., San Diego, CA, USA). The significance of the differences between the individual groups was carried out using the two-tailed Student’s t-test for paired values. Differences with a p-value <0.05 were considered significant.

Results

Preferential expression of AAVCXCR4 in subcutaneous and hepatic MCF-7 tumours compared with non-malignant tissue. A human breast cancer (MCF-7) murine model was used to study tissue specificity of AAV vectors under the control of CMV (AAVCMV) and CXCR4 (AAVCXCR4)
promoters. To establish that it was possible to detect both AAVCMV and AAVCXCR4 in muscle and tumour, and also to determine the expression ratio between tissues over time, subcutaneous MCF-7 tumours and muscle for each mouse were treated with direct administration of AAVCMV or AAVCXCR. AAV vector transduction is often slow due to the necessity of conversion of the single-stranded genome into double-stranded DNA. Previous work by us and others has demonstrated a delay in AAV-related expression following administration; therefore, experimental animals were not imaged prior to day 7 post vector delivery (12). At day 7, AAVCMV-related luminescence was observed in both muscle and tumour with a relative expression ratio of 50±5% in muscle when compared with tumour (Figure 2a). At day 10, AAVCMV expression remained relatively unchanged in tumour, while there was a further increase in expression in muscle (58±25%), surpassing that of the tumour. In the case of AAVCXCR4-related luminescence, tumour levels were markedly higher than in muscle, with expression ratios of 10% in muscle relative to tumour at both days 7 and 10, although differences did not reach statistical significance.

To assess expression levels in a model for metastatic disease of the liver, confined hepatic tumours were established by intrasplenic administration of MCF-7 tumour cells. At 7 days post tumour induction, particles were
Figure 4. Systemic portal delivery and organ distribution of vector expression MCF-7 liver tumours were induced in MF1 nu/nu mice. At 7 days post tumour induction, $5 \times 10^8$ AAV particles were delivered to the liver by splenic portal inoculation. Gene expression was quantified by whole-body bioluminescent (BLI) imaging and subsequent BLI imaging of organs ex vivo. A: While whole body IVIS imaging demonstrated higher total AAVCMV expression in tumour bearing mice when compared with AAVCXCR4, images indicate that AAVCMV expression was primarily localized to the splenic port while AAVCXCR4 expression was localized to the liver. B: Ex vivo analysis of residual splenic and hepatic tissue from tumour bearing mice by IVIS imaging revealed that 98% of AAVCMV expression was in the splenic tissue, while 85% of AAVCXCR4 expression was localized to the liver (>5-fold increase compared with spleen; $p=0.039$). Data are presented as a relative percentage of splenic luminescence for each vector, where luminescence of the splenic tissue is taken as 100%. Representative images of corresponding tissue are shown below each. C: Whole-body IVIS imaging 10 days post systemic portal delivery of AAVCXCR4 to tumour-bearing and tumour-free mice, revealed 5 fold higher gene expression in tumour-bearing mice when compared with tumour-free mice ($p=0.029$). Representative images are shown. D: The ex vivo analysis of splenic and hepatic tissue from both tumour bearing and tumour free mice revealed that gene expression was localized to hepatic tissue (tumour-bearing mice) when compared with splenic tissue while there was no difference in gene expression in either tissue in tumour-free mice. Representative ex vivo images are shown.
injected directly into the liver. AAVCXCR4 demonstrated tumour to normal expression ratios in liver tumours similar to those observed in subcutaneous tumour animals (Figure 2b). AAVCXCR4 muscle expression was 54% that of tumour at day 7, although not statistically significant. Conversely, AAVCMV muscle expression was over 20-fold higher than tumour levels.

Characterization of intratumoural cell populations expressing transgene. To determine transduction efficiencies and to identify cell populations targeted by both viral vectors, subcutaneous MCF-7 tumours were treated with either vector at day 10 post tumour induction. Treated tumours were harvested and dissociated into single cells as described previously (25). The transduction efficiency (luciferase-positive cells) of AAVCMV was greater than that of AAVCXCR4 (Figure 3a). To examine the correlation between reporter gene expression and a CXCR4 protein-positive phenotype, luciferase-positive populations were co-stained with anti-CXCR4 antibodies. Results shown in Figure 3b demonstrate that AAVCXCR4 shows transcriptional selectivity for CXCR4-positive cells (43±5%) when compared with AAVCMV (25±5%) within solid tumours.

Solid tumours are known to be composed of distinct subpopulations including tumour epithelial cells, stromal cells and immune-associated cells. When luciferase-positive cells were co-stained with an epithelial-specific antibody (pancytokeratin), there was a higher percentage of pancytokeratin-positive (tumour) cells expressing luciferase in tumours treated with AAVCXCR4 (67±5%) when compared with tumours treated with AAVCMV (32±7%) (Figure 3c), demonstrating that AAVCXCR4 has transcriptional selectivity for CXCR4 positive and epithelial tumour cells within solid tumours, while AAVCMV, although achieving higher expression levels, mediates transcriptions in all cell types.

