Gene therapy and uterine leiomyoma: a review

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Leiomyomas (fibroids) are common estrogen-dependent uterine tumours that cause significant morbidity for women and a substantial economic impact on health delivery systems. Currently, there is no effective medical treatment option for this condition—hysterectomy is the mainstay of management. This is not an attractive choice for many women, especially patients desiring to preserve their fertility potential. Gene therapy is becoming a clinical reality, with more than 600 clinical trials worldwide. Researchers have recently attempted to develop a gene-therapy-based approach for the ablation of uterine fibroids. The localized nature of this condition and its accessibility using different imaging or endoscopic techniques make it an attractive target for direct delivery of gene-based vectors. Recent work from our laboratory suggests the potential use of a dominant-negative form of estrogen receptor (ER) to inactivate estrogen signalling in leiomyoma cells and induce apoptosis. Our in vivo data in a mouse model demonstrate the ability of an adenovirus-expressing dominant-negative ER to arrest leiomyoma growth. We and others also have described the utility of the herpes simplex virus-thymidine kinase (HSV-TK) plus ganciclovir (GCV) suicide gene-therapy system to effectively eradicate leiomyoma cells by utilizing the bystander effect phenomena and the high expression of gap-junction protein in these tumours. Further work on rat models will pave the way for future leiomyoma gene-therapy clinical trials and allow the realization of gene therapy as a viable non-surgical option for this common problem in women’s health.

Key words: animal model/dominant-negative estrogen receptor/fibroids/gene therapy/uterine leiomyoma

Uterine leiomyoma: the problem

Uterine leiomyomas, known as fibroids, are the most common pelvic tumours in the US and occur in 20–25% of premenopausal women (Healy et al., 1986; Walker et al., 2000; Stewart, 2001). They commonly cause some severe symptoms such as heavy, irregular and prolonged menstrual bleeding and anaemia. Pelvic discomfort and bowel and bladder dysfunction from pressure are also common symptoms. Fibroids have also been associated with infertility and recurrent abortion (Farhi et al., 1995; Eldar-Geva et al., 1998; Hart et al., 2001; Surrey et al., 2001). These tumours tend to grow rapidly during pregnancy and can cause obstructed labour necessitating Caesarean section, fetal malpresentation and fetal anomalies, as well as post-partum haemorrhage secondary to uterine atony. These clinical complications seriously impact women’s health. Uterine leiomyoma is the most important indication for hysterectomy and accounts for 35% of the 600 000 hysterectomies performed in the US, and with this major surgery comes the associated morbidity and mortality, as well as a huge economic impact on healthcare delivery systems (Cramer and Patel, 1990; Calson et al., 1993). Pathologically, uterine leiomyomas are benign tumours that arise from a single uterine smooth muscle cell, and they grow in any part of the uterus (Figure 1) under the influence of local growth factors and sex hormones, including estrogen and progesterone (Coddington et al., 1986; Kawaguchi et al., 1989; Rein et al., 1995; Stewart and Nowak, 1996; Chegini, 2000; Sozen and Arici, 2002; Al-Hendy et al., 2004; Cook and Walker, 2004b; Shozu et al., 2004). Fibroids appear after menarche, proliferate and grow during the reproductive years and stabilize or regress after menopause. They may regrow after hormone replacement therapy (Rein et al., 1995). The diagnosis of fibroids is based on patient symptoms, followed by pelvic examination demonstrating a pelvic mass and confirmation by transabdominal or transvaginal ultrasonic measurements. The aetiology is not clearly understood, although several genetics and environmental factors have been proposed (Linder and Gartler, 1965; Nagele et al., 1996; Morton, 2000; Cook and Walker, 2004a; Gross et al., 2004; Pavlovich and Schmidt, 2004; Walker et al., 2004; Sandberg, 2005; Walker and Stewart, 2005; Wei et al., 2005).
Current therapy for uterine leiomyoma

Few treatment options are currently available to women with symptomatic fibroids (Vilos, 2000; Walker and Stewart, 2005). As no effective medical treatments are currently available (Vilos et al., 2001), the mainstay of treatment is surgery (Lefebvre et al., 2003). GnRH agonists inhibit steroidogenesis and induce chemical menopause and therefore can reduce fibroid volume up to 40% in 3 months, with a dramatic improvement in clinical symptoms (Buttram and Reiter, 1981; Andreyko et al., 1987; Friedman et al., 1991; Thorp and Katz, 1991; Friedman, 1993; Vercellini et al., 1998a). Unfortunately, these effects are short-lived, since the use of GnRH agonists can only be temporary (3–6 months) because of severe side effects and irreversible decrease in bone density (Andreyko et al., 1987). Fibroids tend to rapidly regrow after cessation of GnRH-agonist therapy, with the recurrence of severe clinical symptoms; therefore, their current Food and Drugs Agency (FDA)-approved use is limited to surgical adjuvant.

The two classical surgeries for treatment of uterine fibroids are myomectomy and hysterectomy (Vilos, 2000). Myomectomy, performed either through a laparotomy or through a laparoscopy, aims to remove the fibroid and conserve the uterus (Nezhat et al., 1991; Garcia, 1993; Dubuisson et al., 1996; Seinera et al., 1997; ACOG Committee on Practice Bulletins – Gynecology, 2001). This procedure is usually attempted in young women desiring future fertility (Friedman et al., 1992; Phillips, 1994; Brooks, 1995; Farhi et al., 1995; Goldenberg et al., 1995; Rosenberg, 1995; Nagele et al., 1996; Raders, 1999; Varasteh et al., 1999; Vercellini et al., 1999; Vilos, 1999; Vilos et al., 2000; American Society for Reproductive Medicine, 2001; Munro, 2001; Surrey et al., 2001). Unfortunately, 95% of any type of myomectomy is followed by extensive pelvic adhesions that can also preclude future fertility. In a recent meta-analysis, it was found that approximately two-thirds of women with uterine leiomyoma and otherwise unexplained infertility conceived after myomectomy (Vercellini et al., 1998b), although comparison with expectant management is needed before reaching definitive conclusions on the effectiveness of this time-honoured, conservative surgical procedure. Hysteroscopic myomectomy also appears to enhance fertility when compared with infertile women with normal uterine cavities (Varasteh et al., 1999; Vercellini et al., 1999). Hysterectomy has been the mainstay for the treatment of fibroids (Bachmann, 1990; Friedman and Haas, 1994; Weber et al., 1997; ACOG Committee on Practice Bulletins – Gynecology, 2001; Lefebvre et al., 2002). Between 1965 and 1987, more than $14 \times 10^6$ hysterectomies were performed in the US (Buttram and Reiter, 1981). Uterine leiomyoma accounts for approximately 67% of all hysterectomies performed among middle-aged women (Chryssikopoulos and Loghis, 1986). This surgical approach is extremely costly, especially considering the long post-operative recovery time. Although hysterectomy is a common and safe procedure, it carries a risk of major complications in 15–38% of cases (VeKaut, 1993). Such complications include post-operative haemorrhage, fever or injury to adjacent organs. The risk of death is 0.5 per 1000 cases.

