In-vitro model of uterine leiomyomas: formation of ball-like aggregates

Y.Kobayashi¹, T.Nikaido¹, Y.L.Zhai¹, M.Inuma¹, T.Shiozawa¹, M.Shirota² and S.Fujii¹,³

¹Department of Obstetrics and Gynecology, School of Medicine, Shinshu University, Asahi, Matsumoto 390 and ²Drug Development Research Laboratories, Pharma Research Development Division, Hoechst Japan Limited, Japan
³To whom correspondence should be addressed

To clarify the biological characteristics of uterine leiomyomas, cells explanted and cultured from uterine leiomyomas and from normal myometrial tissue were observed by time-lapse cinemicrography and phase-contrast microscopy. The histological characteristics were evaluated by electron microscopy and immunofluorescence microscopy, and these observations revealed significant differences. By time-lapse cinemicrography, the cells cultured from leiomyomas and myometrium differed in their behaviour. Cells from the myometrium started to grow in parallel with the cell's major axis and formed topographically uniform hills and valleys by day 21 of culture. In contrast, the cells from leiomyomas started to grow irregularly, as if having no contact inhibition, and formed ball-like aggregates of cells by day 21 of culture. The aggregates resembled the nodules of leiomyoma in vivo. Ultrastructurally, cells from both leiomyomas and myometrium had typical features of smooth muscle. Immunofluorescently, a different distribution of α-smooth muscle actin-positive filaments and different staining of cellular fibronectin and N-cadherin between the cells from leiomyomas and myometrium were observed, which may contribute in part to the different behaviour of the cells. Given that the explant cell culture system resembles the features of uterine leiomyomas in vivo, this suggests that it can be used as an in-vitro model.

Key words: ball-like aggregates/hills and valleys/smooth muscle/tissue culture/uterine leiomyoma

Introduction

The development of leiomyomas after menarche, their enlargement and growth during pregnancy, and their regression after menopause suggest that their growth depends on steroids including oestrogen and progesterone (Buttram and Reiter, 1981; Kawaguchi et al., 1989, 1991). This led to the management of uterine leiomyomas by the administration of gonadotrophin-releasing hormone analogue (GnRHa), which induces a reversible hypogonadotropic hypogonadal environment. Numerous studies have demonstrated that GnRHa therapy can shrink the size of leiomyomas and can reduce uterine volume (Filocori et al., 1983; Lumsden et al., 1987; Friedman et al., 1990; Moghissi, 1991; Oguchi et al., 1995). Even so, the pathogenesis of leiomyomas is not well clarified and the differences between smooth muscle cells of leiomyomas and the myometrium are not clearly understood.

Uterine smooth muscle cells can be successfully cultured either by enzymatic isolation methods (Chamley-Campbell et al., 1979; Burnstock, 1981) or by explant methods in vitro (Chen et al., 1973; Moss and Benditt, 1975; Kawaguchi et al., 1985), and the growth of explants of human leiomyomas and myometrium in long-term culture has been reported (Chen et al., 1973; Moss and Benditt, 1975; Kawaguchi et al., 1985). In this study, in-vitro explants of uterine leiomyomas and myometrum were cultured long-term, and the behaviour and morphology of these cultured cells were observed by time-lapse cinemicrography and phase-contrast microscopy. We encountered an unexpected behaviour and morphology of the cultured cells from leiomyomas which were quite different from those cultured from the myometrium. The cultured cells were analysed by electron microscopy and immunofluorescence microscopy using various antibodies to cytoskeletal extracellular matrix and adhesion molecules so as to establish an in-vitro model of uterine leiomyomas.

