Toward gene therapy of uterine fibroids: targeting modified adenovirus to human leiomyoma cells

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BACKGROUND: To circumvent the paucity of the primary adenovirus (Ad5) receptor and the non-specific Ad5 tropism in the context of uterine leiomyoma cells, Ad5 modification strategies would be beneficial. METHODS: We screened several modified adenoviruses to identify the most efficient and selective virus toward human leiomyoma cells to be used as candidate for delivering therapeutic genes. We propagated: wild-type Ad5-luc, fiber-modified viruses: ad5 RGD-luc, Ad5-Sigma–luc, Ad5/3-luc and Ad5-CAV2-luc, as well as transcriptional targeted viruses: ad5 survivin-luc, Ad5-heparanase-luc, Ad5-MSLN-CRAD-luc and Ad5-SLPI-luc, on 293 cells and purified them by double CsCL density centrifugation. Then we transfected primary cultures of human leiomyoma cells derived from fibroids of four different patients, telomerase-immortalized human leiomyoma cell line (huLM), telomerase-immortalized normal human myometrial cell line (HM9) and immortalized normal human liver cells (THLE3) with the viruses at 5, 10 and 50 plaque-forming units (PFU)/cell. After 48 h, luciferase activities were measured and normalized to the total cellular protein content. RESULTS: Ad5-RGD-luc and Ad5-CAV2-luc, Ad5-SLPI-luc and Ad5-MSLN-CRAD-luc at 5, 10 and 50 pfu/cell showed significantly higher expression levels of luciferase activity in both primary and immortalized human leiomyoma cells when compared with Ad5-Luc. Additionally, these modified viruses demonstrated selectivity toward leiomyoma cells, compared with myometrial cells and exhibited lower liver cell transduction, compared with Ad5-luc, at the same dose levels. CONCLUSIONS: Ad5-CAV2-luc, Ad5-RGD-luc, Ad5-SLPI-luc and Ad5-MSLN-CRAD-luc are promising delivery vehicles in the context of leiomyoma gene therapy.

Keywords: uterine leiomyoma; gene therapy; adenovirus targeting strategies

Introduction

Uterine leiomyomas are the most common gynecological tumors and affect more than 25% of reproductive-age women (Buttram et al., 1981; Stewart, 2001). One study revealed prevalence as high as 77% (Cramer and Patel, 1990) of symptomatic fibroids. As fibroids are associated with a host of clinical problems including menorrhagia, pelvic pressure, pelvic pain, spontaneous abortions and infertility (Farhi et al., 1995; Eldar-Geva et al., 1998; Hart et al., 2001; Surrey et al., 2001). Leiomyoma-associated manifestations are responsible for about one-third of all hospital admissions for gynecological services and ~370,000 of all hysterectomies performed annually in the United States alone (Cramer and Patel, 1990; Lepine et al., 1997). Although different treatment modalities for uterine leiomyomas are currently available, including classical surgical options (hysterectomy and myomectomy) (Cramer and Patel, 1990; Lepine et al., 1997; Vilos, 2000), non-invasive options such as myolysis and uterine artery embolization (Vilos et al., 1998; Liu, 2000; Payne and Haney, 2003), as well as medicinal options such as GnRH agonists and the progesterone antagonist, RU 486 (mifepristone) (Buttram and Reiter, 1981; Andreyko et al., 1987; Kettel et al., 1994; Eisinger et al., 2003), unfortunately none of these are optimal and they each imply their own limitations. In general, the surgical approach, the mainstay for leiomyoma treatment (Lefebvre et al., 2003), is costly, especially considering the long post-operative recovery time and in 15–38% of cases (VeKaut, 1993), it carries a risk of major complications such as post-operative hemorrhage, fever, injury to adjacent organs and possible extensive pelvic adhesions and, hence, the preclusion of future fertility (Vercellini et al., 1998; Al Hendy and Salama, 2006). Therefore, the development of novel therapeutic strategies for uterine leiomyoma would be greatly welcomed, especially those that are localized, conservative and do not interfere with hormonal levels.
Gene therapy is a promising approach; it involves delivery of genetic materials to target cells to achieve therapeutic benefits, such as interfering with certain harmful gene functions, restoring lost function or initiating a new function (Al Hendy and Salama, 2006). Although there are many ways to deliver therapeutic genes to target tumors, the adeno viral (Ad) vector is the most commonly used vehicle in gene therapy clinical trials (Hallenbeck and Stevenson, 2000; Nettelbeck et al., 2000). The use of Ad vectors in gene therapy is advantageous because of many positive attributes including the following: (i) Ad has the ability to provide efficient in vivo gene transfer to both dividing and non-dividing cells (Bangari and Mittal, 2006); (ii) Ad has exhibited higher in vivo stability; (iii) Ad stocks can be prepared to ultra-pure high concentrations (10^13 particles/ml), which allow the delivery of large amounts of viral particles in finite volumes (Kozarsky and Wilson, 1993); (iv) Ad is non-oncogenic and stays episomic, which makes it relatively safe (Rein et al., 2006); (v) Ad can accommodate larger transgenes of up to 7.5 kb (Vorburger et al., 2006); (ii) Ad has exhibited higher levels of gene targeting to the nucleus, which results in significant gene expression (Hallenbeck and Stevenson, 2000). However, the gene delivery efficiency of human serotype 5 recombinant adenoviruses (Ad5) in tumor gene therapy clinical trials to date has been limited, mainly due to the development of Ad-neutralizing antibodies within the human population limiting the gene transfer to target cells (Worgall et al., 1997), short life span of gene expression due to local immune response against Ad5 (Yang et al., 1995), the inability of Ad5-based vectors to transduce important therapeutic target cell types (Stone et al., 2005), the paucity of the primary coxsackie/Ad receptor (CAR) in many human tumors, as well as virus dissemination to normal tissues (Yang et al., 1995; Bergelson et al., 1997; Tomko and Philipson, 1997). To circumvent some of these drawbacks, several virus modifications have been performed to augment the infectivity and/or increase the selectivity of Ad toward target tumor cells (Connelly, 1999; Barker et al., 2003; Everts and Curiel, 2004; Breidenbach et al., 2006).

