Establishment of a Novel Xenograft Model for Human Uterine Leiomyoma in Immunodeficient Mice

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Uterine leiomyomas are the most common gynecological benign tumor and greatly affect reproductive health and wellbeing, but the pathophysiology and epidemiology of uterine leiomyoma are poorly understood. One of the major reasons for the slow progress in leiomyoma research is the lack of a good in vivo model system. We therefore aimed to develop a novel model by transplanting human uterine leiomyoma xenografts in an immunodeficient mouse strain (NOD/SCID/γc-null: NOG). Human uterine leiomyoma tissues were cut into small pieces and inserted subcutaneously into the right and left flanks of NOG mice. Estrogen supplementation was needed to maintain the features of uterine leiomyoma in xenografted tissues. After 4 weeks or 8 weeks of transplantation, xenografted tissues were harvested and analyzed regarding tissue morphology, collagen content, and proliferation and apoptosis of uterine leiomyoma smooth muscle cells. The xenografts that were harvested after 4 weeks and 8 weeks retained the histological architecture of original uterine leiomyoma tissue both in cellular and collagen components. The expression profiles of key markers of uterine leiomyoma were also maintained, including estrogen receptor, progesterone receptor, and α-smooth muscle actin, as judged by immunohistochemical staining. The proportion of proliferating cells was significantly increased (1.5-fold) in the xenografts after 8 weeks of transplantation, whereas that of the apoptotic cells remained unchanged. Importantly, the reproducible results were obtained with the tumor tissues derived from six patients. The present in vivo model may provide a useful tool for development of novel therapeutic strategies for uterine leiomyoma.

Keywords: non-obese diabetic; severe combined immunodeficient; γc-null mice; uterine leiomyoma; xenotransplantation; leiomyoma model; estrogen


Uterine leiomyomas are the most common gynecological benign tumor. They cause health problems such as infertility, menorrhagia, anemia and pelvic pain. Uterine leiomyomas are the predominant indication for hysterectomy in premenopausal women and account for 33% of all hysterectomies (over 200,000 annually) in the United States (Wilcox et al. 1994; Lepine et al. 1997). Numerous studies have shown that the growth of leiomyomas depends on the ovarian hormones estrogen (E₂) and progesterone (Rein 2000). Despite its high prevalence, the pathophysiology of leiomyoma is poorly understood (Walker and Stewart 2005). One of the major causes of the slow progress in leiomyoma research is the absence of a good in vivo model system to study these tumors.

The Eker rat is a well-known animal model of uterine leiomyoma. Female Eker rats have spontaneous uterine leiomyomas with a frequency of approximately 65% (Howe et al. 1995). Eker rat leiomyomas share phenotypic, biochemical and genetic characteristics with human uterine leiomyomas (Walker et al. 2003). Although the Eker rat is the most valuable model for uterine leiomyoma, there are critical limitations regarding low penetrance, high cost and sarcomatous histology. Recently, to overcome such limitations, Memy I, a novel human uterine leiomyoma xenograft model, was developed. Human uterine xenografts pretreated with both adenoviral-cyclooxygenase-2 (COX-2) and adenoviral-vascular endothelial growth factor-A (VEGF-A) were implanted subcutaneously into severe combined immunodeficient (SCID) mice (Hassan et al. 2008). In this model, human leiomyoma tissue could be engrafted and survive for at least 1 month. This model has great advantages of low cost and enabling the study of in vivo cultures from human leiomyoma. On the other hand, the disadvantages of this model are that it needs an adenoviral transfection...
tion system and the overexpression of COX-2 and VEGF-A may change the character of human leiomyoma tissue itself.


The aim of this study was to develop a human uterine leiomyoma xenograft model using NOG mice.

Materials and Methods

All procedures for collecting human specimens and animal experiments in this study were approved by the Ethics Committee and the Animal Care and Use Committee of Tohoku University School of Medicine.

