Increased expression of calcium-binding protein S100 in human uterine smooth muscle tumours

Takanobu Kanamori1,2, Kenji Takakura1, Masaki Manda1, Masatoshi Kariya1, Ken Fukuhara1,2, Masakiyo Sakaguchi3, Nam-ho Huh3, Kaoru Saito4, Toshiharu Sakurai2, Jun Fujita2 and Shingo Fujii1,5

1Department of Gynecology and Obstetrics and 2Department of Clinical Molecular Biology, Faculty of Medicine, Kyoto University, Kyoto, 606-8507, 3Department of Cell Biology, Okayama University, Graduate School of Medicine and Dentistry, Okayama, 700-8558 and 4Department of Obstetrics and Gynecology, School of Medicine, St Marianna University, Kawasaki, 216-8511, Japan

5To whom correspondence should be addressed at: Department of Gynecology and Obstetrics, Faculty of Medicine, Kyoto University, 54, Kawaharacho, Shogoin, Sakyoku, Kyoto, 606-8507, Japan. E-mail: sfu@kuhp.kyoto-u.ac.jp

S100 proteins belong to the EF-hand Ca2+-binding protein family and regulate a variety of cellular processes via interaction with different target proteins. Several diseases, including cancer and melanoma, are related to the abnormal expression of S100 proteins, which are expressed in cell- and tissue-specific manners. We investigated the expression of S100 family members in human uterine smooth muscle tumours. Expression of six members of the S100 protein family: S100A1, A4, A6, A7, A10 and A11, was found in human uterine leiomyoma and myometrium tissue, but expression of other members was not detected by RT–PCR. Real-time PCR showed that S100A11 expression was significantly increased in leiomyoma compared with myometrium. Suppression of S100A11 by small interfering RNA (siRNA) led to apoptosis, and the overexpression of S100A11 inhibited apoptosis in human uterine smooth muscle tumour cells. These findings suggest that S100A11 has an anti-apoptotic function and is related to the process of growth of human uterine leiomyoma.

Introduction

Uterine leiomyomas are the most common neoplasm in the female genital tract (Charles and Michael, 2002). Approximately 20–25% of women of reproductive age are afflicted with this disease (Buttram and Reiter, 1981). Although leiomyomas are benign and rarely result in a lethal outcome, they frequently lead to infertility and menorrhagia (Bajekal and Li, 2000). In addition, leiomyomas are the most common indication for hysterectomy in Japan, as well as in the USA (Farquhar and Steiner, 2002). Despite the prevalence and tremendous influence of uterine leiomyomas on reproductive women, their pathogenesis has yet to be elucidated. It has previously been reported that leiomyomas grow under the influence of ovarian steroids (estrogens and progesterone) (Kawaguchi et al., 1985, 1989) and express receptors for these hormones (Kawaguchi et al., 1991). Growth factors, such as epidermal growth factor, insulin-like growth factors (IGF) and their binding proteins, transforming growth factor-β (TGF-β), and the receptors for these ligands have also been reported to affect the growth of leiomyomas (Fayed et al., 1989; Giudice et al., 1993; Vollenhoven et al., 1993; Harrison et al., 1994; Arici and Sozen, 2000). Nonetheless, the factors that promote the initial development of leiomyomas and regulate their growth in vivo remain poorly understood.

Calcium regulates a variety of intracellular processes through calcium-binding proteins (Teratani et al., 2002). The largest family of calcium-binding proteins has a common EF-hand, a helix-loop-helix motif, and the S100 proteins are a superfamily of the calcium-binding proteins with two EF-hands (Teratani et al., 2002). Human S100 proteins comprise 16 members that display amino acid sequence homology ranging from 25 to 65% (Schäfer et al., 1995). Thirteen of these genes (S100A1-A13) are clustered on the chromosome 1q21 (Wicki et al., 1996a,b; Mandinova et al., 1998) and others are distributed on different chromosomes; S100B is located on chromosome 21q22 (Allore et al., 1988), S100P on chromosome 4p16 (Schäfer et al., 1995) and CALB3 on chromosome Xp22 (Howard et al., 1992). Individual S100 proteins are expressed in a cell-specific manner in normal tissues, as opposed to the ubiquitous distribution of the intracellular Ca2+-binding receptor calmodulin (Donato, 2003). The amount of a given S100 protein expressed in cells is not fixed, with some cell types expressing large amounts and other cell types expressing intermediate, low, or no detectable amounts (Donato, 2003). This may reflect the functional role of a given S100 protein. In a few cases, S100 proteins share their target proteins, and hence regulate identical activities, and in still other cases different S100 members take part in the regulation of similar groups of activities. The variety of intracellular target proteins and the cell specificity of the expression of S100 proteins suggest that S100 proteins might have a role in the fine regulation of effector proteins, specific steps of signalling pathways, or cellular functions (Donato, 2003).