To achieve the levels of enhanced transduction efficiency required in the context of uterine leiomyoma, it may be necessary to route the Ad5 vectors via CAR-independent pathways. One such modification is the insertion of short peptide (21 amino acid) composed of arginine, glysine and aspartate (RGD) to the H1 loop of the wild Ad5 fiber knob domain to reroute Ad5 binding to the cellular integrin (Dmitrov et al., 1998; Cripe et al., 2001). Another strategy is to employ knob switching by creating chimeric fibers possessing the knob domains of alternate human Ad serotypes beside the Ad5 fiber ex, Ad5/3 and Ad-sigma, which are created by substituting 1 Ad5 fiber with adeno virus serotype 3 (Ad3) or reovirus fiber, respectively. Functionally, Ad5-sigma utilizes CAR, sialic acid and the junctional adhesion molecule 1 (JAM1) for cell entry (Mercier et al., 2004), whereas Ad5/3 is redirected to bind the putative Ad3 receptors (CD80, CD86 or CD46) (Kanerva et al., 2002). Recently, more radical modifications based on xenotypic knob switching with non-human adenovirus have been exploited, e.g. Ad5-CAV2 in which the Ad5 fiber knob is switched to that of the canine Ad serotype 2 (Soudais et al., 2000; Glasgow et al., 2004).

To achieve a higher level of tumor cell selectivity, transcriptional targeted viruses have been developed. In transcriptional targeting, the expression of the gene of interest is placed under control of a tumor-specific promoter (TSP) that maintains a tumor-on status, a normal tissue-off status and/or liver-off status. Another improvement is the introduction of the conditionally replicating adenovirus (CRAD) concept. In these vectors, the viral genome and subsequent replication is modified in such a way that it will only be complemented by the tumor cell (Alemany et al., 2000; Heise and Kiri, 2000; Hermiston, 2000), e.g. Ad5-MSLN-CRAD. The Ad5 vectors used in this study have been listed and described in Table I.

Uterine leiomyoma is an attractive target for gene therapy because of several inherent biologic features. The disease is localized and well circumscribed in the uterus with fibrous capsules that could conceivably simplify targeting the viral load to the tumor (Al Hendy and Salama, 2006). We have recently applied several gene therapy strategies for uterine leiomyoma; these include the use of an adenovirus-delivered dominant-negative estrogen receptor (Ad DNER) mutant (ER1-536) under a cytomegavirion (CMV) promoter (Ad5-DNER) (Al-Hendy et al., 2004) or an Ad-herpes simplex thymidine kinases/genciclovir (HSV-TK/GCV) (Al-Hendy et al., 2000; Salama et al., 2007). We had considerable success with these approaches in the leiomyoma nude mice model in vitro and recently in the spontaneous Eker rat model (Hassan et al., 2007).

To optimize our approach toward leiomyoma gene therapy, as well as improve the safety profile of this novel treatment, we have presented the utility of several modified Ad vectors in the human leiomyoma cell line. Our goal was to identify the best Ad vector to enable the targeting of therapeutic genes to human leiomyoma cells with minimal effect on normal myometrial cells, as well as normal liver cells.

Material and methods

Recombinant adenovirus

Large-scale production of adenovirus vectors was performed as we have described previously (Al-Hendy et al., 2000) with a typical batch yield of 2 x 10^10 plaque-forming units (PFU)/ml. Ad vectors used in this study are listed in Table I.