Animals

Eight-week-old female NOG mice were purchased from Central Institute for Experimental Animals (Kawasaki, Japan). Serum estrogen levels were maintained at high levels by subcutaneous implantation of 1.5 mg, 60-day sustained-release 17β-estradiol pellets (Innovative Research of America, Sarasota, FL, USA) as described previously (Hassan et al. 2008). The pellets were implanted 2 days before the tissue transplantation. We confirmed the rise of serum concentration of E2 at 8 weeks after implantation using an enzyme-linked immunosorbent assay (Cayman Chemical Co., Ann Arbor, MI, USA). The mean serum concentrations of E2 with and without pellet were 528.5 ± 9.3 pg/ml (n = 6) and 132.4 ± 4.2 pg/ml (n = 6), respectively.

Animals were housed in micro-isolator cages in a barrier facility under well-controlled, pathogen-free conditions. Monitored ambient temperatures were kept constant at 22°C; animals were maintained in a 12-hour light/dark cycle. All housing materials were autoclaved before use. Mice were fed a laboratory diet and water ad libitum.

Tissue harvest and implantation

Human leiomyoma tumor tissues were collected from six Japanese female patients (aged 42-48 years) at the time of hysterectomy. Later, these samples were histopathologically confirmed as uterine leiomyoma by a pathologist. The fibroid tissues used in the study were from a single, large and intramural fundal location. We used tissue samples from the same patient for all animals in one experiment. Fibroid tissues were harvested aseptically and then placed on ice. Tissue samples were cut into square-shaped pieces 3 mm in length and 2 mm in thickness at the broadest end. Squares were randomly divided into experiment groups. For transplantation, three NOG mice were allocated for each group and then anesthetized with Nembutal (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan). Small slits (3 mm) were made in the skin of the right and left flanks of each animal, and 2 squares of tissue were dipped in Matrigel (Becton Dickinson, Franklin Lakes, NJ, USA) and inserted subcutaneously. In addition, 0.05 ml of Matrigel was injected into the insertion region, and the wounds were then closed with tissue glue. Mice were closely moni-
tored after tissue implantation and the developed lesions were measured once per week using caliper squares. The tumor volume was calculated from 3 diameters using the following formula: volume = R1 × R2 × R3 × 0.52. Each experiment was repeated three times using the tissue samples collected from different patients with essentially the same results. Representative data are presented.

Tissue evaluation at 4 weeks and 8 weeks after grafting

At 4 weeks or 8 weeks postimplantation, all mice were killed and the experiment was concluded. Xenograft tissues were harvested and examined for gross evidence of vascularization and tissue morphology. The tissues were formalin-fixed, paraffin-embedded and subjected to hematoxylin and eosin (HE) histological evaluation. The collagen and smooth muscle fiber content of the lesions was evaluated using Elastica Masson’s Trichrome staining.

Immunohistochemical analysis was performed employing the streptavidin-biotin amplification method using a Histofine kit.

Fig. 1. Human uterine leiomyoma explants in NOG mice.

Gross appearance of human uterine leiomyoma explants placed under the skin of NOG mice at 8 weeks after grafting (A). The tumor size was measured once per week with caliper squares without killing the animals as described in Materials and Methods (B). Tumor size was calculated as a percentage of day 0 volume. The values are mean ± s.e. of the samples from 3 animals with 2 xenografts of a representative experiment.
For immunostaining, the slides were heated in an autoclave at 121°C for 5 min in antigen retrieval solution (Nichirei) following deparaffinization for antigen retrieval. The antigen-antibody complex was visualized with 3,3′-diaminobenzidine solution and counterstained with hematoxylin. Expression of the estrogen receptor (ER) and the progesterone receptor (PR) was evaluated with an anti-ER alpha antibody (Invitrogen, Carlsbad, CA, USA) and an anti-PR antibody (Chemicon, Temecula, CA, USA). We also used immunohistochemistry to evaluate typical histological features of leiomyoma, including anti-alpha smooth muscle actin (αSMA; Dako, Carpinteria, CA, USA).