Recently we reported that secreted frizzled related protein 1 (sFRP1) and PEP-19 are overexpressed in uterine leiomyoma and we postulated that their expression may contribute to uterine leiomyoma development (Fukuhara et al., 2002; Kanamori et al., 2003). PEP-19 belongs to a family of calmodulin regulatory proteins that includes RC3/neurogranin and GAP-43/neuromodulin (Slemmon et al., 1996). All such proteins interact with the calcium-free form of calmodulin, sequestering calmodulin from calcium and preventing...
its activation until certain threshold levels of calcium are attained (Gerendasy and Sutcliffe, 1997). As we reported previously (Kanamori et al., 2003), calcium/calmodulin metabolism may be involved in the pathogenesis of human uterine leiomyoma. S100 proteins are also EF-hand Ca2+-binding proteins such as calmodulin, which participates in the regulation of many important biological processes. Based on our previous findings, the present study was performed to verify the involvement of calcium/calmodulin metabolism in the pathogenesis or development of human uterine smooth muscle tumours. Thus, we investigated the expression and explored the possible roles of S100 proteins in human uterine smooth muscle tumours. As S100A11 rather than other S100 protein family members showed distinct expression in leiomyomas, we investigated S100A11 more thoroughly in the present study.

Materials and methods

**RNA and paraffin section samples**

Leiomyoma, matched myometrium, and leiomyosarcoma tissues were obtained from patients who underwent hysterectomy for uterine leiomyoma or leiomyosarcoma in Kyoto University Hospital. Informed consent was obtained from each patient before surgery with the use of consent forms and protocols approved by the Human Investigation Committee of the hospital. All leiomyoma samples were intra-mural type and from patients with regular menstrual cycles. Myometrial tissues were obtained mostly from the uterine fundus, and leiomyoma tissues were from midway between the centre and the periphery of each tumour nodule. The main criteria used to diagnose leiomyosarcoma of the uterus are the presence of nuclear atypia, a high mitotic index, and coagulative tumour necrosis (Charles and Michael et al., 2002). Tissue specimens were immediately frozen in liquid nitrogen after the hysterectomy for total RNA isolation.

**RT–PCR**

Total RNA was isolated from surgically resected samples by homogenization in Trizol reagent (Life Technologies, USA). Each RNA sample was treated with Deoxyribonuclease I Amplification Grade (Life Technologies) in order to remove DNA prior to RT–PCR amplification. First-strand cDNA was then synthesized in a volume of 20 μl containing 1 μg of total RNA, 0.5 μl of oligo (dT) 18 primer, 10 pmol of dNTP, 5 IU of ReverTra Ace (TOYOBO, Japan) and first-strand synthesis buffer (TOYOBO, Japan). Synthesis of cDNA was performed at 36°C for 10 min, 42°C for 1 h and 99°C for 5 min. PCR amplification was performed for the number of cycles indicated below using 1 μl of first-strand cDNA as a template.