Cell cultures

For experimental models, we used primary cultures of human leiomyoma cells (PLM 148, PLM 155, PLM 158 and PLM 144) derived from fibroid tumors removed during hysterectomies of four different patients. Human leiomyoma tissues were collected according to the policies of the Institutional Review Board of the University of Texas Medical Branch, Galveston, TX, USA, and used to establish PLM cells, as described previously (Rauk et al., 1995; Al-Hendy et al., 2004). The immortalized human uterine leiomyoma cell line (huLM), which expresses both estrogen receptors and progesterone receptors, was a kind gift from Dr Darlene Dixon (National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA). The cells were cultured and maintained as described previously.
Ad5-luc | CMV | WILD | E1/E3 deleted, a luc gene under the CMV promoter in place of E1 | Krasnykh et al. (1996)
Ad5-RGD-luc | CMV | Fiber | E1/E3 deleted, a luc gene under the CMV promoter in place of E1, RGD-4C modification in the HI loop of the knob domain | Dmitriev et al. (1998)
Ad5-sigma-luc | CMV | Fiber | E1/E3 deleted, a luc gene under the CMV promoter, chimeric fiber with the tail and shaft from Ad5 and the knob domain of reovirus | Mercier et al. (2004)
Ad5/3-luc | CMV | Fiber | E1/E3 deleted, a luc gene under the CMV promoter in place of E1, chimeric fiber with the tail and shaft from Ad5 and the knob domain of Ad3 | Kanerva et al. (2002)
Ad5-CAV2-luc | CMV | Fiber | E1/E3 deleted, a luc gene under the CMV promoter in place of E1, Ad5 fiber knob switching to that of canine adenovirus serotype 2 | Schagen et al. (2006); Bruner-Tran et al. (2006)
Ad5-survivin-luc | Survivin | Promoter | E1/E3 deleted, a luc gene under the Survivin promoter in place of E1 | Van Houdt et al. (2006)
Ad5-heparanase-luc | Heparanase | Promoter | E1/E3 deleted, a luc gene under the heparanase promoter in place of E1 | Breidenbach et al. (2006)
Ad5-SLPI-luc | Secretory leukocyte protease inhibitor (SLPI) | Promoter | E1/E3 deleted, a luc gene under the SLPI promoter in place of E1 | Barker et al. (2003)
Ad5-MSLN-CRAD-luc | Mesotheolin (MSLN) | Promoter based CRAD | A luc and E1A genes under the MSLN promoter | Tsuruta et al. (2006)

RGD, Arg-Gly-Asp peptide; CAV2, canine adenovirus serotype 2; E1 and E3, early translated region (1 and 3, respectively); luc, luciferase reporter gene.

(Carney et al., 2002). To represent normal cells (controls), we used a telomerase-immortalized human myometrial cell line (HM9); this cell line was cultured and maintained as described previously (Carney et al., 2002; Al-Hendy et al., 2004). We also used the THLE3 cell line, an immortalized liver cell line derived from adult human normal liver cells. The THLE3 cell line was purchased from American Type Culture Collection (Manassas, VA, USA) and was maintained in bronchial epithelial growth cell medium (BEGM Bullet Kit, CC3170, Clonetics Corporation, Walkersville, MD, USA). This medium includes 500 ml of basal medium and separate frozen additives; we discarded the gentamycin/amphotericin and epinephrine and added an extra 5 ng/ml of epidermal growth factor (EGF), 70 ng/ml of phosphoethanolamine, and 10% fetal bovine serum (FBS). The cells were maintained at 37°C and 5% CO2/air.

Transfection with adenovirus vectors and luciferase reporter assays

The transfection with Ad vectors was conducted as we have described previously (Al-Hendy et al., 2004; Salama et al., 2007). Briefly, various cell lines (PLM, huLM, HM9 and THLE3) were cultured in 12-well plates (105 cells/well). The cells were transfected with 5, 10 or 50 pfu/cell of various Ad vectors using transfection medium containing 1% antibiotic and 2% FBS for 4 h with continuous gentle shaking. The media was then replaced with fresh media, and incubation was continued for 48 h. Luciferase transactivation was determined using luciferase enzyme assay systems, according to the manufacturer’s protocol (Promega, Madison, WI, USA). The luciferase activity was normalized against total protein content, as measured with a BCA Kit (Pierce Biotech, Rockford, IL, USA) (Reynolds et al., 1999). The experiments were repeated four times using different patient-derived PLM cells to ensure generalizability of results.

Statistical analysis

The results of the luciferase transactivation were expressed as mean ± standard error of the mean (SEM) of four different experiments. Statistical analysis was determined using two-tailed Student’s t-test to compare groups. A P-value < 0.05 was considered significant.