Proliferation was assessed by staining for the expression of the Ki-67 protein with monoclonal antibody (Dako). Apoptosis was evaluated with TdT-mediated dUTP nick end labeling (TUNEL) staining using In Situ Cell Death Detection Kit, POD (Roche Diagnostics, Basel, Switzerland) according to the manufacturer’s instructions.

Examination of estrogen dependence for implanted tissue

The mice were randomly divided into three groups of three mice: 1) inserted 17β-estradiol pellets [E₂(+)] group, 2) without 17β-estradiol pellets [E₂(−)] group and 3) without 17β-estradiol pellets and administration of gonadotropin-releasing hormone agonist (GnRHa) (buserelin, Sigma Aldrich, St. Louis, MO, USA) [Buserelin group]. The buserelin was dissolved in aqueous solution (0.06 mg/kg body weight) and injected subcutaneously into the skin behind the neck once a day for 4 weeks. E₂(+) and E₂(−) mice were treated with an equal volume of water. After 4 weeks, all mice were killed, and implanted tissues were evaluated with HE, Elastica Masson’s Trichrome, Ki-67 and TUNEL staining.

Statistical analysis

Statistical analysis was performed using Excel 2007 (Microsoft, Redmond, WA, USA) with add-in software Excel Statistic 2006 (SSRI, Tokyo, Japan). The significance of differences between means was evaluated by ANOVA, followed by Scheffe’s F-test. Data are expressed as means±S.E. Statistical significance was set at P < 0.05.

Results

Uterine leiomyoma xenografts could be implanted and their size was maintained

The xenografted human uterine leiomyoma tissues retained their gross appearance and did not change in size up to 8 weeks after tissue implantation (Fig. 1). The gross appearance showed that there were small vessels around the implanted tissues and several subcutaneous vessels were connected to the xenografts.
Next, we evaluated whether the uterine leiomyoma xenografts retained their original histological characteristics after implantation. We performed HE staining of the Original group (fresh human uterine leiomyoma), the 4w group (after 4 weeks postimplantation) and the 8w group (after 8 weeks postimplantation) (Fig. 2A-C). The HE staining analysis of the 4w or 8w group showed similar histological features to that of the Original group. The tumors were composed of whorled, anastomosing fascicles of leiomyocytes. The leiomyoma cells had an eosinophilic cytoplasm with elongated or oval nuclei. Neither nuclear atypia nor significant mitosis was present. In addition, there was no significant difference in cell density between groups (data not shown).

We further analyzed the collagen content of the xenografts. We performed Elastica Masson’s Trichrome staining of the three groups identified above. The distribution of whorled, anastomosing fascicles of leiomyocytes suggested that the tumors were composed of similar collagen content and distribution of smooth muscle fiber were found to be similar in all groups (Fig. 2D-F). The leiomyoma cell bundles (red stain) were surrounded by moderate amounts of connective tissue (green stain).

It is necessary to keep a good blood supply for the survival of the xenografts. The HE staining analysis showed the presence of small blood vessels and red blood cells in these vessels not only around the xenografts (Fig. 2G) but also in the xenografts (Fig. 2H). These results indicated the presence of evident vascularization in the uterine leiomyoma xenografts.

**Histological assessment of uterine leiomyoma xenografts**

Proliferation and apoptosis rates in engrafted tissues

Immunohistochemical analysis with anti-Ki-67 antibodies revealed that the numbers of proliferating cells were slightly increased in engrafted groups (4w and 8w) compared with that of the Original group (Fig. 3A-C). The proportions of Ki-67-positive cells in 4w and 8w groups were significantly increased (~1.5-fold) compared with that of the Original group (P < 0.05, Fig. 3G). Meanwhile, analysis with TUNEL staining revealed that apoptotic cells were similar among the Original group and engrafted groups (4w and 8w).