Two relatively new modalities have been developed for treatment of uterine fibroids: myolysis and uterine artery embolization. Myolysis refers to the technique where an attempt is made to disrupt or abolish the blood supply to the fibroid and cause shrinkage using bipolar or monopolar electrosurgery (Gallinat and Leuken, 1993; Nisolle et al., 1993; Vilos, 1997; Zreik et al., 1998). The procedure is rarely performed and is not recommended for women who wish to get pregnant, since the risk of uterine rupture is very high (Goldfarb, 1992, 1995; Phillips, 1995; Phillips et al., 1997; Vilos et al., 1998). Uterine artery embolization is a procedure in which a radiologist attempts to cut the blood supply to the fibroid (Ravina et al., 1995; Bradley et al., 1998; Goodwin and Walker, 1998; Hutchins et al., 1999; Lee et al., 1999; Burbank and Hutchins, 2000; Liu, 2000; Pron et al., 2003a). This procedure is usually followed by severe pain requiring hospitalization for analgesia. No long-term follow-up is available yet, and several life-threatening complications as well as mortality have been reported (Bradley et al., 1998; Goodwin et al., 1999; Hutchins et al., 1999; Lanocita et al., 1999; Vashisht et al., 1999; Walker et al., 1999; Chrisman et al., 2000; Hurst et al., 2000; Nikolic et al., 2000; Pelage et al., 2000; Ravina et al., 2000; Siskin et al., 2000; Stringer et al., 2000; Amato and Roberts, 2001; American College of Obstetricians and Gynecologists, 2001; De Iaco et al., 2001, 2002; Felemban et al., 2001; Godfrey and Zbella, 2001; Spies et al., 2001; Payne and Haney, 2003; Tropeano et al., 2003; Pron et al., 2003c). Interestingly, in the few published reports so far, a modest decrease in the volume of the uterine leiomyoma (10–30%) was associated with a dramatic improvement in clinical condition and marked patient satisfaction (91–95%) (Ravina et al., 1995; Bradley et al., 1998; Goodwin and Walker, 1998; Hutchins et al., 1999; Pron et al., 2003a). Several concerns have been raised about this treatment, including future fertility as well as the possibility of missing other fibroids or uterine malignancy. Close to 5% of women undergoing uterine artery embolism develop subsequent amenorrhoea and menopause because of inadvertent impairment of ovarian function. Uterine artery embolization appears to be a promising, minimally invasive procedure in appropriately selected cases of symptomatic uterine fibroids (Hutchins et al., 1999; Tropeano et al., 2003). Long-term follow-up studies are being conducted, the results of which will help both gynaecologists and patients utilize this procedure in the treatment of uterine fibroids (SOGC, 2004; Chrisman et al., 2005; Dorenberg et al., 2005; Goldberg, 2005).

In summary, for a woman with symptomatic fibroids who wants to preserve her fertility, there is currently no conservative and safe...
Gene therapy of uterine leiomyoma

A naked DNA injection, without any carrier, into local tissues or into the systemic circulation is the simplest and safest physical/mechanical approach for gene therapy. Relatively little attention is needed on issues of complex formation and its safety assessment. So far, direct injection sites include skeletal muscle, liver, thyroid, heart muscle, urological organs, skin and tumours (Nishikawa and Huang, 2001). Indeed, the use of this approach is limited, at present, by its comparatively low efficiency. Converging evidence demonstrates that the delivery of therapeutic plasmid DNA to the non-proliferating cell nucleus is an inefficient process and is limiting for exogenously added transgene activity (Munkonge et al., 2003). To overcome this hurdle, attempts have been made to use the nuclear targeting motifs to direct the passage plasmid DNA across the nuclear membrane into the nucleus (Mattaj and Englmeier, 1998). Once a well-defined nuclear targeting signal is conjugated to the DNA of interest, the DNA-signal conjugate can cross into the nucleus and exhibit a higher expression rate. In addition, other technical hurdles have been encountered in the direct injection of naked DNA for gene-therapy purpose, such as the rapid degradation by nucleases and fast clearance by the mononuclear phagocyte system, the expression level and the area of tissue treated after a naked DNA injection (Lechardeur et al., 1999; Gallo-Penn et al., 2001). Consequently, great efforts have been made towards physical manipulations to improve the efficiency of gene delivery. These methods have also attracted interest for their potential ability to circumvent various barriers, which significantly compromise the efficiency of gene delivery—including massive dilution of DNA upon injection, accessibility of the target site and entry into the cell and the nucleus. Among the physical methods commonly used for gene delivery are microinjection, particle bombardment (gene gun) and electroporation.

Microinjection

Microinjection is the simplest gene-delivery method. Microinjection of naked DNA directly into the nucleus was shown to bypass cytoplasmic degradation, resulting in a much higher level of gene expression than intracytoplasmic injection. In fact, microinjections are too inefficient in their current state to be used for some in vivo gene-therapy applications. However, these techniques are useful for in vitro application (Hendrie and Russell, 2005). Furthermore, the in vitro application of microinjections is a laborious procedure; only one cell at a time can be injected, typically allowing for only a few hundred cells to be transfected per experiment. Therefore, microinjection, at its current technological level, is impractical for most, if not all, in vivo gene-delivery applications.