<table>
<thead>
<tr>
<th>S100 species</th>
<th>Sense primer</th>
<th>Antisense primer</th>
<th>Annealing temperature (°C)</th>
<th>Amplicon (bp)</th>
<th>GenBank number</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>CTTGGCCATCTGTCCAGAAC</td>
<td>CAATGTTGGCTGTCGTCTCAAA</td>
<td>59</td>
<td>362</td>
<td>NM006271</td>
</tr>
<tr>
<td>A2</td>
<td>CACAGATCCCATATGTCAGTTT</td>
<td>GGAAGTCAAGGATCTGCTTCA</td>
<td>56</td>
<td>327</td>
<td>NM005978</td>
</tr>
<tr>
<td>A3</td>
<td>GCCGTAGCTGCTGCCACGTGTTG</td>
<td>GCAGTCTGTTGAAGACTCTG</td>
<td>54</td>
<td>260</td>
<td>NM002960</td>
</tr>
<tr>
<td>A4</td>
<td>CGACCTGACGAAGTCAAGGAC</td>
<td>TATTTGAACTTGCTACAGT</td>
<td>54</td>
<td>346</td>
<td>NM002961</td>
</tr>
<tr>
<td>A5</td>
<td>CTGCCAGACTGTGATGGAAGAGAC</td>
<td>GAAGTGGTCTTGATGCCTTG</td>
<td>54</td>
<td>272</td>
<td>NM002962</td>
</tr>
<tr>
<td>A6</td>
<td>CATTGGGAGTGAAGAACGACA</td>
<td>AGGTTGTTCTACATTCTCCT</td>
<td>65</td>
<td>239</td>
<td>NM014624</td>
</tr>
<tr>
<td>A7</td>
<td>AGGGCTCAAATAGGACGATAGT</td>
<td>CGAAGAGAGGAAAGCTCAGAA</td>
<td>54</td>
<td>215</td>
<td>NM002963</td>
</tr>
<tr>
<td>A8</td>
<td>ATTTCTATGCGTCTACAGGG</td>
<td>GCGCAATGACTGCTACTCTC</td>
<td>58</td>
<td>223</td>
<td>NM002964</td>
</tr>
<tr>
<td>A9</td>
<td>GTCGACGCTGCTGGAACGCAA</td>
<td>CCTGGGTCTCAGTTACTGG</td>
<td>55</td>
<td>419</td>
<td>NM002965</td>
</tr>
<tr>
<td>A10</td>
<td>GACCACACCATATGCTCCTCT</td>
<td>TATGACTGGAGGTCACATG</td>
<td>58</td>
<td>339</td>
<td>NM002966</td>
</tr>
<tr>
<td>A11</td>
<td>CCGCTCATGTAAGAATTCTGG</td>
<td>TGCTCTGGAGGTCGTTAGT</td>
<td>58</td>
<td>241</td>
<td>NM002962</td>
</tr>
<tr>
<td>A12</td>
<td>CCTCTTAAAGGTGTAGCTGTA</td>
<td>CTGGTGTGTTGGAGGAAAA</td>
<td>59</td>
<td>271</td>
<td>NM005621</td>
</tr>
<tr>
<td>A13</td>
<td>AACAAGTGGTGTAGTTAGTG</td>
<td>GACACTGAGAAATGAGAGA</td>
<td>55</td>
<td>203</td>
<td>NM005979</td>
</tr>
<tr>
<td>A14</td>
<td>GCCACCATCAGCACTGCCCTTT</td>
<td>GACACACAGAGCAGCAGCTTC</td>
<td>58</td>
<td>334</td>
<td>NM004057</td>
</tr>
<tr>
<td>B</td>
<td>GACCCCTCATATGCTCTGAGC</td>
<td>TGCGCTGAGCTCTAAATTCACCT</td>
<td>55</td>
<td>318</td>
<td>NM006272</td>
</tr>
<tr>
<td>CALB3</td>
<td>TCTAGCTGTTCCTACATTGGG</td>
<td>GACGACAGACAGACAGGCCTCTT</td>
<td>58</td>
<td>383</td>
<td>NM005980</td>
</tr>
</tbody>
</table>

Sixteen species of S100 cDNA fragments were amplified by PCR using cDNA of human tissues as templates and S100 protein-specific sense and antisense primers at the indicated annealing temperature.