**Fig. 3.** Apoptosis and proliferation in engrafted leiomyoma tissues.

The distribution of proliferative cells was identified with antibody to Ki-67 (dark brown nuclear staining) (A-C), and apoptotic cells were identified by TUNEL staining (dark brown nuclear staining) (D-F). 1. Freshly processed human uterine leiomyoma (Original). 2. Implanted uterine leiomyoma retrieved after 4 weeks in vivo in NOG mice (4w). 3. Implanted uterine leiomyoma retrieved after 8 weeks in vivo in NOG mice (8w). Original magnification was × 20. The degree of proliferation or apoptosis was scored by counting the Ki-67-positive cells (G) or TUNEL-positive nuclei (H), respectively, against the total number of cells in 4 random high-power fields for each tissue section. The proportions were calculated for 3 animals per group and were included in these calculations. Data are presented as mean ± s.e. * indicates significant difference (p < 0.05).
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The proportions of TUNEL-positive cells were not significantly different between the Original group versus 4w and 8w groups (Fig. 3H).

Steroid receptors and smooth muscle actin are expressed by engrafted tissues

We performed immunostaining of ER, PR and αSMA on Original, 4w and 8w engrafted groups. Expression of αSMA was analyzed as a specific marker for smooth muscle. All protein (ER, PR and αSMA) levels were comparable among the three groups with respect to total staining intensity and staining pattern (Fig. 4). The nucleus of the leiomyoma cells was strongly stained with ER and PR. The strong reaction to αSMA was detected in the cytoplasm.

Estrogen supplementation is needed for the survival of engrafted leiomyoma tissues

Next, we analyzed whether estrogen supplementation is necessary for the maintenance of leiomyoma xenografts. At 4 weeks after implantation, we evaluated the histopathological changes of leiomyoma xenografts with or without estrogen pellet using HE staining and Elastica Masson’s Trichrome staining (Fig. 5A-B, D and E). In HE-stained sections, uterine leiomyoma xenografts without estrogen supplementation were partially necrotic and exhibited little cellularity (Fig. 5B). Xenografts without estrogen supplementation retrieved after 8 weeks in vivo showed complete necrosis (data not shown). No hosts in this study were ovariectomized, so these results indicated that the ovarian hormones secreted from mouse ovaries were insufficient to sustain the xenografts. At the same time, we analyzed the effect of GnRHa (buserelin) on leiomyoma xenografts to suppress the ovarian hormones secreted from mouse ovaries. Buserelin treatment without estrogen supplementation seemed to enhance tissue necrosis, which was detected in the xenograft without estrogen supplementation (Fig. 5C). In Elastica Masson’s Trichrome-stained sections, we could detect a decrease in smooth muscle fiber and the appearance of collagen degradation in the xenografts without estrogen supplementation (Fig. 5E). Buserelin treatment seemed to enhance these changes to some extent (Fig. 5F).

We further studied the effect of estrogen supplementation and GnRHa treatment on proliferation and apoptosis indexes in xenografts. The proportions of Ki-67-positive cells in xenografts were significantly decreased without estrogen supplementation and buserelin treatment further repressed them (Fig. 5G). The proportions of TUNEL-positive cells were significantly increased without estrogen

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**Fig. 4.** Expression of the estrogen receptor, progesterone receptor and smooth muscle actin in engrafted human uterine leiomyoma tissues.

Immunohistochemistry with antibody against estrogen receptor α (ER) (A-C), progesterone receptor (PR) (D-F) and α-smooth muscle actin (αSMA) (G-I) of histological sections. 1, Freshly processed human uterine leiomyoma (Original). 2, Implanted uterine leiomyoma retrieved after 4 weeks in vivo in NOG mice (4w). 3, Implanted uterine leiomyoma retrieved after 8 weeks in vivo in NOG mice (8w). Original magnification was × 20.
supplementation, but buserelin treatment did not enhance the apoptosis (Fig. 5H).