Gene gun

The concept of gene-mediated particle bombardment, gene gun, is to move naked DNA plasmid into target cells on an accelerated particle carrier with a device called the gene gun (Nishikawa and Huang, 2001). Microparticles (e.g. gold and tungsten) coated with plasmid DNA are accelerated by a force (e.g. helium pressure) to penetrate the cells and to deliver the DNA. The gold particles are typically 1–3 μm in diameter, and plasmid DNA is attached to the gold particles by precipitation in the presence of polyvinylpyrrolidone, the polycation spermidine and CaCl2. Because of their small size, the DNA-loaded particles penetrate the cell membrane and carry the bound DNA into the cell. The DNA dissociates from the gold particles and is expressed intracellularly (Benn et al., 1996).
Gene gun can achieve direct gene delivery into tissues or cells. Shooting gold particles coated with DNA allows direct penetration through the cell membrane into the cytoplasm and even the nucleus, bypassing the endosomal compartment. Among the advantages of particle-mediated bombardment are ease and speed of preparation of the delivery vehicle, the stability of the DNA preparation, the absence of (viral) antigens, the ability to target the projectiles to different tissue depths and areas and the rapid shedding of both particles and DNA if they are targeted to the epidermis (Davidson et al., 2000). The major application of this technology is genetic immunization, with the most obvious target being the skin. DNA immunization appears to be advantageous as it activates all pathways of immunity, especially cytotoxic T-cell responses, which have been difficult to induce with protein vaccines. Skin delivery technologies, including the gene gun, provide potentially safer alternatives to needle injection and promises increased efficacy in the prevention and/or therapy of infectious diseases, allergic disorders and cancer. The approach has been used for genetic vaccination, immunomodulation and suicide gene therapy to treat cancer.

Advantages of this gene-therapy approach are the applicability to different cell types and tissues and the ability to deliver large DNA molecules. The high loading capacity of the microprojectiles can allow the introduction of multiple genes into target cells. Another significant advantage is the applicability of this technology to in vivo gene transfer. Bombardment of many tissues including the skin, liver, pancreas, kidney and muscle resulted in readily detectable transgene activities (Andree et al., 1994; Benn et al., 1996; Mahvi et al., 1996; Sun et al., 1997). Current limitations of this technique are comparable with those of other non-viral transfection methods: gene expression is transient, cellular uptake is moderate and the frequency of stable gene integration is low.

Electroporation

Electroporation designates the use of electric pulses to transiently permeabilize the cell membrane. It has been shown that DNA can be transferred to cells through a combined effect of electric pulses causing permeabilization of the cell membrane and an electrophoretic effect on DNA, leading the polyanionic molecule to move towards or away from the electric field. Delivering the DNA using an electric field is now referred to as DNA electrotransfer or electro-gene transfer. There is strong evidence suggesting that the electro-gene transfer can be highly efficient, with low variability both in vitro and in vivo (Andree et al., 1994; Mir et al., 2005). Furthermore, the use of this electrogenethrapy provides a means for targeting the gene therapy, as the area transfected is restricted by the placement of the electrodes and is therefore highly controllable. Thus, there is an increasing use of electrogenethrapy to transfer therapeutic genes to various tissues (Belehradek et al., 1993; Mir et al., 1998; Gothelf et al., 2003). It is worth mentioning that the application of DNA electrottransfer in vivo is also interesting because it allows the transfer of genes into tissues without using a virus. Moreover, no chemical method works better in vivo than the direct electrottransfer of the naked DNA (Andre and Mir, 2004).

Synthetic vectors

Synthetic vectors provide opportunities for improved safety, greater flexibility and more facile manufacturing (Pack et al., 2005). In general, synthetic vectors (cationic lipids and polymers), complex with DNA, condense the genetic material into small complexes of plasmid DNA with cationic lipids and polymers are known, respectively, as lipoplexes and polyplexes. These small particles mediate their cellular entry and protect the delivered genes by sterically blocking the access of nucleolytic enzymes (Abdelhady et al., 2003). Technically, these vectors are relatively straightforward and easily scaled up and produced in large amounts (Worgall, 2005). In addition, lipoplexes and polyplexes have many advantages: their capacity to complex relatively large amounts of DNA, their versatility of use with any size or type of DNA/RNA, their ability to transflect even non-dividing cells, their stability and the fact that cellular transfection does not require specific receptors (Huang and Viroonchatapan, 1999; Worgall, 2005). Furthermore, they are likely to present minimal toxic or immunological problems, which allows for repeated administration without adverse immunologic reaction (Barron and Szoka, 1999).

Although the present forms of these synthetic vectors generally do not have the capacity for cell-specific targeting, the chemistry of the synthetic vectors can allow for the attachment of targeting moieties that allow both increased cell uptake and cell specificity (Pack et al., 2005). Ligand-directed tumour targeting of lipoplexes shows promise for targeted gene delivery and systemic gene therapy. Lipoplexes directed by ligands such as folate, transferrin or anti-transferrin receptor single-chain antibody (TfRscFv) showed tumour-targeted gene delivery and expression in human breast, prostate, head and neck cancers (Xu et al., 2001).

Although lipoplexes and polyplexes have numerous advantages, they suffer from inefficient gene transfer. In addition, the expression of foreign genes tends to be transient, precluding their use in many clinical applications requiring sustained and high-level expression of the transgene (Verma and Weitzman, 2005).

Viral vectors

The basic concept of viral vectors is to utilize the innate ability of the virus to deliver genetic material into the infected cells. Viral vectors are derived from viruses with either RNA or DNA genomes and are represented as both integrating and non-integrating vectors. The viral vectors include retrovirus, adenovirus, AAV, lentivirus and HSV. They are different in their immunogenicity, vector tropism, maximum size of gene that can be packaged and integration into the host genome leading to persistence of transgene expression in dividing cells. The characteristics of each of these viral vectors make them appropriate for specific applications.

Retrovirus

Retroviruses were one of the early vectors used to introduce DNA into cells, and they remain one of the most commonly used vectors for clinical trials (Culver et al., 1995). The basis for their enormous success includes the relative simplicity of their genomes, ease of use and long-term transgene expression in the transduced cells or their progeny. These characteristics render them ideal vectors for a stable correction of genetic defects.

