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Gene therapy targeting Leiomyoma: Adenovirus mediated delivery of dominant negative estrogen receptor gene shrinks uterine tumors in Eker rat model

Memy H Hassan, MSc^{a,b}, Salama A. Salama, Ph.D.^a, Dong Zhang, Ph.D.^{c,d}, Hossam M.M. Arafa, Ph.D.^b, Farid M.A. Hamada, Ph.D.^b, Hala Fouad, MD,Ph.D.^a, Cheryl C. Walker, Ph.D.^c, and Ayman Al-Hendy, MD,PhD. [FRCSC Professor and Vice Chair]^{a,d,*}

Memy H Hassan: ; Salama A. Salama: ; Dong Zhang: ; Hossam M.M. Arafa: ; Farid M.A. Hamada: ; Hala Fouad: ; Cheryl C. Walker: ; Ayman Al-Hendy: Ahendy@MMC.edu

^a Department of Obstetrics and Gynecology, University of Texas Medical Branch, Galveston, Texas, USA

^b Department of Pharmacology and Toxicology, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt

^c Department of Carcinogenesis, M.D. Anderson Cancer Center, University of Texas, Texas

^d Center for Women Health Research, Department of Obstetrics and Gynecology, Meharry Medical College 1005 Dr. D.B. Todd Jr. Blvd. Nashville, Tennessee 37208

Abstract

Objective—To evaluate the utility of gene therapy for uterine fibroids in the Eker rat model using an adenovirus-mediated delivery of a dominant negative estrogen receptor gene (Ad-DN-ER).

Design—Animal study.

Setting—University animal laboratory.

Animal(s)—27 female Eker rats

Interventions—We randomized Eker rats with MRI-confirmed uterine leiomyomas to a single treatment of direct intra-fibroid injection with Ad-DN-ER, Ad-LacZ, or vehicle.

Main Outcome Measures—The tumor volumes determined by MRI scanning and caliber measurement. Samples of serum, fibroid tumors, and various organs were collected at 8, 15 30 days post-treatment to assess treatment safety and efficacy.

Results—Ad-DN-ER treatment significantly decreased uterine fibroid volume by 45%, 80% and 77.4% of pretreatment volume at days 8, 15 and 30 respectively and modulated the expression of apoptosis-, proliferation- and extracellular matrix related genes' when compared to control animals. Ad-DNER did not produce any toxic effects to non-target tissues.

*Reprint Requests: Ayman Al-Hendy MD PhD FRCSC Professor and Vice Chair, Center for Women Health Research, Department of Obstetrics and Gynecology, Meharry Medical College 1005 Dr. D.B. Todd Jr. Blvd. Nashville, Tennessee 37208, Tel. 615-963-3148 Fax. 615-963-3125.

Capsule: Interception of ER signaling via direct intra-fibroid injection with Ad-DN-ER shrinks Eker rats' fibroids. This gene therapy approach presents a promising conservative treatment option for women with symptomatic uterine fibroids.

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Conclusion—Ad-DN-ER treatment shrinks Eker rats' fibroids, in part, via modulation of several estrogen regulated genes. This safe gene therapy approach presents a promising conservative treatment option for women with symptomatic uterine fibroids.

Keywords

Uterine leiomyoma; Gene therapy; Dominant negative estrogen receptor

Introduction

Uterine leiomyomas (fibroids) are benign neoplasms of the myometrium, frequently found in reproductive age women (1-3). Uterine leiomyomas commonly cause significant symptoms such as anemia resulting from heavy irregular uterine bleeding, Pelvic discomfort, and bowel/bladder dysfunction from pressure. Fibroids have also been associated with infertility and recurrent abortion (4-7). The genesis of uterine leiomyoma involves somatic mutations and a complex interaction with trophic sex hormones and growth factors (8) whereas estrogen is considered a major promoter of neoplastic growth (9-14). The pivotal role of estrogen in leiomyoma development and growth has been established through both clinical observations and research studies (15-17). For example fibroids only appear after menarche, proliferate and grow during the reproductive years and stabilize or regress after menopause, and they tend to re-grow after hormone replacement therapy (8). The effects of estrogens in leiomyomas cells are mediated via the estrogen receptor (ER), of which both subtypes, ER α and ER β , were found in fibroids (17-18). We and others have reported that uterine leiomyomas express ER at higher level compared to normal myometrium (19-21)