Each S100 cDNA was amplified by PCR from human tissue cDNA using the S100 cDNA-specific sense and antisense primers shown in Table I. Amplification was performed in a volume of 25 μl containing 1 μg of cDNA, 100 μmol/l of dNTP, 5 pmol of primers, 0.5 μl of Taq and Taq buffer (TaKaRa Bio Inc., Japan). PCR was performed by 35 cycles of incubation at 98°C for 1 min, 55°C for 2 min and 72°C for 2 min in the GeneAmp PCR system 2400 (Applied Biosystems, USA). The PCR product was electrophoresed on a 2% agarose gel with the marker Ready Load 100 bp DNA Ladder (Life Technologies) and visualized by staining with ethidium bromide and examination using a UV illuminator.

**Immunohistochemistry**

The immunohistochemical localization of S100A11 proteins was studied by a modified avidin–biotin complex technique. Tissue sections were deparaffinized in xylene and dehydrated through a graded ethanol series. The endogenous peroxidase activity was blocked for 30 min in 1% methanolic hydrogen peroxide solution, followed by incubation with 20% normal horse serum to minimize non-specific binding of the secondary antibody. The sections were incubated at 4°C for 24 h with the anti-S100A11 antibody (Sakaguchi et al., 2000) diluted 1/200.

After the sections had been washed three times in phosphate-buffered saline (PBS), they were incubated for 30 min at room temperature with biotinylated horse anti-mouse IgG (H + L) (Vector Laboratories, USA) and then incubated with the avidin–biotin–peroxidase complex (Vector Laboratories) for 30 min in the dark. The sections were again washed in PBS before development with diaminobenzidine. The slides were finally washed in distilled water and counterstained with haematoxylin.

**Western blotting**

Normal myometrium and leiomyoma tissues were homogenized at 4°C in the lysis buffer containing 150 mmol/l NaCl, 50 mmol/l Tris–Cl, 5 mmol/l EDTA, 1% Triton X-100, 1% Na-deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 1 mmol/l phenylmethylsulphonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A. The homogenates were subsequently centrifuged at 15 000 g for 30 min, and the supernatants were collected for protein analysis. The protein concentration of each sample was determined using a commercial protein assay kit (DC Protein Assay; Bio-Rad Laboratories, Inc., USA). Proteins resolved by SDS–PAGE were transferred to Immobilon-P membranes (Millipore Corp., USA). After brief washing in TBST [25 mmol/l Tris–HCl (pH 7.5), 50 mmol/l NaCl, 0.1% Tween 20], the membranes were blocked with 10% skim milk in TBST for 1 h at room temperature and washed three times with TBST. The membranes were incubated at 4°C for 24 h with S100A11 antibody diluted 1/1000 in 10% skim milk/TBST. After three washes with TBST, the membranes were incubated for 2 h with horse-radish peroxidase conjugated to anti-goat antibody (Dako Corp., Denmark).
After three washes with TBST, bound antibodies were visualized by chemiluminescence (ECL; Amersham Pharmacia Biotech Ltd, UK).

Quantitative real-time PCR
Real-time PCR (TaqMan) analysis was performed using Applied Biosystems 7000 Prism (Applied Biosystems). Matching primers and fluorescence probes (siRNA) were selected for S100A11 genes according to the Primer Express Program provided by Applied Biosystems. Primer and probe concentrations were optimized with a pool of cDNA from uterine leiomyoma and myometrial tissues.

The PCR reaction was performed in 50 µl with Taqman Universal PCR Mastermix (Applied Biosystems) and cDNA templates. β-Actin primers and probes were obtained from Applied Biosystems. The following primers and probes were used: CCGTATCCCGCTTGCTTGAAAGA (forward primer), CTTGACCGCAATACTTTGAG and GGACACCAACA-GTGATGGTCAGCTAGATT (probe).

To quantify the amount of specific mRNA in the samples, a standard curve was generated for S100A11 using leiomyoma cDNA. In addition, a standard curve was generated for beta-actin cDNA. This enabled standardization of the initial RNA content of a tissue relative to the amount of β-actin mRNA.

siRNA preparation
To design S100A11 (Gen Bank NM005620)-specific small interfering RNA (siRNA) duplexes, we selected the sequence spanning positions 228–246 with symmetric 2 nt 3' overhangs of identical sequence. We used 2'-deoxynucleotides instead of uridine residues in the 3' overhangs to reduce the cost of RNA synthesis and to enhance nuclear resistance. The selected siRNA sequence was also submitted to BLAST search against the human genome sequence to ensure that only one gene of the human genome was targeted. The 21 nt RNA were purchased from Japan Bio Service (Japan) in a desalted form.