Although the ability of retroviruses to integrate into the cell genome permits long-term transgene expression in the transduced cells or their progeny, it brings up the concern about their safety in clinical trials. The safety records of retroviruses have been challenged, since recent reports linked two cases of childhood leukaemia to retrovirus-based gene therapy (Check, 2003). In addition,
the limited cellular tropism of the natural envelop of the wild-type virus represents a barrier to retrovirus transduction and limited its utility in many applications (Somnia and Verma, 2000).

There are other serious limitations to the usefulness of retroviral vectors in the clinical application of gene therapy: (i) low vector titre (10^7 infection particles/ml); (ii) low transfection efficiency, demonstrated through in vitro experiments; (iii) particle instability and difficulty to concentrate and (iv) inability to transduce non-dividing post-mitotic cells, particles infecting only proliferating cells and the potential generation of replication-competent particles (Barquinero et al., 2004).

Retroviral vectors are suitable for ex vivo gene therapy, for example, for transducing CD34+ bone marrow haematopoietic stem cells (Hacein-Bey-Abina et al., 2002) or peripheral blood lymphocytes. Despite the disadvantages, retroviral gene-delivery systems have already been used in many clinical trials (Rainov and Ren, 2003). The inability of retroviruses to transfect non-dividing cells has restricted the in vivo applications to gene delivery in actively dividing cells such as stem cells and cancer cells (Verma and Weitzman, 2005).

Many attempts have been made, however, to circumvent these problems. Retroviral vectors have been re-engineered so that the retroviral insertion takes place only at the desired specific sites of the host cell chromosome. Using chromatin insulators can reduce the chances for retrovirus-mediated oncogenesis by inhibiting non-specific activation of nearby cellular proto-oncogenes (Yi et al., 2005). Furthermore, the use of self-inactivating (SIN) retroviral vectors or complete removal of the coding sequences for gag, pol and env genes can be utilized to virtually eliminate the possibility of generation of replication-competent retroviruses (Yi et al., 2005). Re-engineering retroviral vectors based on their ability to incorporate env glycoproteins from related and unrelated viruses or chimeric envelop protein allowed pseudotyping with alternative glycoproteins. This pseudotyping approach allows specific tropism to be conferred to retrovirus vectors for particular tissues (Mazarakis et al., 2001). In addition, targeted retrovirus-mediated gene transfer with high tissue specificity can be achieved by fusing part of the env gene to a coding sequence for the ligand to a tissue-specific receptor. Cotransduction of a suicidal gene under the control of an inducible promoter could also be an important safety feature, since destruction of transduced cells can be triggered if abnormal growth is observed. Additionally, conditional expression of the transgene only in appropriate target cells via the combination of targeted transduction, cell type-specific expression and targeted local administration will increase the overall safety of the retroviral systems (Yi et al., 2005). Furthermore, splitting the viral genome, or complete deletion of the coding sequences for gag, pol and env genes, can be implemented to virtually eliminate the possibility of generation of replication-competent retroviruses.

**Adenovirus vectors**

Replication-incompetent adenoviral vectors (Ad) have emerged as a very popular and safe vector for human gene therapy and will soon be the most commonly used vehicle in clinical trials (Hallenbeck and Stevenson, 2000; Journal of Gene Medicine, 2005). The use of adenovirus vectors in gene therapy is advantageous because of many positive attributes: (i) Ad has the ability to provide efficient in vivo gene transfer to both dividing and non-dividing cells; (ii) Ad has exhibited higher in vivo stability; (iii) Ad stocks can be prepared to high concentrations (10^13 particles/ml) and are purified with cesium chloride gradient ultracentrifugation or chromatography, which will allow the delivery of large amounts of viral particles in finite volumes (Kozarsky and Wilson, 1993); (iv) Ad is non-oncogenic and stays episomic, thus ensuring less disruption of vital cellular genes and its relative safety; (v) transgenes of up to 4.7–4.9 kb can be incorporated, and the cloning capacity of Ad can be further increased to 8.3 kb by deleting additional dispensable sequences from Ad genome; (vi) Ad has the advantage of supporting high levels of gene targeting to the nucleus—resulting in significant gene expression [see (i)] (Hallenbeck and Stevenson, 2000) and (vii) Ad tropism can be manipulated by inserting of receptor-binding ligands into the major capsid proteins, permitting cell-specific transfection.

The utility of adenovirus in tumour gene therapy is substantiated by the concept of transductional targeting, which aims at specific transduction of the target cells. Transductional targeting decreases the tropism of adenovirus towards the normal cells and enhances the ability of the virus to infect tumour cells (Kanerva and Hemminki, 2005). Transcriptional targeting can be achieved by placing the transgene under the control of tissue- or tumour-specific promoters. Another improvement is the recent introduction of conditionally replicative adenoviral vectors for tumour gene therapy (Gomez-Navarro and Curiel, 2000). In this instance, the viral genome and subsequent metabolism will be modified in such a way that it will only be complemented by the tumour cell. The virus will only be able to replicate within the tumour and not in normal tissues. Several adenovirus examples have been developed for diseases such as cervical and prostate cancer (Alemany et al., 2000; Heise and Kim, 2000; Hermiston, 2000).

There are several challenges facing the large-scale application of Ad as a method for gene delivery. The capacity of the Ad vector to transfect target cells depends on the levels of expression of integrin and coxsackievirus and adenovirus receptor (CAR). Thus, cells expressing CAR and integrins below the threshold level are refractory to Ad infection, for example, smooth muscles, endothelial cells, skeletal muscles, fibroblasts and haematopoietic cells (Barnett et al., 2002). In addition, it has been observed that most of the primary tumour cells express low level of CAR compared with normal tissues. Thus, most of the tumour cells are poorly transfected with Ad. An additional factor that add to this difficulty is that part of the delivered vectors will be sequestered by CAR-expressing non-target cells (Kim et al., 2002).

Extensive attempts have been made to circumvent such negative attributes that limit the expansion of Ad vector deployment in gene therapy studies. For instance, new serotypes of Ad vectors have been developed. These new serotype Ad vectors transduce the target cells utilizing CD46 as a cellular receptor rather than the CAR utilized by the Ad5-based vector. Thus, these new serotypes of adenovirus vectors are able to efficiently infect cells by expressing no or low levels of CAR (Stone et al., 2003; Vogels et al., 2003).