Although uterine fibroid seriously impact women health, there are only a few conservative treatment options currently available to women with symptomatic uterine fibroids (22-25) and surgery has been the mainstay (25,26). The surgical approach is costly, especially considering the long post-operative recovery time and might carry a risk of major morbidity and mortality complications (27-28). Even more critical, most of available surgical intervention will preclude or diminish future fertility potential of affected women many of whom might still desire to preserve their ability to procreate (23,28-33). Since current treatments for uterine leiomyoma are far from satisfactory and leave much to be desired, the development of a safe, effective, non-surgical and localized method of treatment for uterine fibroids would greatly benefit many women, especially when this localized method of treatment is able to ablate uterine leiomyoma without interfering with ovulation, uterine blood supply or the systemic hormonal milieu. Dominant-negative ERs (DNER) are ER-mutants that intercept the estrogen-signaling pathway. These mutants form heterodimers with wild-type ER, making it unable to bind the estrogen-responsive element (ERE) and hence unable to activate transcription (34). We previously demonstrated that adenoviral vectors (Ad) are able to infect fresh uterine leiomyoma tissues directly removed from hysterectomy specimens (28). In addition, we reported that Ad-DNER severely inhibited both rat and human leiomyoma cell proliferation and resulted in a marked increase in the number of apoptotic cells as well as significantly inhibited rat leiomyoma cells-developed lesions in nude mice (14).

Such data obtained from our previous studies does indeed prepare the stage for further preclinical studies using experimental models that are more relevant to human leiomyoma. In the current work we assess the efficacy and safety of in vivo gene therapy of uterine leiomyoma using adenovirus mediated delivery of DN-ER in the immune-competent Eker rats that spontaneously develop uterine fibroid lesions which share many anatomical, histological and biological features with human leiomyoma (35-37).

Materials and Methods

Recombinant Adenovirus

The adenoviral vector carrying the dominant-negative ER mutant ER1-536 under cytomegalovirus promoter (Ad-DNER) used in this work has been described in our earlier work (38,14). An Adenovirus expressing a marker gene coding for bacterial β -galactosidase (Ad-LacZ) was a kind gift from Dr. Savio Woo (Mount Sinai School of Medicine, NY) and was used as negative control for the Adenovirus genome back bone effect. Large-scale production of adenovirus vectors was performed as we have described earlier (39) with a typical batch yield of 2×10^{10} plaque-forming units (PFU)/ml.

Adenovirus Dose Calculation, Dilution and Tumor Injection

The virus stock was diluted in serum-free medium and injected directly into the visible leiomyoma lesions at laparotomy. The calculated virus inoculum was divided equally among the four quadrants of the fibroid tumor. We used the optimal MOI of 100 pfu/ELT3 cells as previously determined from *in vitro* studies (14). The number of cells per tumor was determined using the general rule of 3×10^8 cells/cm³ of leiomyoma tumor (40). The tumor volume was determined from the 3 measured diameters (length, width, and height) using the ellipsoid formula: volume = $R1 \times R2 \times R3 \times 0.52$ (41).

Animals

Female Eker rats with mutation in the tuberous sclerosis 2 (*Tsc-2*) tumor suppressor gene were obtained from M.D. Anderson Cancer Center, Smithville, Texas. The presence of uterine tumors were detected with MRI scanning as described earlier (42,43). Rats harboring MRI-measurable tumors were randomized to one of the following three groups: i) a single treatment with direct intratumor injection of Ad-DN-ER, ii) a single treatment with direct intra-tumor injection of Ad-Lac Z or ii) serum free media. The last two groups served as negative controls for therapeutic gene or Adenovirus genome back bone effects respectively. The tumor volume was determined by serial MRI scanning and confirmed with caliber measurement at times of injection and euthenization. Animals were observed daily for any post-treatment complications or adverse events. Sample rats were selected randomly and killed at the following time points post inoculation treatment: 8, 15 and 30 days (the end of the experiment). Blood and tissue samples were collected from tumors as well as several other organs. The rats were cared and handled in accordance with the National Institutes of Health guidelines and they were housed in facilities accredited by the Association for the Accreditation of Laboratory Animal Care. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas Medical Branch (UTMB), Galveston, Texas, USA.

Evaluation of Adenovirus Transfection Efficiency and Transgene Distribution within Eker Rat Leiomyomas

To evaluate the distribution and gene expression of adenovirus, random representative fresh leiomyoma tissue from the Ad- lac Z treated Eker rat group were collected and subjected to X-Gal staining using In situ β -Galactosidase staining kit (Stratagene, La Jolla, CA) according to supplier instructions. The appearance of blue color indicates that the Adenovirus particles have indeed transfected and delivered at least one copy of the transgene to the nucleus (14).