The RNA oligonucleotides used for S100A11 were: sense (5'-GACAGA-GUCCUACAGCGCGCC-3') and antisense (5'-GAAGUAGAGACUCUGCUCC-3') and antisense (5'-CCACUACAAUUCUCAGUGT-3'). The siRNA duplex formation (annealing) was performed as previously described (Elbashir et al., 2001a).

Cell culture and siRNA transfection
The human leiomyosarcoma cell line SKN was maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 15% fetal bovine serum (FBS) in a 37°C incubator ventilated with 5% CO2 in air. The medium was changed every 3 days, and cells were subcultured after 4 days. Twenty-four hours after transfection, the cells were trypsinized, diluted with fresh medium without antibiotics and transferred to 3.5 cm dishes. Transient transfection of siRNA was performed using Opti-MEM medium (Life Technologies) and 16 µl of Opti-MEM. The entire mixture was added to the cells in each dish. Four hours after transfection of siRNA, FBS was added and the culturing of the cells was then continued.

Detection of apoptosis
The induction of apoptosis induced by serum deprivation was evaluated using an enzyme-linked immunosorbent assay (ELISA) kit for apoptosis detection (Cell Death Detection ELISA Plus; Roche Molecular Biochemicals, Germany). The proportions of mono- and oligo-nucleosomes in the cytoplasmic fraction of the cell lysate were determined based on a quantitative sandwich enzyme immunoassay principle using mouse monoclonal antibodies directed against DNA and histones. SKN cells were transfected with S100A11 siRNA or non-specific siRNA. SKN cells were plated at a density of 7.0 × 104 cells in 6-well flat-bottomed tissue-culture plates (Iwaki, Japan). Three days after transfection, the medium was replaced by fresh DMEM without FBS. After 24 hr of serum deprivation, the cell lysate was prepared and placed in a streptavidin-coated microtitre plate, and apoptosis was evaluated using the kit in accordance with the manufacturer’s instructions.

Fluorescence-activated cell sorting analysis of S100A11-overexpressing SKN cells
The cDNA of S100A11 was made from human leiomyoma tissue by RT-PCR (sense primer: GCGGCCGCTAGTGGAAACTCTCAGGGCCT; antisense primer: GCTCGAGGGTCTTGTTGCGGAAGGG). The NotI/Xhol fragment of S100A11 cDNA was cloned into the pIREs-hrGFP-1a vector (Strategene, USA) together with GFP, and the resultant plasmid was used for the transfection. SKN cells were transfected with either pRES-GFP-S100A11 plasmid or empty vector plasmid according to the Ca-phosphate method. Twenty-four hours after transfection, the cells were treated with 1.7 µmol/l doxorubicin for 24 h. The treated cells and control cells transfected with the empty vector were harvested with 4 mmol/l EDTA/PBS, fixed in 70% ethanol and stained with 5 µg/ml propidium iodide (PI) (Sigma–Aldrich, USA). The DNA content of the cells was analysed by using a flow cytometer. Only the fraction of cells manifesting a high level of GFP fluorescence was chosen to selectively analyse the cells expressing transfected cDNA (Sakurai et al., 2004). Apoptotic cells appeared as a hypodiploid peak (sub-G1) due to nuclear fragmentation and loss of DNA.

Statistical methods
Statistical significance was assessed by the pair-sample test, the Wilcoxon signed ranks test (Figure 3B) and the paired t-test (Figure 5A, C), Figure 6C).

Results
Transcriptional expression of S100 members
The cDNA fragments of 16 species of S100 were amplified by PCR using cDNA of human tissues as templates, and the specific sense and antisense primers for each S100 family member listed in Table I. We used three RNA samples of leiomyoma and myometrium from three individual patients and three RNA samples of leiomyosarcoma from another three individual patients for the analysis. Every sample showed a similar expression pattern: S100A1, A4, A6, A7, A10 and A11 mRNA were detected in myometrium, leiomyoma and leiomyosarcoma tissues. However, S100A2, A3, A8, A9, A12, A13, B, CALB and S100P mRNA were not detected. A representative case is shown in Figure 1.