An alternative approach to overcome the problem of low transfectability of most tumour cells because of lower CAR expression is to modify the Ad vector tropism. Strategies to modify Ad tropism have focused on alterations to the fibre component of the viral capsid to allow CAR-independent gene transfer. The initial step of Ad infection involves an interaction between the Ad fibre and CAR. However, this interaction is not essential, per se, for infection and only serves as a means of bringing the viral particle into intimate contact with the cellular surface (Barnett et al., 2002). Other tumour-specific receptors could serve the same purpose. Thus, modification of the Ad vector to ablate CAR binding and to redirect binding to other tumour-specific receptors...
would allow for a CAR-independent, targeted vector. It has been reported that by using a bifunctional conjugate consisting of a blocking anti-adenoviral knob Fab linked to tumour-specific receptor, the gene transduction of tumour cells was increased by 7.7- to 44-fold (Goldman et al., 1997a,b). In addition to enhancing the transfectability of tumour cells, this approach has been shown to decrease the dose of Ad needed to mediate the infection of a given number of tumour cells (Rancourt et al., 1998).

Despite the widespread application of the adenovirus, particularly serotype Ad5, broad potential therapeutic applications are limited by the development of vector-neutralizing antibodies within the human population and the inability of Ad5-based vectors to transduce important therapeutic target cell types (Stone et al., 2005). Ad causes an initial non-specific host response, followed by a specific response of cytotoxic T lymphocytes directed against virus-infected cells. In addition, there is activation of B cells and the necessary CD4+ T cells, leading to a humoral response. Serologic surveys found antibodies against Ad serotypes 1, 2 and 5 in 40–60% of children. The cellular immunity eliminates transduced cells, whereas humoral immunity precludes the repeated administration of the Ad vector (Dai et al., 1995; Tripathy et al., 1996; Kafri et al., 1998). This immune response of the host against adenoviral proteins is the major hurdle to the efficient and safe use of adenoviral vectors.

Several attempts have been made to minimize the immunological reaction to Ad. One approach has been to use Ad vectors derived from non-human adenovirus. For example, a simian viral vector has been developed that avoids the pre-existing immunity found in humans (Xiang et al., 2002). Another approach to avoid antibody-mediated neutralizing immunity, as well as clearance mechanism, has been to coat the vector with a polymer such as polyethylene glycol or poly-[N-(2-hydroxypropyl)methylacrylamide]. Incorporation of targeting ligands on the polymer-coated virus produces ligand-mediated, CAR-independent binding and uptake into cells bearing appropriate receptors. This retargeted virus is resistant to antibody neutralization and can selectively infect receptor-positive target cells (Croyle et al., 2000; Fisher et al., 2001). In an alternative approach to the derivation of cancer cell-specific vectors, Ad vectors have been targeted at the level of transcription by placing the therapeutic gene under the control of transcriptional regulatory sequences that are activated in tumour cells but not in normal cells; therefore, the expression is selectively targeted to the tumour cell. A higher degree of specificity for cancer cells can be achieved by combining the complementary approaches of transductional and transcriptional targeting (Glasgow et al., 2004; Lu et al., 2005).

Worldwide, over 600 clinical trials using gene therapy have been conducted or are underway, and Ad vectors are the most commonly used vectors in these trials (Verma and Weitzman, 2005).

**AAV**

AAV vectors are single-stranded DNA human parvoviruses that are dependent on a helper virus, usually an adenovirus, to proliferate. They are capable of infecting dividing and non-dividing cells. AAV is unique among eukaryotic DNA viruses in its ability to integrate at a specific site within the human chromosome (19q13.3-pter). Integration of AAV into the genome of the transduced cells is a double-edged sword: (i) it maintains the expression of the therapeutic gene in progeny cells and (ii) it may increase the risk of mutations that are deleterious to the host. Because of the prolonged transgenic expression and the non-pathogenic nature of AAV, they become increasingly attractive as a vector for gene delivery (Rutledge and Russell, 1997). Several serotypes of AAV have been developed with different tropism to different tissues. Interestingly, different serotype capsids can be combined to generate novel tropisms. This will allow AAV to transfect tissues that have not been easily infected with the current AAV serotypes and may become amenable to AAV-based gene transfer in the future. In contrast to adenovirus vectors, AAV vectors do not cause a significant host inflammatory response (Zaiss et al. 2002). No human disease has been associated with AAV infection. A significant limitation of AAV as vectors, however, is that the viral genome can only accept DNA inserts up to 4.7 kb in length (Smith, 1995).

Other viral systems are at various stages of development, and each has its own unique features including HSV (Bowers et al., 2003), measles (Fielding, 2005), parvovirus (Ponnazhagan, 2004) and many others. Each of these vectors has certain advantages and disadvantages, and they can be used accordingly for certain applications.

**Tumour gene-therapy strategies**

Advances in molecular and tumour biology have contributed greatly to our understanding of the genetic alterations associated with tumour transformation. Thus, gene-therapy strategies have been proposed, which target alterations specific to tumour cells and tumour pathophysiology. These treatment strategies include mutation compensation, molecular chemotherapy, immunopotentiation and alteration of drug resistance.

**Mutation compensation**

Mutation compensation involves correction of the genetic lesions that are aetiologic for neoplastic transformation (Rosenfeld and Curiel, 1996). This gene-therapy strategy is also known as correctional gene therapy and focuses on functional ablation of expression-dysregulated oncogenes, replacement or augmentation of the expression of tumour-suppressor genes or interferences with signalling pathways of some growth factors or other biochemical processes that contribute to the initiation or the progression of the tumour (Elledge and Allred, 1994; Barnes et al., 1997; Seth, 2005; Watanabe et al., 2005). The purest examples of this gene-therapy strategy are the restoration of normal function of tumour-suppressor genes and the blocking of oncogene activity. Several approaches have been used in the mutation compensation gene-therapy strategy. These include antisense oligonucleotide, catalytic ribozymes and small oligonucleotides, dominant-negative gene mutation and, most recently, small interfering RNA (siRNA) technology.