Materials and methods

Cell culture of uterine leiomyomas and myometrium

Myometrial tissue and leiomyomas were collected from 28 leiomyoma-bearing uteri. The respective tissues were divided into two blocks. One block was fixed in 10% phosphate-buffered formalin and embedded in paraffin for histological diagnosis. The second block was placed in sterilized ice-cold calcium–magnesium-free Dulbecco's phosphate-buffered saline (PBS; pH 7.4, Nissui Pharmaceutical Company, Tokyo, Japan) and brought immediately to the laboratory for explant tissue culture. These were washed in PBS and cut further into small pieces of -1-2 mm. For each explant tissue culture dish, seven to nine pieces of the tissue were placed in a 35 mm Petri dish (Nunc Laboratories, Roskilde, Denmark) and incubated at 37°C, 5% CO₂ in air with Dulbecco's modified Eagle medium (DMEM; Nissui Pharmaceutical Company), 10% newborn calf serum (Biological Industries, Kibbutz Beth-Haemek, Israel) and 1% antibiotic–antimycotic solution (Gibco Laboratories, Grand Island, NY, USA). The medium was changed every 3 days. Steroid concentrations in the medium were determined by radioimmunoassay. This revealed concentrations of 17β-oestradiol to be <10 pg/ml and progesterone to be <15 pg/ml. The cells growing from the explants were observed by phase-contrast microscopy (Olympus IMT-2, Tokyo, Japan) every day for 6 weeks. To prepare the subcultures (secondary cultures), cells from the primary cultures at confluence were freed from the surface of the dishes using calcium–magnesium-free PBS supple-
In-vitro model for uterine smooth muscle and leiomyomas

mented with 0.125% trypsin (Gibco Laboratories) and 0.02% EDTA (pH 7.4; Sigma Chemical Company, St Louis, MO, USA), and transferred to other Petri dishes as secondary cultures. The sections were stained with haematoxylin-eosin, and the histology of the tissues was assessed to determine whether they were from the myometrium or leiomyomas.

As the 28 leiomyoma-bearing uteri were removed from premenopausal patients, endometrial dating was performed using the method of Noyes (1973) employing endometrial tissues fixed in 10% phosphate-buffered formalin and embedded in paraffin. Of the 28 patients, 15 were in the proliferative and 13 in the secretory phase of their menstrual cycle.

Time-lapse cinemicrography

In addition to phase-contrast microscopy, the behaviour of cells in the explant cultures was recorded on 16 mm film at a rate of one shot every 5 min by time-lapse cinemicrography.

Electron microscopic studies

Explant cells from the myometrium and leiomyomas after 21 days of culture were fixed in 4.0% glutaraldehyde with 0.1 M cacodylate buffer (pH 7.4) for 2 h, rinsed in buffer and post-fixed in 0.1% osmium tetroxide for electron microscopic study. They were dehydrated in ethanol gradients and propylene oxide, and embedded in Epon 812 with polyethylene capsules [modified from La Vail's methods (1968)]. Cells at the areas of cellular outgrowth 8–10 mm apart from the explants were selected and used in this study. Ultrathin sections were cut parallel to the plane of the coverslip on a Porter-Blum MT-2 ultramicrotome, stained with uranyl acetate and lead citrate, and examined using a Hitachi HU-11D electron microscope.

Immunofluorescence microscopic studies

Explant cells from the myometrium and leiomyomas were cultured in Petri dishes for 21 days, washed with calcium-magnesium-free PBS and fixed immediately with 3.5% paraformaldehyde for 20 min at room temperature. Immunofluorescence microscopic studies were performed on these cells using monoclonal antibodies to cytoskeleton [α-smooth muscle actin, desmin and vimentin (Dako, Glostrup, Denmark), myosin (BioMakor, Rehovot, Israel) and β-tubulin (ICN Biochemicals Inc., Lisle, IL, USA)], intercellular adhesion molecules [E-, N- and P-cadherin (R&D Systems Inc., Minneapolis, MN, USA), β1-integrin (Biotech OY, Helsinki, Finland) and extracellular matrix [cellular fibronectin and laminin (Biotech OY)]. The Petri dishes were washed twice in PBS and exposed to dilutions of the primary antibodies for 1 h, followed by exposure to fluorescein isothiocyanate-conjugated secondary antibodies for 1 h in the dark. The cells were washed twice with PBS and viewed with an Olympus 13H microscope equipped for fluorescence microscopy.

Effect of anti-N-cadherin antibodies on cultured cells from the myometrium and leiomyomas

Explant cells from the myometrium and leiomyomas after 14 days of culture were treated without and with anti-N-cadherin antibodies (0.6 and 2.0 μg/ml). Morphological changes were observed for 5 days by phase-contrast microscopy.