Tumor Tissue Evaluation—Tumor tissues collected from all groups were typically divided into 2 parts: one was immediately preserved in liquid nitrogen, and the other was formalin-fixed, paraffin-embedded, and subjected to hematoxylin and eosin (H&E) histological analysis as we have described previously (44). The histological sections were graded semi-quantitatively for cell density (cellularity), the absence or presence of mitosis per 10 high-

power fields, degree of variation in nuclear size, and hyaline changes as described previously (45). Apoptosis was evaluated with the ApopTag kit (Intergen, Purchase, NY, USA) using the manufacturer's instructions as we described earlier (14). Cell proliferation was assessed in the tissue preparations by detection of PCNA (proliferating cell nuclear antigen) and cyclin D1 proteins expression using rat specific monoclonal antibodies (Santa Cruz biotech, Santa Cruz, CA, USA) at dilution 1: 6000 and 1: 3000 respectively as described previously (46,47,2). The percentage of immunoreactive cells in these assays was calculated by counting positive stained nuclei (brown color) against the total number of cells in 3 random high-power fields for each tissue section. At least 2000 cells were counted per specimen. Three animals were included from each group. Data were presented as mean $\pm$ standard error (SE) of these 3 animals.

Western Blot Analysis

Parts of the liquid nitrogen preserved tumor tissues were washed twice with PBS, and whole tissue lysates were prepared with RIPA lysis buffer. The following antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, Calif., USA) and used at the indicated dilutions: active p53 (1: 2000); Bcl2 (1:1000); Poly (ADP-ribose) polymerase (PARP), (1:2000) and Bax (1: 500) for detection of their corresponding protein as we described previously (14,48,49). The protein expression levels were normalized against the housekeeping β - Actin protein levels.

Real time Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was isolated from liquid nitrogen preserved tumor tissues using RNAqueous kit (Ambion Inc, Austin, Texas). First-strand cDNA was synthesized from 2 μ g total RNA using TaqMan reverse transcription Kit (Applied Biosystems, Foster City, USA). PCR amplification of transforming growth factor (TGF β 3), FAS ligand and death associated protein (DAXX) were performed using rat specific TaqMan gene expressions assay (applied Biosystem, Foster City, USA) according to supplier protocol. Glycerol-3-phosphate dehydrogenase (G3PDH) was used as endogenous control. The potential effects of Ad-DNER on individual target gene expression were calculated as described earlier (49-51). Target gene expression levels were normalized against GAPDH mRNA and change due to Ad-DNER treatment presented as fold change compared to those of control animals.

Caspase-3 Assay and Tunnel Test

The caspase-3 enzyme activity was measured in uterine leiomyoma tissues collected from Eker rats in the three treatment groups (Ad-DNER, Ad-LacZ or vehicle) using the CaspACE Assay system (Promega Corp. WI, USA) based on the ability of the caspase 3 enzyme to release the yellow chromophore *p*-nitroaniline (pNA) from the colorimetric substrate (Ac-DEVD-pNA) provided in the CaspACE™ assay system. The tissue lysates were centrifuged at 13,000 rpm at 4°C for 5 min and supernatants were used for caspase-3 assay. Fluorescence intensity was determined at 405(ex)/510 (em) using the 96 plate Spectra max Gemini spectrofluorometer (molecular devices, Sunnyvale, California, USA). Relative caspase activities for each sample and sample plus inhibitor were calculated from the standard curve. As described previously (14), caspase 3 activity values were normalized against total tissue protein content measured by BCA kit and presented as percentage of control value.

Safety and Toxicity Studies in Eker Rats—All rats were examined daily for any complications/adverse events or unexplained death. At the time of euthanization, the ratio of uterine horn weight to total body weight was calculated, and samples from various organs including the uterus (myometrium and endometrium), ovary, cervix, vagina, liver, spleen, lung, kidney, and brain were collected and examined macroscopically and microscopically (H&E staining) by a pathologist who was blinded to the treatment assignment. Serum liver function

tests (aspartylaminotransferase [AST], alanine aminotransferase [ALT], and total bilirubin) were also evaluated using standard techniques (52).

Evaluation of Adenovirus Dissemination

To examine possible dissemination of Adenovirus particles to adjacent or distant rat organs after a single direct intra-fibroid injection, total DNA was isolated from various rat organs including the brain, lung, heart, liver, spleen, ovary, uterus, vagina, and cervix, as well as the inoculated uterine leiomyoma tumors, using the DNeasy Tissue kit (Qiagen Sciences, Germantown, MD, USA). The presence of Adenovirus DNA was determined by performing sensitive polymerase chain reaction (PCR) amplification for the essential E4 region of the Adenovirus genome as described previously (46,53). The presence of Adenovirus DNA was evident by the presence of the expected 714 bp fragment.