**Molecular chemotherapy**

Molecular therapy, which is known also as cytoreductive gene therapy or suicide gene therapy, refers to the delivery of genes that kill cells by direct or indirect effects. Strategies include gene-directed enzyme prodrug therapy, proapoptotic, anti-angiogenic and gene-directed radioisotopic therapy. Gene-directed enzyme prodrug therapy involves delivering a gene that encodes an enzyme, which then converts an innocuous prodrug to a toxic agent within tumour cells. The two most widely used gene-directed enzyme prodrug systems are HSV-thymidine kinase (HSV-TK) plus ganciclovir (GCV) and Escherichia coli bacterial cytosine deaminase plus 5-fluorocytosine (Greco and Dachs,
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There are certain criteria for the ideal gene-directed enzyme prodrug therapy. The HSV-TK system has many attributes that make it the frontrunner of cytoreductive gene-therapy strategies. Most importantly, the active GCV metabolite diffuses to adjacent untransduced tumour cells to mediate a bystander effect.

**Immunopotentiation**

Modulation of immune response is particularly attractive as a modality for cancer gene therapy. A key focus of tumour gene therapy is the enhancement of the immune system’s ability to destroy tumour cells. Passive immunopotentiation involves boosting the natural immune response to make it more effective. Active immunopotentiation requires the initiation of an immune response against a previously unrecognized tumour. The immunopotentiation gene therapy capitalizes on strategies such as the expression of cytokine genes which may enhance the activity of antigen-presenting cells and T cells (Tepper and Mule, 1994), the expression of co-stimulatory molecules, such as B7.1 and B7.2, which facilitate the recognition and killing of tumour cells (Dohring et al., 1994) or the delivery of exogenous immunogens, which generate local inflammatory reaction that increases the ability of antigen-presenting cells to recognize tumour-associated antigens (Dalgleish, 1996).

**Alteration of drug resistance**

A major hurdle in cancer chemotherapy is anti-cancer drug-related toxicity, mainly the myelosuppression. The development of myelosuppression necessitates the discontinuation of chemotherapy, which may result in failure of cancer treatment. Multidrug resistance gene (MDR1) confers multidrug resistance towards several anti-cancer drugs. Gene-therapy approaches involving retroviral transfer of MDR1 gene into haematopoietic stem cells and progenitor cells before autologous transplantation have been used in the treatment of human germ cell tumours (Abonour et al., 2000) and metastatic breast cancer (Cowan et al., 1999). For cytotoxic drugs that are not substrates for MDR1, such as cyclophosphamide and methotrexate, retroviral transfer of aldehyde dehydrogenase class 1 gene (ALDH-1) and mutated dihydrofolate reductase gene to haematopoietic progenitor cells protects against the myelosuppression induced by these drugs (Takebe et al., 2001).

**Gene therapy of uterine leiomyoma**

Current treatments for uterine leiomyoma are far from satisfactory and leave much to be desired. This is particularly relevant for women with symptomatic uterine fibroids who want to preserve their procreation potential and would not accept the fertility-precluding option of hysterectomy. Clearly, the development of a safe, effective, non-surgical and localized method of treatment for uterine fibroids would greatly benefit many women, especially with more women delaying childbirth into the age range when symptomatic fibroids are most prevalent. Applying gene-therapy approaches to uterine leiomyoma could possibly provide such a treatment.

Development of fibroids involves complex interactions among genes and the environment. Studies of the underlying pathophysiology are beginning to show how specific genes might reverse or retard disease processes at the cellular level. This general approach may prove to be clinically efficacious despite genetic aetiology and without the need to completely replace a single, missing gene product. 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 (Figure 1), which conceivably could simplify targeting the viral load to the tumour by direct ultrasound-guided or endoscopic-mediated intratumour injection using existing technology. This type of approach could avoid the easily identified surrounding healthy myometrium. Using intratumour injections of gene-therapy vectors will minimize the systemic toxicity and avoid the development of an immunologic reaction. Subsequently, this will permit a prolonged expression of transgenes, allow for repeated administration of Ad vector and minimize the amount of vector required to reduce the tumour size. Alternatively, the viral vector can be delivered by transarterial injection by administering the inoculum through a uterine artery catheter threaded to the artery feeding the fibroid. This technology is currently available and is increasingly used for uterine artery embolization (Lee et al., 1999; Burbank and Hutchins, 2000; Liu, 2000). This method would ensure homogenous distribution of the injected virus and may improve the efficiency of cell transfection. Another favourable feature is that uterine fibroids are slow-growing tumours, and marked clinical improvement of fibroid-related symptoms (e.g. irregular vaginal bleeding, pelvic pain and infertility) does not necessitate complete resolution of the fibroid but rather a modest decrease in their size (Vilos, 2000). Uterine artery embolization literature clearly documents this, where a modest decrease in the volume of the uterine leiomyoma was associated with marked improvement in clinical conditions and patient satisfaction (Ravina et al., 1995; Bradley et al., 1998; Goodwin and Walker, 1998; Hutchins et al., 1999). Unlike cancer gene therapy, gene delivery does not have to be achieved in every leiomyoma cell to attain clinical improvement. This is a great advantage because with current gene-therapy vectors, it is virtually impossible to achieve 100% gene transfer in vivo (Niu et al., 1998).

The first attempt to apply the gene-therapy approach to uterine leiomyoma was reported by the Christian group (Niu et al., 1998). They described a non-viral-mediated transfer of the suicide gene for TK into Eker leiomyoma tumour-derived cell-line 3 (ELT3) rat leiomyoma cells and human leiomyoma cells. In this study, a TK-GCV-mediated ‘bystander effect’ was demonstrated, with 48.6% (human) and 65.6% (rat) cell death occurring when 5% of the leiomyoma cells were transfected with the pNGVL1-TK vector; 0.84 and 1.9% of the cells were expected to express TK based on the 16.7 and 65.6% (rat) cell death occurring when 5% of the leiomyoma was reported by the Christman group (Niu et al., 1998). These findings are of particular significance for the uterine leiomyoma, which conceivably could simplify targeting the viral load to the tumour by direct ultrasound-guided or endoscopic-mediated intratumour injection using existing technology. This type of approach could avoid the easily identified surrounding healthy myometrium. Using intratumour injections of gene-therapy vectors will minimize the systemic toxicity and avoid the development of an immunologic reaction. Subsequently, this will permit a prolonged expression of transgenes, allow for repeated administration of Ad vector and minimize the amount of vector required to reduce the tumour size. Alternatively, the viral vector can be delivered by transarterial injection by administering the inoculum through a uterine artery catheter threaded to the artery feeding the fibroid. This technology is currently available and is increasingly used for uterine artery embolization (Lee et al., 1999; Burbank and Hutchins, 2000; Liu, 2000). This method would ensure homogenous distribution of the injected virus and may improve the efficiency of cell transfection. Another favourable feature is that uterine fibroids are slow-growing tumours, and marked clinical improvement of fibroid-related symptoms (e.g. irregular vaginal bleeding, pelvic pain and infertility) does not necessitate complete resolution of the fibroid but rather a modest decrease in their size (Vilos, 2000). Uterine artery embolization literature clearly documents this, where a modest decrease in the volume of the uterine leiomyoma was associated with marked improvement in clinical conditions and patient satisfaction (Ravina et al., 1995; Bradley et al., 1998; Goodwin and Walker, 1998; Hutchins et al., 1999). Unlike cancer gene therapy, gene delivery does not have to be achieved in every leiomyoma cell to attain clinical improvement. This is a great advantage because with current gene-therapy vectors, it is virtually impossible to achieve 100% gene transfer in vivo (Niu et al., 1998).