Results

Morphological differences between cells from uterine leiomyomas and myometrium in vitro

Phase-contrast microscopic and time-lapse cinemicrographic findings

In the long-term culture of tissue explants, time-lapse cinemicrographic and phase-contrast micrographic studies revealed significant differences in behaviour between the spindle-shaped cells from the myometrium and from leiomyomas. However, there was no difference in behaviour of either type of tissue between the proliferative and the secretory phase of the menstrual cycle. Therefore, the following results are irrespective of the menstrual phase under observation.

Figure 1. Phase-contrast micrographs of the cultured cell explants from the myometrium. From the original explant piece (on the left-hand side), spindle-shaped cells started to grow out radially by day 7 of culture (A), becoming arranged in parallel with the cell's major axis (B). By day 21 of culture, a structure of hills and valleys was formed (C and D) (original magnification ×210)
Figure 2. Phase-contrast micrographs of the cultured cell explants from leiomyomas. The spindle-shaped cells had large projections from the cell membrane, spread quite irregularly from the original explant piece by day 10 of culture (A) and were arranged irregularly (B). By day 21 of culture, the leiomyoma-derived cells had undergone contraction and formed focal ball-like aggregates (C and D) (original magnification: A, B and D, ×210; C, ×82).

Culture of explants from myometrial tissues. From the explant pieces of the myometrium, spindle-shaped cells started to grow out radially by day 7 of culture (Figure 1A). These cells usually grew in parallel with the cell's major axis. The spindle-shaped cells had a few projections from the cell membrane.

Figure 3. Electron micrographs of the cultured cell explants from the myometrium (A; bar = 1 mm) and leiomyomas (B; bar = 0.2 mm) showing bundles of filaments with dense bodies (A and B) and pinocytotic vesicles along the cell membrane (A) (C) (bar = 20 mm) shows a vertical section of ball-like aggregates composed of whorled bands of interlacing spindle-shaped cells with filaments.
By day 14 of culture, the number of cells had increased markedly and a sheet of cells arranged parallel to the cell’s major axis was formed (Figure 1B). By day 21 of culture, as a result of focal contraction along the cell’s major axis, a ‘hill and valley’ topography was formed. Further contraction often tore off the cells in the valleys and formed aggregations of cells resembling a structure of hills (Figure 1C and D). By day 30 of culture, the cells became confluent.

Culture of explants from leiomyomas. Spindle-shaped cells from the explant pieces of leiomyomas usually started to grow by day 10 of culture. The spindle-shaped cells had large projections from the cell membrane and spread quite irregularly from the original explant pieces (Figure 2A). The sizes of both cytoplasm and nucleus of the leiomyoma-derived cells were larger than those from the myometrium. The arrangement and behaviour of leiomyoma-derived cells were irregular, as if there was no contact inhibition. By day 21 of culture, the number of cells had increased markedly (Figure 2B) Focal contractions then occurred in the sheet of cells. In contrast to the contraction of the myometrium-derived cells, the leiomyoma-derived cells underwent contraction with clockwise rotation to form ball-like aggregates (BLA) in the sheet of cells (Figure 2C and D). These resembled the nodules of leiomyomas in vivo. BLA continued to undergo contraction and relaxation of the cells. The size of the BLA did not usually exceed 1 mm in diameter. By day 30 of culture, the spindle-shaped cells had become confluent.

Electron microscopic findings

Culture of explants from myometrial tissues. By day 21 of culture, the spindle-shaped cells which had grown around the original explant pieces contained large and ellipsoid nuclei with dispersed nuclear chromatin, several large nucleoli and numerous pores of nuclear membrane. In the cytoplasm, organelles such as rough endoplasmic reticulum, Golgi apparatus and free ribosomes were well developed and located in the perinuclear region. Bundles of myofilaments with dense bodies were also observed (Figure 3A) Along the cell membrane, pinocytotic vesicles were observed. A few basal lamina were present around the cell membrane. These findings were consistent with the ultrastructural features of smooth muscle.