Evaluation of serum Anti-Adenovirus serotype5 antibody titers—Anti-Ad5 neutralizing antibody titers were measured by the ability of serum to prevent Ad5 transfections of cultured cells as described earlier (54-56). Briefly, the sera were serially diluted using heat inactivated OPTI medium, incubated with Ad-LacZ for 1 hour at 37°C, and then used for transfection of human leiomyoma cells. After 24 hours, the cell cultures were fixed and stained with X-gal staining using *in situ* β -Galactosidase staining kit (Stratagene, La Jolla, CA) according to supplier instructions. The number of blue-stained nuclei were counted on four random high power fields and calculated as a percentage of total cells in the field. Antibody titer was calculated as the highest serum dilution that blocks >75% of Ad-LacZ transfection. Ad-LacZ without Eker rat serum was used as a positive control. Eker rat serum known to be negative for Ad-antibodies was used as negative control.

Statistics

The data are presented as mean $\pm$ SE. Group differences were analyzed using one way analysis of variance (ANOVA) followed by Tukey as post ANOVA multiple comparison test. Caspase 3 assay results were analyzed using two tailed unpaired t test. $P < 0.05$ was considered statistically significant.

Results

Adenovirus Efficiently Transfect Eker Rat Uterine Leiomyoma After A Single Intratumor Injection

As shown in Figure 1, Adenovirus was able to optimally transfect eker rat uterine leiomyoma cells after a single intrauterine leiomyoma injection. The efficiency of gene delivery to surrounding uterine leiomyoma tissue was very good and was well distribute around the injection site.

Ad-DNER Shrinks eker rat uterine leiomyoma volume

As shown in Figure 2A & B, the Ad-DNER injected animals showed significant shrinkage in total tumor volume compared to either Ad-lac Z treated or vehicle treated control groups. Ad-DNER produced dramatic shrinkage of the total uterine fibroid volume by -45% ($p < 0.05$), -80% ($p < 0.01$) and -77.4% ($p < 0.01$) of pretreatment volume at days 8, 15 and 30 respectively. The tumor size in control animals receiving Ad-LacZ continued to grow by +26%, +66%, +102% at same time points when compared to pretreatment size. Similarly the negative control group receiving medium alone continued to grow by +20%, +70% and +110% at the same time points. There was no significant difference in the fibroid tumor size between the two control groups (Fig. 2).

Histological Changes In The Eker Rat Uterine Leiomyoma After Treatment With Ad-DNER

As shown in Figure 3 (c-d), histological specimens collected from eker rat fibroid lesions at 8, 15 and 30 days post treatment with Ad-DNER, showed several time dependent cellular changes. Fibroid sections from Ad-DNER treated group showed distorted, sparsely distributed nuclei with smudged chromatin pattern, and ill-defined nuclear and cytoplasmic outline. Mitotic activities were rare, and progressive nuclear degeneration and diminished cellular density, indicated by the wide spacing between nuclei, were frequently seen. Nuclear pyknosis, karyorrhexis, and karyolysis were also seen. Hyalinization was also wide spread especially at 15 and 30 days post-treatment.

Conversely, rat in the two control groups showed the typical interlacing bundles of smooth muscle cells (Fig. 3 a, b). The cells have indistinct cell borders and abundant pale eosinophilic cytoplasm. The nuclei were uniform, crowded, and tightly packed, in some regions overlapping each other, with finely granular chromatin pattern. Mitotic figures were present and hyaline changes were infrequent (Fig. 3a, b).

Ad-DNER Treatment Induces Apoptosis In Eker Rat Uterine Leiomyoma

As shown in figure 4A, Ad-DNER treatment of eker rat uterine leiomyoma exhibited a definite decrease in both antiapoptotic Bcl2 and the death substrate, Poly (ADP-ribose) (PAR) polymerase (PARP1) protein expressions. On the other hand, Ad-DNER treatment produced a clear increase in the proapoptotic Bax protein and the gate keeper gene (p53) at all tested time points (8, 15 and 30 days) when compared to Ad-lac Z treated samples at the same time points. All these changes are indicative of strong induction of apoptosis in fibroid lesions treated with Ad-DNER.

Moreover, Ad-DNER treatment significantly increased the extrinsic apoptotic pathway regulating genes, FasL and Daxx, in Eker rat leiomyoma tissue. As shown in Figure 4B, Ad-DNER induced DAXX mRNA expression by 1.8, 1.6 and 1.4 folds of vehicle control ($p < 0.05$) at 8, 15 and 30 days post treatment. Additionally, FasL mRNA level was also induced to 1.6, 2.1 and 2.7 folds of vehicle control at the same time points (Fig. 4B). Interestingly Ad-Lac Z did not produce any significant changes in these genes' expression compared to vehicle control at all time points.

We also evaluated the induction of apoptosis in Ad-DNER-treated fibroid lesions, by measuring caspase-3 activity. There was marked increase in caspase-3 activity in Ad-DNER compared to vehicle control group, 2669%, 3656% and 10736 % at 8, 15 and 30 days respectively (Fig. 4C). These values were significantly ($p < 0.001$) higher than the values obtained in Ad-lacZ treated section showed caspase-3 activity of 144%, 171 % and 277 % of corresponding control group value at the same time points.