**Discussion**

This is the first report about human uterine leiomyoma xenograft model implanted subcutaneously into NOG mice. In this work we have shown that (1) implanted leiomyoma xenografts retain histological characteristic and indexes of apoptosis and proliferation, (2) ER, PR and αSMA are expressed by engrafted tissues, and (3) estrogen supplementation is needed for the maintenance of engrafted leiomyoma.

The greatest advantage of our model is that there is no need to transfer proangiogenic genes to leiomyoma xenograft. Adenovirus-mediated gene transfers of COX-2 and VEGF-A were necessary to maintain the engrafted leiomyoma in the Memy I model (Hassan et al. 2008). These procedures may change not only angiogenesis but also the original phenotype of uterine leiomyoma. It is also troublesome to use virus-mediated gene transfer methods because bio-safety regulation applies to the use of such viral vectors. It has been reported that vascularization was evident in leiomyoma xenograft of the Memy I model. The vascularization of xenograft in our model was also evident because of the presence of small vessels and red blood cells in these vessels. It was not necessary to overexpress the proangiogenic factors in our leiomyoma xenograft to maintain its vascularization.

During preparation of this manuscript, another type of human uterine leiomyoma xenograft model was reported. Uterine leiomyoma tissues and cultured uterine leiomyoma cells were grafted beneath the renal capsule of NOD/scid/IL-2R gamma null (NSG) immunodeficient mice (Ishikawa et al. 2010). There are three types of common IL-2R γ-chain mutations in immunodeficient mice. NOG mice and NSG mice have different IL-2R common γ-chain mutations and genetic background (NOG mice are the NOD/Shi-scid strain and NSG mice are the NOD/LtSz-scid strain).
(Agliano et al. 2008). Recently it has been reported that NSG mice were more efficiently engrafted with human cord blood cells in bone marrow than NOG mice (McDermott et al. 2010). However, there are no reports about the crucial difference in tissue engraftment potential between NOG mice and NSG mice.

There are several disadvantages of an NSG-based leiomyoma xenograft model. As a subrenal capsule graft site was chosen, a laparotomy was required. This increases the size and numbers of xenografts must be limited because this site is very narrow. Co-administration of E2 and progesterone was shown to be essential for maintenance and growth of xenografted uterine leiomyoma. According to the reported data, E2 and progesterone treatment enlarged the xenografted leiomyoma by 6 to 7 times in volume at 8 weeks after grafting (Ishikawa et al. 2010). In a clinical setting, such a growth rate is too high considering that the growth of human uterine leiomyoma is generally slow, so it is unclear whether the NSG-based model reflects the original characteristic features of human uterine leiomyoma. On the other hand, E2 and progesterone treatment in our model enlarged the xenograft by 1.3 times in volume at 8 weeks after grafting (data not shown). Such a slow growth rate is in accord with the clinical setting.

Moreover, our model has several advantages. Subcutaneously implanted xenografts could be measured easily with calipers without killing the animals. We could implant two xenografts from one patient in one mouse and there was no significant difference in size between two implanted sites (data not shown). This data showed the possibility of generating a mouse bearing multiple xenografts derived from samples from different patients. This enables large-scale experiments for many patients using a few mice.

It may be possible that the site of implantation affects the growth of uterine leiomyoma xenografts. The site of the uterus may be a more physiologically appropriate microenvironment for the implantation of uterine leiomyoma xenografts. This model may induce the symptoms of uterine leiomyomas such as menorrhagia and infertility. Establishment of the uterine implanted leiomyoma model should be the final goal in this field and will provide new insights into the regulation of leiomyoma cell proliferation.

Using this model, we are now undertaking in vivo study of the repressive effects of several drugs that could be uterine leiomyoma therapeutic agents. Indeed, some of these drugs show promising effects. Our model represents a novel and useful tool for in vivo study of human uterine leiomyoma. This will contribute to the development of novel therapeutic strategies for uterine leiomyoma.

References

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