Culture of explants from leiomyomas. The BLA formed by day 21 of culture was composed of whorled bands of interlacing spindle-shaped cells (Figure 3B) These cells contained fewer bundles of filaments (Figure 3C). The bundles were associated with dense bodies and located in the periphery of the cell. Lysosomes and lipid droplets were also observed in the cytoplasm. Along the cell membrane, pinocytotic vesicles were observed. The perinuclear organelles were less developed than in the spindle-shaped cells from the myometrium. The cells grown from leiomyomas had ultrastructural features consistent...
with smooth muscle. The whorled bands of interlacing spindle-shaped cells of BLA resembled the features of leiomyomas in vivo.

**Immunofluorescence microscopic findings**
The spindle-shaped cells grown from the myometrium and leiomyomas were immunostained using monoclonal antibodies to cytoskeleton (α-smooth muscle actin, myosin, desmin, vimentin and β-tubulin), intercellular adhesion molecules (E-, N- and P-cadherin, β1-integrin) and extracellular matrix (cellular fibronectin and laminin). Myosin, desmin, vimentin, β-tubulin, β1-integrin and laminin staining patterns revealed no differences between the cells grown from the myometrium and leiomyomas. However, there was a difference in immunostaining for α-smooth muscle actin between the myometrial and leiomyoma cells. Those from the myometrium showed positive staining for α-smooth muscle actin in filaments running parallel to the cell’s major axis (Figure 4A). Those from leiomyomas also showed positive staining for α-smooth muscle actin filaments, but they often overlapped and crossed intricately (Figure 4B). Immunostaining for cellular fibronectin was positive around the cell membrane of the spindle-shaped cells grown from both the myometrium and leiomyomas. However, the staining was more intense on the cells grown from leiomyomas than from the myometrium (Figure 4C and D). Immunostaining for N-cadherin was negative in the cells grown from the myometrium, but was positive in the cytoplasm and around the cytoplasmic membrane of cells grown from leiomyomas (Figure 4E and F).

**Effect of anti-N-cadherin antibodies on cultured cells from the myometrium and leiomyomas**
Cultured explant cells from the myometrium treated for 5 days with anti-N-cadherin antibodies (0.6 and 2.0 μg/ml) did not show significant morphological changes (Figure 5A). In contrast, cultured cell explants from leiomyomas treated for 5 days with anti-N-cadherin antibodies (0.6 and 2.0 μg/ml) showed significant morphological changes. After 3 days of treatment with anti-N-cadherin antibodies, the cells from leiomyomas lost their spindle shape, gradually became disordered and developed into either cytoplasm-nch or cytoplasm-scant cells with many string-like cytoplasmic protrusions (Figure 5B). The majority of these cells became detached from the culture dish by day 5 of the treatment.

**Discussion**
This study revealed that the cultured cell explants from the myometrium and leiomyomas can grow and form a structure of either hills and valleys or BLA by day 21 of culture. Ultrastructurally, the cultured cell explants grown from both the myometrium and leiomyomas had characteristics consistent with smooth muscle cells (Kawaguchi et al., 1985), although a mixture of synthetic phenotype may indicate that either fibroblasts or myofibroblasts could be grown from the explants of both the myometrium and leiomyomas. A report on the ultrastructural features of uterine smooth muscle cells during the first few days of culture suggested that they went through a transition from contractile to synthetic phenotypes (Palmberg and Thyberg, 1986). This process involves the loss of myofilaments, and the formation of a widespread rough endoplasmic reticulum and a prominent Golgi apparatus as an initiation of the synthetic process. As a result, the smooth muscle cells acquire a fibroblast- or myofibroblast-like appearance. It has been reported that cultured cells from both the myometrium and leiomyomas are analogous to the dedifferentiated stage (synthetic phenotype) of smooth muscle cells by day 14 of culture, and resemble the redifferentiated stage (contractile phenotype) by day 21 of culture (Kawaguchi et al., 1985; Fujii et al., 1990). In our study, cultured cells from the myometrium which had a contractile phenotype by day 21 of culture showed focal synchronized contractions that resulted in the formation of hills and valleys characteristic of normal smooth muscle cells in vitro (Burnstock, 1981). In addition, almost all cultured cells contained a large amount of α-smooth muscle actin-positive filaments in the cytoplasm. These findings suggest that the majority of cultured cell explants in this study are derived from smooth muscle.