We further evaluated apoptosis induction using the TUNNEL test. The Ad-DNER-treated fibroid lesions exhibited strong positive tunnel reaction at $57\% \pm 3$, $43\% \pm 4$ and $35\% \pm 3$ at all tested time point 8, 15 and 30 days respectively. This was significantly higher ($p < 0.05$) compared to vehicle treated group ($2.5 \pm 0.7\%$, $2.8 \pm 1.5\%$ and $2\% \pm 1$) and Ad-LacZ treated control ($4.7 \pm 2.8\%$, $4.5 \pm 1.5\%$, $7\% \pm 2$) at same time points (Figure. 4D).

Ad-DNER Inhibits The Expression of Proliferation and Extracellular Matrix Formation-Related Genes

As shown in Figure 5A; immunohistochemical analysis using rat specific antibodies against PCNA showed that treatment of Eker rat leiomyoma with Ad-DNER induced a significant ($P < 0.05$) decrease of PCNA protein expression compared to control and lacZ treated fibroid lesions. The percentages of PCNA positive cells in tumor sections prepared from Ad-DNER

treated animals euthanized after 8, 15 and 30 post treatment were $30\% \pm 3$, $47\% \pm 2.7$ and $22\% \pm 1.8$ compared to $73\% \pm 4$, $81\% \pm 7$ and $77\% \pm 6$ in control animals and $71.5\% \pm 8$, $77\% \pm 9$ and $67\% \pm 5.3$ in sections prepared from Ad-LacZ treated tumors, respectively ($P < 0.05$). Interestingly cyclin-D1 expression was also significantly ($P < 0.05$) decreased in Ad-DNER treated fibroid sections. These sections exhibited the following percentages of cyclin-D1 positive cells $25\% \pm 3$, $9\% \pm 2$ and $5.5\% \pm 1.5$ at 8, 15 and 30 days post treatment compared to the following percentages in sections from vehicle control $49\% \pm 5$, $53\% \pm 8$ and $51.6\% \pm 7.3$, as well as from Ad-lacZ treated controls $46\% \pm 7$, $42.5\% \pm 7.8$ and $42\% \pm 4.3$ respectively, at the same time points (Fig. 5B).

Additionally, as shown in Figure 5C, Ad-DNER significantly, ($p < 0.05$), decreased the mRNA expression of TGF β 3 gene to 0.6 and 0.5 fold of control at 15 and 30 days post treatment respectively while at 8 days there is no significant effect (1.1 fold). Conversely, in Ad-lacZ and vehicle controls did not exhibit in TGF β 3 mRNA expression at all tested time points (Fig. 5).

Humoral Immune Response to Adenovirus Treatment in Eker Rats

As shown in Figure 6, direct injection of Ad-lac Z or Ad-DNER into uterine leiomyoma significantly, $P < 0.01$, elicited systemic anti-adenovirus antibodies that was detected in Eker rat serum. Antibody titers of 67.5 ± 7.5 and 87.5 ± 12.5 were detected in Ad-LacZ and Ad-DNER treated Eker rat at 30 days post treatment, respectively.

Safety and Toxicity evaluation of Ad-DNER treatment in Eker rat

Our daily monitoring indicated that all animals well tolerated the virus inoculation and survived the experiment without any apparent sign of toxicity and no unexplained death.

As shown in Figure 7A; PCR amplification of Adenovirus E4 region showed that the delivered virus particles did not disseminated to most of the tested organs and localized mainly in tumor tissue. Faint bands of the E4 region of adenovirus DNA was also detected in uterine and liver tissues of 40% and 30% of animals respectively (Fig 7. and Table 1).

Careful histological evaluation of tissue section obtained from various Eker rat organs including liver and uterus at different time point (8, 15 and 30 days) using H&E staining revealed no signs of tissue damage or necrosis in all collected tissues (data not shown). Moreover, Ad-DNER treatment did not cause any significant effect on Eker rat liver function tests (AST, ALT and total bilirubin) at 30 days post-treatment, compared to vehicle or Ad-lac Z treated controls (Fig. 7B).