Results

Fiber-modified virus showed higher luciferase transactivation compared with wild-type adenovirus in human leiomyoma cells

We evaluated the gene delivery efficiency of fiber-modified adenoviruses (infectivity-enhanced Ad5 vectors) at three dose levels (5, 10 and 50 pfu/cell) by assessing the mediated luciferase activity of the modified virus panels in 2-cell models of human uterine leiomyoma: the immortalized leiomyoma cell line (huLM) and the primary leiomyoma cell line (PLM) prepared from four different patients. As shown in Fig. 1, Ad5-RGD-luc, Ad5-sigma-luc, Ad5/3-luc and Ad5-CAV2-luc supported higher luciferase transactivation than wild-type Ad5-Luc in both huLM and PLM cells. Ad-RGD-luc at 10 pfu/cell had 700% and 450% of Ad5-luc (wild type) activity in huLM and PLM cells, respectively (P < 0.001) (Fig. 1A). Ad-sigma-luc at 10 pfu/cell showed 330% (P < 0.05) and 240% (P < 0.001) when compared with Ad5-luc in huLM and PLM cells, respectively (Fig. 1A). Ad5/3-luc at 10 pfu/cell showed 424% and 254% of Ad5-luc activity in huLM and PLM cells, respectively, in all of its results (P < 0.001) (Fig. 1A), whereas Ad5 CAV2-luc at 10 pfu/cell exhibited 800% and 680% of Ad5-luc activity in huLM and PLM cells, respectively (P < 0.001) (Fig. 1A). The same trend was obtained at both 5 pfu/cell and 50 pfu/cell for all fiber-modified viruses included in this study (data not shown). As shown in Fig. 1B, the modified adenoviruses (Ad5-RGD-luc, Ad5/3-luc and Ad5 CAV2-luc) at the low dose (5 pfu/cell) still demonstrated higher transduction efficiency (225%, 173
Targeting of fiber-modified adenovirus to leiomyoma versus normal myometrium

We evaluated the fiber-modified viruses to determine if they are selective toward leiomyoma compared with normal myometrium cells. We screened the transduction efficiency of these fiber-modified viruses in immortalized normal human myometrium cells (HM9) versus huLM cells. Interestingly, Ad5-CAV2-luc showed the best selective profile toward uterine leiomyoma versus HM9 cells. Luciferase activity occurred at a greater level in huLM cells (489%) than the corresponding luciferase activity in HM9 cells at 10 pfu/cell ($P < 0.001$) (Fig. 2). Ad5-RGD-luc was second in showing selectivity toward leiomyoma cells, as it expressed 357% of HM9 luciferase activity ($P < 0.001$) (Fig. 2), whereas Ad5-sigma-luc and Ad5/5.1-luc showed 205% ($P < 0.001$) and 180% of their corresponding luciferase activity in HM9 cells, respectively, at 10 pfu/cells (Fig. 2). These results were comparable with Ad5-luc, which showed luciferase activity in uterine leiomyoma cells of 130% of its corresponding HM9-luc value at 10 pfu/cell. The same order of selectivity was shown at 5 and 50 pfu/cell (data not shown). In conclusion, Ad5-CAV2-luc and Ad5-RGD-luc are highly selective toward huLM cells compared with HM9 cells.

Figure 1: Evaluation of fiber-modified Adenoviruses for transduction of human leiomyoma cells

Fiber-modified viruses showed higher luciferase expression levels in human primary (PLM) and immortalized leiomyoma cells (huLM) compared with Ad5-luc at 10 pfu/cell (A). Moreover, Ad5-CAV2-luc, Ad5-RGD-luc and Ad5/5.1-luc, at the lowest doses used (5 pfu/cell), had higher luciferase expression levels compared with Ad5-luc at 10 pfu/cell, which is the dose reported in our previous work to deliver therapeutic genes to 95–100% of the target cells using Ad5-luc (B). Luciferase activity (RLU) was normalized to the protein content and expressed as mean ± standard error of the mean (SEM) of four separate experiments and plotted as percentage of Ad5-luc activity. $^a$ and $^b$ indicate significant differences compared with corresponding Ad5-luc ($P < 0.001$ and $P < 0.05$, respectively)

$P < 0.001$; 148% and 250%, $P < 0.001$, respectively) when compared with Ad5-luc at 10 pfu/cell, a dose we have previously shown to mediate optimal transfection by Ad5-luc (Al-Hendy et al., 2004). Collectively, Ad5-CAV2-luc had the highest transduction level, followed by Ad5-RGD-luc and Ad5/5.1-luc in various huLM cells (Fig. 1).

Figure 2: Transduction targeting of fiber-modified Adenoviruses to human leiomyoma cells versus myometrium cells

The fiber-modified viruses, Ad5-CAV2-luc and Ad-RGD-luc, significantly expressed higher transduction efficiency in immortalized uterine leiomyoma cells (huLM) compared with immortalized normal myometrium cell (HM9) at 10 pfu/cell. Luciferase activity (RLU) were normalized to the protein content and expressed as mean ± standard error of the mean (SEM) of four different experiments and plotted as percentage of corresponding luciferase activity in HM9 cells at the same dose level. $^a$ indicates a significant difference from corresponding Ad5-luc activity ($P < 0.001$)
Evaluation of the activity of four TSPs in human leiomyoma cells compared with adenovirus wild type

Three TSPs showed significantly higher luciferase activity in both PLM and huLM cells when compared with Ad5-luc (Fig. 3). Ad5-SLPI-luc showed the highest luciferase activity, as it had 1400% and 1100% of Ad5-luc activity at 10 pfu/cell in huLM and PLM cells, respectively ($P < 0.001$) (Fig. 3). Ad5-MSLN-CRAD-luc was the second modified virus from this panel that expressed higher lucifirase activity, exhibiting 870% and 730% of corresponding wild Ad5 luc activity in the same cell order at 10 pfu/cell ($P < 0.001$) (Fig. 3A).