This study also revealed that smooth muscle cells from the myometrium, after long-term culture of explants and observed by time-lapse cinemicrography, grew in parallel with the cell’s major axis and formed a structure of hills and valleys as the result of cell contractions. In contrast, cells from leiomyomas grew irregularly as lacking contact inhibition, and showed contraction of the cells with clockwise rotation forming BLA. Ultrastructurally, the BLA was composed of whorled bands of...
interlacing spindle-shaped cells which resembled the features of leiomyomas in vivo. This unexpected behaviour and morphology of cultured cells from leiomyomas was quite different from cultures of the myometrium in vitro. The structures (BLA) observed in the cells from leiomyomas in vitro resembled leiomyoma nodules residing in the myometrium in vivo. The distinctive behaviour and morphology formed by cultured cells from leiomyomas may enable us to use it as a model of leiomyomas in vitro. Although several methods, including culture of enzyme-dispersed cells, have been attempted with cells from leiomyomas and the myometrium, differences in the behaviour of the cultured cells are not clearly described (Chen et al., 1973; Moss and Benditt, 1975, Chamley-Campbell et al., 1979, Burnstock, 1981; Kawaguchi et al., 1985). Some of our findings are a result of the use of time-lapse cinemicrography.

Our results showed that myosin, desmin, vimentin, b-actin, fibronectin, laminin and a-smooth muscle filament actin stained positively with no apparent differences between the cells from the myometrium and leiomyomas. However, a difference in the staining of a-smooth muscle actin, laminin, and a-smooth muscle filament actin-positive filaments was observed in the cells from leiomyomas and not in those from the myometrium. Because the expression of the cadherin-α was significantly different between the cultured cells from the myometrium and leiomyomas, the immunohistochemical reactivity of the cadherin-α antibody was studied on tissue biopsies. However, N-cadherin was not expressed in biopsies of either the myometrium or leiomyomas (data not shown). This discrepancy of the expression of N-cadherin in leiomyoma tissues in vivo and in vitro may be partly because of the chicken-derived antibodies for N-cadherin used for these experiments. However, N-cadherin has been identified in neural and non-neural tissues, including developing muscle (Gilbertson-Beading and Fisher, 1993). Moreover, we have obtained experimental data from Western blotting which suggests that leiomyoma tissues both in vivo and in vitro are expressing b-catenin, unlike tissues from the myometrium (unpublished observations). This suggests that some type of cadherin/b-catenin system may exist in leiomyomas. Interestingly, the addition of anti-N-cadherin antibodies to leiomyoma cultures induced cell-cell dissociation but did not affect the cells from the myometrium. Moreover, the addition of tumour necrosis factor (TNF)-α, which is reported to induce the disordered expression of cadherin/b-catenin at the site of cell-cell contact (Tabibzadeh et al., 1995), induced significant dyshesion (cell-cell dissociation) of cultured cells from leiomyomas but did not affect those from the myometrium (unpublished observations). This suggests that some type of cadherin/b-catenin system expressing antigenicity for N-cadherin was probably acquired in the cultured cells from leiomyomas, and that the system might be affected by TNF-α. We can speculate that N-cadherin-related cell-cell contact may contribute to the formation of cell aggregations in leiomyoma-derived cells. Together with the differences in the intracytoplasmic filaments, these different arrangements of extracellular matrices and intercellular adhesion molecules may partly contribute to the difference in behaviour in vitro of cells derived from the myometrium and from leiomyomas.

Currently, the reason that leiomyoma-derived culture cells form BLA is unknown. Further research is necessary to elucidate the mechanisms of formation of BLA in vitro. However, because our explant-derived cell culture system is similar to uterine leiomyomas in vivo, we would like to propose this as an in vitro model for the study of uterine leiomyomas to elucidate the biological characteristics of formation of uterine leiomyomas as well as to evaluate the efficiency of drugs that are currently used for their therapy.

Acknowledgements

The authors thank Dr. Henry J. Norris (Arnold Palmer Women and Children Hospital, Orlando, FL, USA) for his critical review of this manuscript, and T. Sugio, Y. Yoshikawa and H. Fujimoto for their technical assistance. This work was supported in part by a grant from the Ministry of Education, Science, Sports and Culture of Japan (grant no. 06771328).

References


Received on December 8, 1995; accepted on May 8, 1996