Discussion

It is well established that estrogen plays a pivotal role in leiomyoma growth (8-14,57,58) and ERs *expressions* are higher in human uterine leiomyoma compared to adjacent normal myometrium (19-21), Thus it is expected that interruption of ERs signaling pathway would be a viable target for many therapeutic modalities. One of these approaches exploits dominant negative ERs (DNER) to inactivate endogenous wild type ER (38,59). Recent reports from our lab demonstrated that Adenovirus can optimally transfect eker rat uterine leiomyoma cells at 100 pfu/cell, Moreover Ad-DNER treatment resulted in suppression of cell proliferation and induction of apoptosis, as well as regression of *in vivo* lesions in nude mice (14,28). Furthermore, our prior work has demonstrated that such treatment modulated the expression of several genes implicated in leiomyoma growth, apoptosis, estrogen metabolism and extracellular matrix formation in cultured human leiomyoma cells (48). In this report, we aimed to further pursue our goal to develop an alternative localized nonsurgical conservative

treatment approach for uterine leiomyoma. To that end, it was paramount to evaluate this therapeutic modality in Eker rat, the only immunocompetent animal model that spontaneously develops uterine fibroid lesions (37). Our goal was to assess both treatment safety and efficiency in preparation for a future clinical trial.

Our studies revealed that Ad-DNER was able to markedly shrink Eker rat uterine leiomyoma volume compared to vehicle and Ad-lacZ controls (Fig. 2), the shrinkage reached a peak of 80% and was sustained for 30 days after a single Ad-DNER inoculation. Furthermore, Ad-DNER modulated the expression of several estrogen regulated genes controlling apoptosis, proliferation and extracellular matrix formation. This study provides potential mechanisms of action of this Ad-vector mediated DNER gene therapy approach and explains the rapid and impressive tumor shrinkage (Fig. 4-5). Noteworthy, this treatment approach was safe and did not induce any macroscopic or microscopic tissue damage nor caused any significant changes in serum liver function tests (Fig. 6-7).

As a pertinent step, we initially investigated whether gene transfer using an adenoviral vector expressing β -galactosidase gene (Ad-lacZ) could transfect eker rat fibroid tissue after direct intrauterine leiomyoma injection in vivo. Our study demonstrated that Adenoviral vectors efficiently transfected uterine leiomyoma tissue in vivo and transgene expression was well distributed around the injection sites (Fig. 1). Ad-lacZ injection did not produce any significant change in the tumor volume compared to vehicle only control animals, which expelled the possibility that any of the observed effects of Ad-DNER is due to the Adenovirus genome backbone in this system.

Furthermore, we investigated the potential mechanisms for the Ad-DNER mediated fibroid tumor shrinkage by evaluating cellular pathways that have been implicated in leiomyoma pathogenesis such as proliferation pathway (PCNA and cyclin-D1) (Fig. 5 A&B), apoptosis represented by (P53, Bax, Bcl2, PARP, FasL and DAXX as well as caspase 3 activity and tunnel assay) (Fig. 4), and extracellular matrix formation represented by TGF β 3 (Fig. 5C). All of these selected genes are in part regulated by estrogen (12, 13, 60-62).

The tumor suppressor p53 controls multiple downstream targets that regulate variable cellular pathways such as cell-cycle, apoptosis, DNA repair and replication (63,64). Therefore, p53 has been categorized as both a caretaker and gatekeeper tumor suppressor gene (65). Interestingly, estrogen (E2) stimulates leiomyoma growth, in part, by decreasing the p53 protein levels in the nuclei and suppressing normal p53 functions, conversely GnRH agonist remarkably increase leiomyomas p53 tumor suppressor content (61). P53-mediated apoptosis have been documented by two separate pathways (64). The extrinsic death-receptor pathway and the intrinsic, mitochondrial pathway shift the balance in the Bcl-2 family towards the pro-apoptotic members (BAX). Both pathways lead to activation of caspases as a common final pathway (64). Among p53-downstream genes, we selected two genes for further evaluation; Fas ligand (Fas L), a key component of the extrinsic death pathway (66,67) and DAXX (Death-Domain Associated protein), a Fas-binding protein (68). Fas ligand is a transmembrane protein of the tumor necrosis factor family of ligands. Fas ligand engages Fas receptors triggering a cascade of subcellular events including binding cytoplasmic regions of Fas receptors by adaptor proteins such as DAXX that culminates in the activation of upstream caspases 8 and 10 which via a series of intermediates result in activation of executor caspases such as caspase 3 that disassemble the cell (69,70). DAXX interacts directly and activates ASK1 (Apoptosis Signal Regulating Kinase 1) triggering the c-Jun N-terminal kinase (JNK) cascade, culminating the activation of transcription factors such as c-Jun which directly or indirectly may counteract the expression of survival factors, such as Bcl-2 (71). Uterine leiomyomas showed a decreased Fas L and caspase3 protein expression, both of which are significantly increased with GnRH α treatment (62).

In Intrinsic pathway, Bcl-2, a mitochondrial protein, inhibits apoptotic process and promotes cell survival. Bcl-2 protein prevents the release of cytochrome *c* from the mitochondria and subsequent activation of caspase cascade (72-74). Over-expression of Bcl-2 can block p53-dependent dissipation of the mitochondrial membrane potential (68,75). On the other hand Bax, a proapoptotic protein, has been reported to mediate the opposite effect of Bcl2 protein (72,76). Interestingly, human leiomyoma cells has been reported to express higher amount of Bcl-2 protein and lower amount of BAX protein compared to normal myometrial cells (77, 78).