As shown in Fig. 3A, the heparanase promoter was third in showing higher luciferase activity, as it expressed 307% ($P < 0.001$) and 257% ($P < 0.001$) at 10 pfu/cell of Ad-5 luc activity in huLM and PLM cells, respectively. Survivin promoter exhibited the lowest activity (18.2% and 1.7%, $P < 0.001$) of Ad5-luc activity (10 pfu/cell) in huLM and PLM cells, which suggests that the survivin promoter achieved a uterine tumor-off profile. This panel of modified viruses exhibited the same trend of luciferase activity in huLM and PLM cells at both 5 and 50 pfu/cell (data not shown).

Figure 3B demonstrates a very impressive result: Ad5-SLPI-luc, Ad5-MSLN-CRAD-luc and Ad5-heparanase-luc at the lowest dose (5 pfu/cell) still exhibit significantly higher luciferase activity (800%, $P < 0.001$; 380%, $P < 0.001$; 150% $P < 0.05$, respectively) when compared with Ad5-luc at 10 pfu/cell, which is the dose that we have previously demonstrated to mediate optimal transduction efficiency by Ad-5 in huLM cells (Al-Hendy et al., 2004).

Transcriptional targeting of TSPs to uterine leiomyoma cells versus normal myometrium cells

We tested the transductional efficiency of the transcriptional targeted viruses in huLM cells versus HM9 cells to evaluate their selectivity toward leiomyoma compared with normal myometrium. As shown in Fig. 4, Ad-MSLN-CRAD-luc, Ad-SLPI-luc, Ad-heparanase-luc and Ad5-survivin-luc showed a higher selectivity profile toward human uterine huLM cells, as they expressed the following luc activities: 838% ($P < 0.001$), 473% ($P < 0.001$), 180% ($P < 0.01$) and 199% ($P < 0.05$), respectively, of their luc activity in HM9 at 10 pfu/cell compared with Ad5luc, which expressed 130% of its luc activity in HM9 (Fig. 4). The same selectivity profile was exhibited by these viruses at 5 and 50 pfu/cell (data not shown). Conclusively, among the transcriptional targeted virus panel, Ad5-MSLN-CRAD-luc and Ad5-SLPI-luc were the most selective toward huLM cells compared with HM9 cells.

Evaluation of modified viruses in normal human liver cells

Because Ad5-luc has a higher liver tropism and causes hepatotoxicity, which is considered to be a major obstacle in adenovirus gene therapy and virotherapy (Huard et al., 1995; Hemminki and Alvarez, 2002), we evaluated potential liver infectivity caused by the two panels of viruses (fiber-modified panel: ad5-RGD-luc, Ad5-sigma-luc, Ad5-3-luc and Ad5-CAV2-luc; and transcriptional targeted panel: ad5-SLPI-luc, ad5-MSLN-CRAD-luc, ad5-heparanase-luc, ad5-SLPI-luc, and ad5-survivin-luc). The activity of these modified viruses in huLM cells was compared with that of Ad5-luc, Ad5-galactosidase-luc, and Ad5-luc at 10 pfu/cell in huLM and PLM cells, respectively ($P < 0.001$) (Fig. 3). Ad5-SLPI-luc showed the highest luciferase activity, as it had 1400% and 1100% of Ad5-luc activity at 10 pfu/cell in huLM and PLM cells, respectively ($P < 0.001$) (Fig. 3). Ad5-MSLN-CRAD-luc was the second modified virus from this panel that expressed higher lucifirase activity, exhibiting 870% and 730% of corresponding wild Ad5 luc activity in the same cell order at 10 pfu/cell ($P < 0.001$) (Fig. 3A).