Systemic delivery of AAVCXCR4 mediates tumour-specific transgene expression in marine livers. The ideal cancer therapeutic strategy would selectively target malignant cells throughout the body, while having minimal effect on healthy cells. To examine the possibility for AAV to be used in a systemic cancer therapeutic strategy (previously unreported), mice bearing localized hepatic tumours were administered either vector through a splenic port. While luminescence from AAVCMV was significantly higher than that from AAVCXCR4, AAVCMV expression was concentrated at the site of administration (splenic port), unlike AAVCXCR4, where expression was detected only in the liver (Figure 4a). This pattern was confirmed by ex vivo BLI imaging of the excised tissues (Figure 4b). The tumour-specific nature of AAVCXCR4 was further confirmed by examining its expression in both hepatic tumour-bearing and tumour-free animals. AAVCXCR4 gene transcription was up-regulated in liver bearing tumour, with only baseline levels evident in the livers of tumour-free mice (Figure 4c). Ex vivo analysis confirmed that gene expression of AAVCXCR4 was confined to tumour bearing organs (Figure 4d).

Tumour-selective expression from AAVCXCR4 is maintained in patient tissue. To assess the translational aspect of using CXCR4 as a tumour specific promoter in an AAV vector, the transcriptional patterns of AAVCMV and AAVCXCR4 were assessed in ex vivo cultured normal (liver) and tumour (breast) patient tissue samples (n=3) and BLI carried out five days post transduction. Analysis revealed AAVCXCR4 expression was 4 times greater in tumour tissue than in normal liver tissue, although not statistically significant, while expression of AAVCMV was comparable in both normal and tumour tissue (Figure 5). This confirms the tumour selectivity of AAVCXCR4 in patient tissue, a fact which, when combined with the systemic murine data from this study, supports the concept of utilising AAVCXCR4 as a reliable tumour-targeting systemic vector for treatment of metastatic cancer.

Discussion

The concept of developing gene delivery vectors that localize gene expression to tumour sites via systemic delivery is important for the advancement of cancer gene therapies. A systemically delivered targeted therapy allows biodistribution of therapeutic to metastatic disease in distant organs, with the advantage of reduced toxicity and improved therapeutic index and with a convenient and generally well-tolerated administration. This work provides proof of concept for the
possibility of selective tumour targeting via AAV vectors following systemic delivery. We demonstrated reduced expression of AAVCXCR4 in normal tissue in which standard AAV vector (AAVCMV) is strongly expressed, which translated to a reduction in non-specific transgene expression. The AAVCXCR4 vector demonstrated efficient tumour-selective expression when locally administered to subcutaneous tumours and normal tissue (muscle). Population analysis by FACS showed that AAVCXCR4 preferentially targets epithelial tumour and CXCR4-expressing cells in subcutaneous MCF7 tumours, while the CMV promoter demonstrated majority expression in non-tumour and non-CXCR4-positive cell types.

AAV is known to mediate long-term expression in both normal and tumour tissue, unlike other widely used gene delivery vectors such as adenovirus, which only maintains minimal gene expression within days after delivery. Comparison of the expression kinetics of CMV with CXCR4 revealed that the CXCR4 promoter retained high expression in tumours while maintaining low expression in normal tissue for a period of up to 10 days, unlike AAVCMV. AAVCMV expression in tumours decreased by 65% from day 7 to day 10. CMV promoter activity has previously been demonstrated to be silenced in tumour cells via methylation of ‘foreign’ (microbial) DNA CpG sequences (26-28). Therefore, even though the CMV promoter may provide higher expression levels initially, use of a native mammalian promoter sequence like that of CXCR4 is likely to provide long-term expression.

Our intravenous delivery experiments in the hepatic model demonstrated enhanced gene expression for AAVCXCR4 in tumour-bearing livers with minimal expression in tumour-free livers. Several serotypes of AAV exist and corresponding tissue tropism varies considerably (11). The AAV2 serotype examined here is known to transduce a wide range of tissue types, including liver and muscle, albeit at a lower efficiency than other serotypes more specific for individual cell types. Therefore, use of an AAV serotype with a high tropism for the target tissue would be expected to produce efficiencies higher than reported here for AAV, in addition to providing a further level of selectivity in terms of vector safety. In particular, it has been demonstrated that AAV8 has higher efficiency for delivery to liver, while AAV1 and AAV6 to lung (6, 29). Therefore, employment of other serotypes, or pseudotyping (cross-packaging AAV2 DNA with other serotype capsids) may further improve expression in liver and other distant metastatic sites (30).

CXCR4 expression represents an ideal tumour-selective system, as it has been shown to be up-regulated in breast tumour cells and suppressed in normal epithelial cells. Furthermore, this up-regulation of CXCR4 may contribute to metastatic progression through chemokine networks (18, 19). Therefore, the use of CXCR4 as a TSP would potentially enable selective targeting of CXCR4-positive tumour cells which are developing or already possess the potential for metastasis. Furthermore, ex vivo studies with patient tissue have demonstrated the tumour-selective nature of AAVCXCR4 over AAVCMV, confirming the translational potential of this vector.

Due to their distinctive properties, AAV vectors have the potential to play a significant role in the advancement of cancer care. Here we have demonstrated an AAV-based tumour targeting strategy using a tumour-selective promoter strategy. The transcriptional targeting approach displays significantly improved tumour to normal transgene expression ratio, allowing for the efficient targeting of AAV vectors to cancer cells and the sparing of normal tissue in both subcutaneous and metastatic liver tumours using both local and systemic delivery routes. Intra-tumoural analysis emphasised the specific tropism for tumour cells. This study demonstrates the tumour-targeting flexibility which AAV-based vector systems offer and the ability to administer AAV systemically and achieve a high level of systemic tumour targeting. This vector strategy stands to play an important role in targeting distant, inaccessible, or undetected metastatic disease.

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