In the present study, the caspase3 activity and the protein levels of active P53 and the proapoptotic BAX protein were remarkably increased while Bcl-2 protein level was decreased by treatment with Ad-DNER (Fig. 4 A & C). Also Ad-DNER treatment induced a significant increase in the expression both Fas ligand and DAXX at m RNA level (Fig. 4B), suggesting that both intrinsic and extrinsic pathways are induced by DNER treatment of Eker rat leiomyoma. These results are similar to observation obtained using GnRh α in women with symptomatic uterine fibroids followed by hysterectomy (18). Clearly, Ad-DNER gene therapy would have the distinctive advantage of being a localized treatment targeted to the fibroid lesions with no systemic side effects or complications. In addition, unlike GnRHa, the Ad-DNER approach should not interfere with the ability of the treated subject to ovulate and conceive (28).

Accumulating evidence indicate that uterine leiomyoma growth is in part regulated by the balance between genes controlling proliferation and apoptosis (60,79). PCNA and cyclinD1 have been reported as a marker of cellular proliferation (61,80,81) and expressed at higher level in leiomyoma compared to normal myometrium compartment (60). As shown in Figure 5 A-B, Ad-DNER exhibited marked decrease in PCNA and cyclinD1 expression which might reflect a cell cycle arrest (60,80,81) probably mediated through active p53 over-expression (Fig. 4A). Taking these results together one can conclude that Ad-DNER treatment shrinks eker rat leiomyoma tumors in apart through induction of apoptosis and inhibition of proliferation.

Transforming growth factor- β (TGF β) family are pleiotrophic cytokines with key roles in tissue morphogenesis and growth (82). TGF β increases the expression of extracellular matrix (ECM) proteins (83,84). Leiomyomas contain abundant quantities of ECM, and have been reported to contain increased levels of estrogen-induced TGF- β 3 relative to adjacent myometrium (13). In the present study we demonstrate that the interception of estrogen signaling pathway with Ad-DNER resulted in a marked decrease in the expression of TGF- β 3 mRNA level (Fig. 5C) which possibly has led to in a decrease in leiomyoma ECM formation and ultimately has contributed to a decrease leiomyoma volume in the Ad-DNER group.

Although it well established that Adenovirus are the one of most efficient vectors for gene transfer (85), anti-adenovirus immune response constitutes a major hurdle that might limit the gene transfer to target cells (86,87) so it is of particular interest in this study to evaluate the interplay between Adenovirus treatment and immune system exploiting the immune competent nature of Eker rat. Our results here have demonstrated a moderate induction in the level of anti adenovirus serum antibodies after a single intratumor injection (Fig. 6). However, we do not expect a significant effect of such antibodies on gene transfer using repeated adenovirus injection, because of (1) Ad-DNER is delivered locally by direct injection into a solid tumor, several reports have demonstrated that in such scenarios, the effect of serum neutralizing antibodies is minimal (39,74,87-89), (2) Characteristic features of uterine leiomyoma such as being discreet and well-circumscribed lesions with a defined fibrous capsule will facilitate direct adenovirus targeting inside tumor tissue while avoiding possible neutralization with preexisting serum antibodies (28,90).

From clinical point of view, Adenovirus mediated gene therapy for uterine leiomyoma has to avoid toxic side effects to other normal organs especially liver, a common forager for systemically injected Adenoviral particles (91,92). Interestingly careful examination of different Eker rat organ histology specially hepatic and uterine section after treatment with Ad-DNER, did not reveal any evidence of organ damage. Furthermore the liver function test did not reveal any significant changes compared to control animals (vehicle treated) which solidify the safety profile of this treatment regimen (Fig. 7B). The issue of potential Ad5 dissemination was also evaluated in all adenovirus treated animals. Adenovirus E4 DNA was detected, albeit as faint bands, only in 40% and 30% of the treated animals in the liver and uterus respectively (Fig. 7A & Table 1). Interestingly, we did not find an evidence for significant dissemination of our target gene to any other adjacent or distal body organs. In this work, we have utilized first generation unmodified adenovirus vector. Recently, we have developed and tested several Adenoviral vectors that have been modified at the fiber level as well as at the promoter level and can be retargeted towards human uterine leiomyoma cells (93). We are actively pursuing these modified adenoviral vectors to ultimately develop the safest and most efficient adenoviral vectors for uterine leiomyoma gene therapy (93).