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Ad5-heparanase-luc, Ad5-survivin-luc and Ad5-MSLN-CRAD-luc) in immortalized normal human liver cells (THLE3) by utilizing the luciferase reporter gene activity. In this regard, fiber-modified Ad5-sigma-luc and Ad5/3-luc from the first panel showed higher liver transduction as they mediated luciferase activity of 228% ($P < 0.001$) and 435% ($P < 0.001$), respectively, at 10 pfu/cell when compared with Ad5-luc at the same dose (Fig. 5A). Conversely, Ad5-RGD-luc showed a non-significant decrease in luciferase activity in THLE3 cells compared with Ad5 wild type at 10 pfu/cell, as it exhibited 93% of Ad5-luc activity at the same dose level; Ad5-CAV2-luc expressed the lowest luciferase activity in this virus panel, showing 60% of Ad5-luc value at 10 pfu/cell ($P < 0.01$) (Fig. 5A). As shown in Fig. 5B, the transcriptional targeted viruses (Ad5-SLPI-luc, Ad5-heparanase-luc, Ad-survivin-luc and Ad5-MSLN-CRAD-luc) exhibited a general trend of lower liver cell transduction compared with Ad5-luc at 10 pfu/cell (70%, $P < 0.01$; 43%, $P < 0.001$; 4.3%, $P < 0.001$ and 65% $P < 0.01$, respectively, of Ad5-luc activity). The same trend was expressed by all viruses included in this study at both 5 and 50 pfu/cell (data not shown).

Interestingly, when compared with Ad5-luc at 10 pfu/cell, all transcriptional targeting viruses at 50 pfu/cell (Ad-survivin-luc, Ad5-heparanase-luc and Ad5-MSLN-CRAD-luc) showed lower liver transduction than Ad5-luc (Fig. 5B). The transcriptional targeted viruses at 50 pfu/cell had 16.4%...
(P < 0.001), 70% and 92%, respectively, of luc activity mediated by Ad5-luc at 10 pfu/cell; however, Ad5-SLPI-luc at 50 pfu/cell exhibited a non-significant increase in luc activity compared with Ad5 at 10 pfu/cell (110%). Three of the fiber-modified viruses had significantly higher liver cell transduction efficiency at 50 pfu/cell (highest dose) when compared with Ad5 at 10 pfu/cell. Interestingly, at 50 pfu/cell, Ad5-CAV2-luc did not have significantly higher luc activity (135% of Ad5-luc at 10 pfu/cell) (Fig. 5B).

In conclusion, transcriptional targeted viruses showed lower liver infectivity, whereas fiber-modified viruses had a higher liver transduction infectivity. The exceptions were Ad5-CAV2-luc, which expressed a lower liver transfection level, and Ad5-RGD-luc, which was similar to Ad5 wild type (Fig. 5A and B).

Discussion

The development of novel therapeutic strategies would be a welcome addition for uterine leiomyoma, especially if it is conservative, relatively safe and implies a localized method of treatment that would ablate the uterine fibroid without interfering with ovulation, local blood supply to uterus and systemic sex hormonal milieu and, hence, fertility, which are major side effects of current available treatment options for uterine leiomyomas (Andreyko et al., 1987; VeKaut, 1993; Kettel et al., 1994; Vercellini et al., 1998; Vilos et al., 1998; Vilos, 2000; Eisinger et al., 2003; Payne and Haney, 2003; Al Hendy and Salama, 2006).

In this regard, adenovirus-mediated gene therapy of leiomyoma is a potentially promising approach. We have recently demonstrated this potential by applying Ad5-DNER and Ad5-HSV-TK/GCV gene therapy to the treatment of uterine leiomyoma in mice (Al-Hendy et al., 2004; Salama et al., 2007). These modalities severely inhibited cell proliferation and resulted in a marked increase of apoptotic cells, as well as regression of in vivo lesions in nude mice (Al-Hendy et al., 2004) and Eker rats (Hassan et al., 2007). These strategies, however, had some limitations, e.g. the non-specific tissue tropism of native Ad5. It is well-established that Ad5-luc is CAR-dependent for cell entry (Kanerva et al., 2000; Eisinger et al., 2003; Payne and Haney, 2003; Al Hendy and Salama, 2006). Of note, although the primary cell cultures were prepared from four different patients, the behaviors of the modified viruses were fairly similar in all samples (data presented as mean ± SEM from the four patients) and gave a similar trend of efficiency that was observed in huLM cells (Figs 1 and 3). Interestingly, the modified Ad5 vectors showed higher luciferase activity in the immortalized human leiomyoma cell line versus primary leiomyoma cells which might conceivably reflect variability in both primary CAR and modified Ad specific receptors which has been observed before in other cell line models (Stoff-Khalili et al., 2005).

The two adenovirus panels used in this study exploit two main strategies: Ad5-transduction targeting and Ad5-transcriptional targeting. Transductional targeting aims at deletion of the broad tropism of Ad5 toward normal epithelial cells and/or enhances virus infectivity of CARdeficient tumor cells (Noureddini and Curiel, 2005). In this study, we explored the possibility of achieving higher transduction levels in the context of human leiomyoma using certain transductionally enhanced Ad5, including Ad5-RGD-luc, Ad5-CAV2-luc, Ad5-sigma-luc and Ad5/3-luc. We showed that Ad-RGD-luc and Ad-CAV2-luc support significantly higher reporter gene activity in leiomyoma cells than unmodified Ad-5-luc at all tested multiplicities of infection (MOIs) (5, 10 and 50 pfu/cell; 10 pfu/cell is represented in Fig. 1).