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The exact mechanisms underlying the bystander effect are not clear. It has been reported, however, that the transfer of phosphorylated GCV through direct cell–cell contact (gap junctions) or through apoptotic vesicles between TK-expressing and TK-non-expressing tumour cells is involved (Freeman et al., 1993). This efficient bystander effect manifested in leiomyoma cells was substantiated by our recent finding that the expression of connexin 43, a protein that forms gap junctions between cells in various mammalian tissues, is high in leiomyoma tissues compared with the adjacent normal myometrium (Figure 2) (Marwa et al., 2005); similar findings were also previously reported by Andersen (2000). These findings...
suggest that the bystander effect will likely be operational when TK-GCV is applied in vivo and would greatly enhance the efficacy of this gene-therapy approach. Additionally, the efficient intercellular gap-junction communication in leiomyoma compared with the normal myometrium would also provide a safety margin for the utility of this suicidal gene-therapy strategy—thus limiting the transfer of the toxic GCV-triphosphate to the normal myometrium exhibiting lower gap-junction expression and physically separated from the tumour tissue by the well-developed avascular capsule. Our recent application of the HSV-TK/GCV to human and rat leiomyoma cells in vitro using an adenoviral delivery system confirmed the above finding (Marwa et al., 2005). We are currently testing this approach in different animal models for uterine leiomyoma.

We have also recently applied a different gene-therapy strategy for uterine leiomyoma. Since steroid hormones, estrogen and progesterone play a pivotal role in the development and progression of leiomyoma, it is conceivable that the signalling pathway of these hormones may represent a potential target for gene therapy of leiomyoma.

We proposed to use a dominant-negative estrogen receptor (ER) to intercept the estrogen-signalling pathway. These mutants work by forming heterodimers with wild-type ER, which make it unable to bind the estrogen-responsive element (ERE) and unable to activate transcription when bound to ERE (Ince et al., 1993). First, we tested whether adenoviral vectors are able to infect leiomyoma cells. We assessed the ability of Ad-LacZ, an adenovirus expressing a marker gene, to infect human and rat leiomyoma cells. LM-15, a human leiomyoma cell line, was successfully transduced with Ad-LacZ, with maximal (100%) transduction achieved at a multiplicity of infection (MOI) of 10 PFU/cell (Figure 3) (Al-Hendy et al., 2004). Rat leiomyoma cells reached optimal transduction at an MOI of 100 PFU/cell. We also assessed the ability of adenovirus to infect fresh uterine leiomyoma tissue disks 2–3 mm in diameter directly removed from hysterectomy specimens. The tissues were incubated with an adenovirus expressing a marker gene (Ad-LacZ) and followed by X-gal staining. A clear nuclear blue colour (indicating virus transduction) was noticed in the smooth muscles of the leiomyoma tumour (Figure 4).

To perturb the estrogen-signalling pathway, we used adenoviral vectors expressing a dominant-negative ER mutant (ER1–536) under a cytomegalovirus (CMV) promoter (Ad-ER-DN) (Al-Hendy et al., 2004). In an in vitro system using Ad-ER-DN to transduce both human and rat leiomyoma cell lines, Ad-ER-DN induced an increase in both caspase-3 levels and the Bax/Bcl-2 ratio, with evident apoptosis in the TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labelling (TUNEL) assay (Figure 5). In nude mice, rat leiomyoma cells ex vivo transduced with Ad-ER-DN supported significantly smaller tumours compared with Ad-LacZ-treated cells 5 weeks after implantation. In mice treated by direct intratumour injection into pre-existing lesions, Ad-ER-DN caused immediate overall arrest of tumour growth (Figure 6). In addition,
the Ad-ER-DN-treated tumours demonstrated severely inhibited cell proliferation (BrdU index) (Figure 7) and a marked increase in the number of apoptotic cells (TUNEL index) (Figure 8). Our study clearly demonstrated that dominant-negative ER gene therapy may provide a non-surgical treatment option for women with symptomatic uterine fibroids who want to preserve their uterus.

Figure 4. Adenovirus-infected human leiomyoma explants. Fresh uterine leiomyoma tissue disks 2–3 mm in diameter were incubated for 5 h with Ad-LacZ, 109 PFU/ml, followed with X-gal and haematoxylin–eosin staining. Clear blue colour was demonstrated in the nuclei of smooth muscle tumour cells. [Reprinted from Al-Hendy et al. (2004) with permission from Elsevier.]

Figure 5. Adenovirus with dominant-negative estrogen receptor (ER) induced positive TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labelling (TUNEL) reaction in human leiomyoma cells. Human LM-1 cells were infected with the corresponding virus and assayed for TUNEL 5 days later. In Ad-ER-DN-treated cells, 40–50% of nuclei demonstrated positive TUNEL staining compared with less than 4% in Ad-LacZ-treated cells. [Reprinted from Al-Hendy et al. (2004) with permission from Elsevier.]