In conclusion, Adenovirus-mediated delivery of a dominant negative estrogen receptor gene by direct intra-tumor inoculation shrinks uterine leiomyoma tumor in Eker rats. This effect might be mediated in part through modulation of some estrogen regulated genes that control apoptosis, proliferation and extracellular matrix formation. Eker rats tolerated Ad-DNER without apparent toxicity or change in liver function tests. These studies provide essential preclinical data for the development of gene therapy as an alternative safe non-surgical treatment option for uterine leiomyoma.

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Figure 1.

Adenovirus distribution in eker rat uterine leiomyoma tissue after a single direct injection of Ad-lac Z 3×10^{10} pfu/cm³ of tumor.

Eker rat uterine leiomyoma tissue stained for X-gal: (a) 8 days post treatment versus (b) vehicle treated. Presence of blue stain indicates delivery and expression of β -galactosidase gene.

Figure 2.

Ad-DN-ER injection into eker rat uterine leiomyoma significantly shrinks total leiomyoma volume (compared to both vehicle control and Ad-LacZ treated animals). Tumor volume was calculated as a percentage of its corresponding day zero (pretreatment) volume and presented as $M \pm SE$ of at least three animals at each time point. a,b indicate significant difference from vehicle control and Ad-LacZ treated animals, respectively, at $p < 0.05$.

Figure 3.

Representative H&E micrographs from vehicle control (a), Ad-LacZ (b), and Ad- DN-ER treated fibroid lesions collected from Eker rats at 8, 15, 30 days (c-e) post treatment respectively [Original magnification 40 $\times$].

Ad-DN-ER treatment induces histological changes in eker rat fibroid tissues manifested by increase in apoptotic cells decreased cellularity, distorted and spare nuclei with smudged chromatin pattern increased hyalinized intercellular matrix.

Figure 4.

Ad-DN-ER treatment of fibroid lesions in Eker rats induces modulation of several estrogen regulated genes controlling both (A) intrinsic (p53, BAX, Bcl2 and PARP) and (B) extrinsic (DAXX and fas L) apoptotic pathways. Data represent uterine leiomyoma tissues collected at 8, 15 and 30 days post Ad-DNER treatment compared to either vehicle control or Ad-lacZ treated group: (A) Protein levels assessed by western blot; (B) DAXX and FAS L mRNA expression; (C) demonstrate increased Caspase 3 activity; (D) increase in the percentage of positive TUNNEL cells in the Ad-DN-ER treated lesions. The letters a or b indicate significant difference from vehicle control or Ad-lac Z treated lesions respectively at $p < 0.05$ using two tailed student T test in (C) caspase 3 experiment and Tukey test as post ANOVA test in (B) RT-PCR and (D) TUNNEL assay experiments.

Figure 5.

Ad-DNER decreases the expression of proliferation- and extracellular matrix formation-related genes

Immunohistochemical analysis of proliferation related gene expression (A) PCNA and (B) Cyclin D1 in eker rat uterine leiomyoma tissue sections collected from the animals treated with vehicle (control) (a), Ad-lacZ (b) or Ad-DN-ER collected 8 (c), 15 (d) and 30 days (e) post-treatment. (C) Expression TGFβ3 gene at mRNA level detected by RT-PCR amplification. The letters a, and b indicate significant difference in Ad-DNER from vehicle control and lacZ treated lesions at $p < 0.05$ using Tukey test as post ANOVA test.

Figure 6.

Ad-DN-ER induces humoral immune response in eker rat after direct intrauterine leiomyoma injection. Data are presented as Mean and Standard error of three animals results per group. ^aIndicates significant difference from negative control (no virus-treated group) at $p < 0.05$ using the Tukey test as a post-ANOVA test.

Figure 7.

Safety studies of the Ad-DNER gene therapy approach after a single direct intra-fibroid treatment of eker rat.

(A) Assessment of adenovirus particles dissemination into Eker rat body organs by PCR amplification of the specific Ad5 E4 region detected by 714pb DNA band on 1% agarose gel. The band was detected mainly in tumor tissues while faint bands were detected also in uterus (lane 11) and liver (Lane 12) respectively, in 40% and 30% of the treated animals. (B) Although Ad-DNER particles were detected in the liver of 40 % of eker rats injected with adenovirus, Ad-DNER did not produce any significant change in liver function tests (AST, ALT and total bilirubin) evaluated 30 days post adenovirus injection, compared to vehicle control animals.

Evaluation of Adenovirus dissemination in Eker rat organs after a single intrauterine leiomyoma injection of 3×10^{10} PFU/cm³ tumor.

Table1

Evaluated Vagina Organs	Cervix	Brain	Heart	Lung	Liver	Spleen	Kidney	Ovary	Uterus
% of E4 (+) organ Per Total Animals	0%	0%	0%	0%	30%	0%	0%	0%	40%