Ad5-CAV2-luc is an infectivity-enhanced adenovirus that shows expanded tropism created by switching the xeno knob of the original Ad5 knob with that of canine adenovirus serotype 2 (Glasgow et al., 2004). The receptor for this particular virus has not yet been identified but is thought to be different from the receptors for CAR and integrins (Soudais et al., 2000; Noureddini and Curiel, 2005), and it displays a distinct tropism not exhibited by human Ad5 (Soudais et al., 2000). Ad5-CAV2-luc was reported to enhance gene delivery to various tumor cell types such as primary ovarian cells (Soudais et al., 2000; Glasgow et al., 2004). Our data agree with these reports and demonstrate that Ad5-CAV2-luc consistently exhibited the highest gene transfer of all fiber-modified viruses used in this study in both PM and huLM cells at all dose levels (Fig. 1). Our data, however, are different from data obtained by Stoff-Khalili et al. (2005) in breast cancer cells, as they reported that Ad5-CAV2-luc mediated only a modest increase. These results are probably due to the paucity of Ad5-CAV2-luc receptors in breast cancer cells (Stoff-Khalili et al., 2005).

Ad-RGD-luc is an expanded tropism adenovirus that contains a targeting peptide (Arg-GlyAsp) attached to the HI
loop of its fiber to redirect its entry through integrin receptors on the cell surface (Dmitriev et al., 1998; Reynolds et al., 1999). Previous reports have shown significantly increased transductional efficiency mediated by this virus when compared with unmodified Ad-5-luc in integrin expressing ovarian cancers (Kanerva et al., 2002), squamous cell carcinomas of the head and neck (Dehari et al., 2003) and CAR-negative cell lines (Wu et al., 2002). Our data are in agreement with these reports, as Ad5-RGD-luc showed higher transduction efficiency in uterine leiomyoma cells compared with Ad5-luc viruses. Leiomyoma tissues express various types of integrins (Taylor et al., 1996). The results were also not unexpected, considering both the smooth muscle nature of uterine leiomyoma (Chegini et al., 2002) and a study by Curiel (1999), who demonstrated that the RGD-containing adenovirus consistently exhibits enhanced gene delivery to endothelial and smooth muscle cells in vitro.

The Ad5-transcriptional targeting strategy also represents a powerful molecular tool to achieve leiomyoma cell-specific expression of transgenes encoded with viral vectors. We evaluated four TSPs for their activity in huLM cells: survivin, secretory leukoprotease inhibitor (SLPI), heparanase and mesothelin (MSLN) promoters. The activity of these four promoters has not been evaluated previously in huLM cells. Their TSP activity, however, has been reported to be highly expressed in many tumor types (Chang and Pastan, 1996; McElvaney et al., 1997; Barker et al., 2003; Zhu et al., 2004); therefore, they were considered potential candidates for the development of leiomyoma-specific transcriptional targeting of adenovirus mediated gene expression. According to our results, Ad-SLPI-luc and Ad-MSLN-CRAD-luc showed the highest reporter gene activities in uterine leiomyoma cells at MOIs of 5, 10 and 50 pfu/cell when compared with Ad5-luc (10 pfu/cell is represented in Fig. 3).

SLPI is a potent inhibitor of serine proteases such as elastase and cathepsin G from neutrophils, trypsin and chemotrypsin from pancreatic cancer and chymase/trypase from mast cells (Thompson and Rabinovitch, 1996; McElvaney et al., 1997). SLPI plays a role in the protection of mucosal surfaces by helping to prevent injury associated with inflammation (Eisenberg et al., 1990). The SLPI promoter is regulated by the estrogen in the cycling and pregnant rat endometrium (Chen et al., 2004). It is also highly expressed in some estrogen-dependent conditions such as breast cancer (Garver et al., 1994; Barker et al., 2003) and endometriosis (Suzumori et al., 1999). The SLPI protein is detected in higher amounts in cervical mucus secretion (Wallner and Fritz, 1974; Casslen et al., 1986) and in pregnant uteri of different species such as the pig (Farmer et al., 1990), horse and cow (Zhang et al., 2002), rhesus monkey and human endometrium during the menstrual cycle (King et al., 2000; Okulicz and Ace, 2003). SLPI also inhibits the production and activity of matrix metalloproteinases (MMPs) in monocytes and macrophages (Zhang et al., 1997). Furthermore, SLPI knockout mice showed enhanced elastase activity and increased activation of MMPs, which then leads to reduced matrix deposition and impaired cutaneous wound healing (Ashcroft et al., 2000). Collectively, these reports are consistent with a role of SLPI in the regulation of proteolytic cascades and inflammatory events. To our knowledge, this is the first report that suggests enhanced SLPI-promoter activity in huLM cells. On the basis of the reported functions of SLPI, we speculate that SLPI expression is increased in leiomyoma tissue, since leiomyoma is an estrogen-dependent neoplasm (Chen et al., 2005) with lower levels of MMP activity (Dou et al., 1997) and an abundant accumulation of extracellular matrix and collagen deposition (Kawaguchi et al., 1985; Stewart et al., 1994). Our results support this concept, as Ad-SLPI-luc showed significantly higher luciferase activity in leiomyoma cells (Fig. 3); this suggests that SLPI can be used as a uterine leiomyoma TSP for adenovirus gene therapy targeting.