Figure 6. Direct intratumour injection of adenovirus with dominant-negative estrogen receptor (ER) inhibited tumour growth. Direct intratumour injection of the corresponding agent was performed on day 16 after cell implantation. (A) Ad-ER-DN treatment caused immediate overall arrest of tumour growth. The difference among treatment and control groups was highly significant ($P = 0.007$). Results are mean ± SD ($n = 10$) of two independent experiments. (B) Tumours treated with Ad-ER-DN demonstrated inhibition of tumour progression. [Reprinted from Al-Hendy et al. (2004) with permission from Elsevier.]
It is worth mentioning that the two studies reporting on fibroid gene therapy were conducted using the ELT3 cell line (Niu et al., 1998; Al-Hendy et al., 2004). ELT3 cells may exhibit quantitative and qualitative differences compared with the human leiomyoma cells. However, data obtained from these two studies does indeed prepare the stage for preclinical studies using experimental models more relevant to human leiomyoma. Furthermore, in vivo animal data obtained thus far have been generated in the immune-deficient nude mouse model. Although leiomyoma nodules developed in this model resemble the human disease in many features (Howe et al., 1995; Hodges et al., 2001), the lack of cell-mediated immunity might present an advantageous environment for the final efficacy of adenovirus transfection and longevity of gene expression (Al-Hendy et al., 2000). Experiments are currently underway in our laboratory to extend these experiments to the Eker rat—the only immune-competent rat model for uterine leiomyoma (Everitt et al., 1995). Such experiments will provide valuable preclinical data, particularly since the fibroid lesions in these rats are in the uterine horns or cervicouterine junction and share various anatomical, histological and biological features with human leiomyoma (Houston et al., 2001; Cook and Walker, 2004a).

Conclusions and future directions

Current non-surgical management options for uterine leiomyomas are frequently insufficient, and new treatment strategies are therefore needed. Novel therapies for disabling such diseases should improve safety, efficacy and cost. Since successful gene therapy relies on the delivery of the therapeutic gene to the target organ or disease site, gene-based therapeutic approaches for management of uterine leiomyomas may represent future viable alternatives, especially for women who want to preserve their fertility. In

![Figure 7. BrdU incorporation of subcutaneous leiomyoma specimens from nude mice. Tissue samples of leiomyoma tumours were collected from mice treated by direct intratumour injection with Ad-LacZ, medium, or Ad-ER-DN at 2 weeks after treatment and processed for BrdU immunostaining. Representative BrdU-labelled tumour sections were counterstained with haematoxylin and eosin (×200) for each treatment option. (A) Ad-LacZ (negative control). (B) Medium alone (negative control). (C) Ad-ER-DN. (D) Significantly less BrdU staining = proliferation was detected in tumours treated with Ad-ER-DN (P < 0.0001). Results are mean ± SD of three different experiments. [Reprinted from Al-Hendy et al. (2004) with permission from Elsevier.]](http://humupd.oxfordjournals.org/).
Gene therapy of uterine leiomyoma

In general, gene therapy requires identification of therapeutic gene(s) and the method for delivering the transgene(s) to the target cells with high efficiency for an appropriate time period. Further understanding and uncovering of the genetic and environmental mechanisms responsible for uterine leiomyoma will aid in designing rational gene-therapy protocols. Additional research is needed to improve our understanding of the genetic and cellular mechanisms of leiomyomas. The development of uterine fibroids involves complex interactions among genes and the environment. Studies of the underlying pathophysiology are beginning to show how specific genes might reverse or retard disease processes at the cellular level. This general approach may prove to be clinically efficacious despite genetic aetiology and without the need to completely replace a single, missing gene product. For instance, dominant-negative ER (delivered via an adenovirus) and Ad-HSV-TK/GCV provide two promising approaches to induce apoptosis and eventually shrink leiomyoma, and these approaches could conceivably constitute the basis for future clinical trials.

Several other biological pathways in leiomyoma represent potential targets for gene-therapy application. For instance, several lines of evidence have suggested that the apoptotic mechanisms are down-regulated in leiomyoma cells compared with normal myometrium (Hoffman et al., 2004). Thus, gene therapy of leiomyoma can be executed by switching on the apoptosis mechanism through both extrinsic and intrinsic pathways [by delivering apoptosis-inducing ligands, such as tumour-necrosing factor (TNF), TNF-related apoptosis-inducing ligand (TRAIL) and FasL or by delivering proapoptotic members of the Bcl-2 family, such as Bax or active caspase molecules]. Angiogenesis, which is essential for leiomyoma growth, is driven by different growth factors such as basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and platelet-derived endothelial

Figure 8. TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labelling (TUNEL) reaction on subcutaneous leiomyoma specimens from nude mice. Tissue samples of leiomyoma tumours were collected from mice treated by direct intratumour injection with either Ad-LacZ, medium, or Ad-ER-DN at 2 weeks after treatment and processed for TUNEL assays. Fluorescent staining indicates TUNEL, which signifies DNA fragmentation and apoptosis. (A) Ad-LacZ (negative control). (B) Medium alone (negative control). (C) Ad-ER-DN. (D) Significantly more apoptotic nuclei were detected in tumours treated with Ad-ER-DN ($P < 0.0001$). Results are mean ± SD of three different experiments. [Reprinted from Al-Hendy et al. (2004) with permission from Elsevier.]
growth factor (PDGF) (Hyder et al., 2000; Gentry et al., 2001; Hong et al., 2001; Di Lieto et al., 2005). Each of these angiogenic factors represents a potential gene-therapy target.

To optimize gene therapy of leiomyoma, the expression of the transgene should be restricted exclusively to leiomyoma. Selective expression of transgenes in leiomyoma can be achieved by transductional and/or transcriptional targeting. Fortunately, the localized nature of leiomyoma allows transductional targeting of gene therapy. For transcriptional targeting, however, the biological uniqueness of leiomyoma (such as leiomyomas-specific transcription factors or proteins) should be identified. This identification will allow the design of leiomyoma-specific promoter/vectors driving therapeutic gene expression only in the leiomyoma tumour cells. Generating improved delivery systems and refining the mechanisms that control gene expression will be a key step in the development of safe clinical protocols of gene therapy of uterine leiomyoma.

Gene therapy is a promising treatment strategy that might find a place among management options for women with uterine fibroids, either alone or as an adjuvant to existing treatment modalities. Because of the unique, localized and tumour-targeting features of the gene-therapy approach, it might be particularly suited for women with symptomatic fibroids who are keen on future fertility.

References


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