Ad-MSLN-CRAD is a type II promoter-inducible conditionally replicative adenovirus (CRAd), in which mesothelin (MSLN), TSP, replace endogenous viral promoters to control early translated genes (E1A) of the virus (Haviv and Curiel, 2003). This restricts viral replication to target tissues, actively expressing the transcription factors that stimulate this TSP and lead to virus replication, oncolysis and subsequent release of the virus progeny. Normal tissue with little or no TSP activity is spared due to lack of replication (Rocconi et al., 2007). This replication cycle is important because it allows dramatic local amplification of the input dose, and, in theory, a CRAd could replicate until all tumor cells are lysed (Haviv and Curiel, 2003). MSLN, one of the most attractive candidates in tumor gene therapy and virotherapy, is overexpressed in different tumor types such as ovarian cancer, mesotheliomas and different squamous cell cancers of the cervix, vulva, lungs and esophagus (Chang and Pastan, 1994, 1996). MSLN is not present in normal tissues, except in mesothelial cells, and is not shed into the bloodstream in significant amounts (Breidenbach et al., 2005). This limited tissue distribution of the MSLN gene could represent a useful TSP candidate for targeting the adenovirus to uterine leiomyomas. Our results indicate that the MSLN promoter is transcriptionally active in uterine leiomyoma cells (PM and huLM). The MSLN promoter exhibited higher luciferase activities compared with the CMV promoter of unmodified Ad5 at all tested doses (Fig. 3).

We targeted adenoviruses that showed high activity in immortalized human leiomyoma cells (huLM) to evaluate the modified adenovirus specificity in leiomyoma gene therapy, and we also evaluated them against normal immortalized human myometrial cells (HM9). Ad5-CAV2-luc, Ad5-RGD-luc, Ad5-SLPI-luc and Ad5-MSLN-CRAD-luc achieved significantly higher activity in huLM than in HM9 cells (Figs 2 and 4) when compared with Ad5-luc at 5, 10 and 50 pfu/cell. This indicates the significant tissue selectivity of these viruses toward the neoplastic leiomyoma cell versus the normal myometrial cells.

The adenovirus has a propensity to localize to the liver (Huard et al., 1995; Sullivan et al., 1997), and leakage of low Ad5 titer into bloodstream results in detectable gene expression in the liver (Hiltunen et al., 2000). Furthermore, the liver sequesters the majority of systemically administered adenovirus particles via hepatic macrophage (Kupffer cell) uptake (Alenmey et al., 2000; Tao et al., 2001). This is compounded by the fact that hepatocytes express higher amounts.
of CAR and integrin (Einfeld et al., 2001). This localization to the liver can potentially lead to adenovirus-mediated inflammation and liver toxicity (Lieber et al., 1997; Hemminki and Alvarez, 2002). We decided to evaluate the ability of various modified Ad5 at different dose levels (5, 10 and 50 pfu/cell) to transduce the normal human THLE3 liver cell line. Our results demonstrated that Ad5-CAV2 from the fiber-modified panel and all of the transcriptional targeted viruses supported lower liver luciferase transactivation compared with Ad5-luc (10 pfu/cell represented in Fig. 5A). This is in agreement with previous reports that used either human liver tissue slices (Rein et al., 2004; Stoff-Khalili et al., 2005; Breidenbach et al., 2006) or animal models (Lu et al., 2005). Implementation of targetted Ad5 gene therapy in uterine leiomyoma cells provides great potential to achieve higher gene transfer at lower Ad5 doses. It also affords a wider safety range, as the targeted adenoviruses can be utilized at higher dose levels to maximize therapeutic impact with lower liver side effects compared with Ad5. Additionally, serotype switching, such as Ad5-CAV2-luc, could circumvent any pre-existing neutralizing Ad5 antibodies (Wu et al., 2002).

In conclusion, Ad5-CAV2-luc, Ad5-RGD-luc, Ad-SLPI-luc and Ad5-MSLN-CRAD-luc showed higher reporter gene activity in huLM cells, and lower activity in both HM9 and THLE3 cells compared with unmodified Ad5-luc. These modified adenoviruses are promising vectors for targeted adenovirus-mediated gene therapy in uterine leiomyoma. Such modified (improved) adenoviral vectors will need to be tested in Eker rat, an experimental animal model for uterine leiomyoma (Everitt et al., 1995) before contemplation of a human clinical trial.

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