<table>
<thead>
<tr>
<th>S100</th>
<th>Myometrium</th>
<th>Leiomyoma</th>
<th>Leiomyosarcoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A2</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>A3</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>A4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A5</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>A6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A8</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>A9</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>A10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A12</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>A13</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>B</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>CALB</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>S100P</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
</tbody>
</table>

Figure 1. Transcriptional expression of S100 family in human myometrium, leiomyoma, and leiomyosarcoma. The cDNA band of each of the indicated S100 family members was PCR-amplified from myometrium, leiomyoma, and leiomyosarcoma using the primers described in Table I. N = no expression.

737
**S100A11 protein expression in human uterine leiomyoma and myometrium**

Among the S100 family members, S100A11 showed distinct expression in leiomyoma compared with myometrium by RT–PCR (Figure 1). Therefore, we investigated S100A11 most thoroughly among these family members. S100A11 protein was expressed in the cytoplasm, as shown using anti-S100A11 antibody (Figure 2A). S100A11 protein was also detected as an 11 kDa protein band by Western blotting in leiomyoma and myometrium (Figure 2B).

**S100A11 mRNA expression in human uterine leiomyoma and myometrium**

S100A11 mRNA expression was quantified more precisely by real-time PCR. S100A11 mRNA was more highly expressed in leiomyoma compared with the matched myometrium in 23 samples of the 28 samples analysed (Figure 3A). This pattern was not seen in five samples (four samples in proliferative phase, and one in secretory phase). The expression in each sample was standardized relative to β-actin expression. A significant increase S100A11 expression was observed in leiomyoma compared with myometrium (Wilcoxon signed ranks test; P < 0.05) (Figure 3B).

**S100A11 expression in human uterine leiomyosarcoma**

Immunostaining of S100A11 protein in human uterine leiomyosarcoma and SKN were performed using anti-S100A11 antibody. S100A11 expression was observed in the cytoplasm of leiomyosarcoma as well as leiomyoma (Figure 4A). In SKN cells, S100A11 expression was observed clearly in the cytoplasm, not the nucleus, and SKN had conspicuously strong S100A11 expression compared with leiomyosarcoma and leiomyoma (Figure 4B).

We performed the real-time PCR to confirm this S100A11 expression tendency provided by the immunohistochemistry. The expression of S100A11 mRNA was more prominent in SKN cells and leiomyosarcoma tissue compared with that in leiomyoma tissue (Figure 4C).

**Effect of siRNA for S100A11**

S100A11 mRNA expression in SKN cells was reduced by half, 3 days after transfection of siRNA for S100A11, as shown in Figure 5A. There was no significant difference in the cell number between the control and the siRNA-treated groups, suggesting that siRNA did not affect the cell growth (Figure 5B). To evaluate the effect of RNA interference on cell apoptosis, FBS was removed from the culture medium to induce apoptosis from the 4th day after transfection. The cell counts showed no significant difference between controls and the siRNA-treated group irrespective of the starvation treatment (Figure 5B). In the absence of FBS, the degree of apoptosis was significantly higher in the siRNA-treated group than in the control group, as shown using a cell death detection ELISA kit. The results were analysed by the paired t-test, and this siRNA effect was confirmed as statistically significant (Figure 5C).

**Effect of overexpression of S100A11**

More than 7% of SKN cells transfected with pRES-GFP-S100A11 plasmid actually expressed S100A11 together with GFP. The control SKN cells transfected with empty vector expressed GFP at a similar rate. To assess whether S100A11 has anti-apoptotic function, we measured the DNA content in these cells by flow cytometry after treatment with doxorubicine. The hypodiploid peak (subG1 area), containing cells with degraded DNA, was decreased in the cells transfected with GFP-S100A11 vector (Figure 6A), compared with the cells transfected with GFP alone (Figure 6B). A significant decrease in the percentage of the cells in sub-G1 DNA stage was observed in the S100A11-transfected cells compared with the control group after doxorubicine treatment. The transfection experiments were performed four times, and the results were analysed by
Figure 3. Real-time PCR analysis of \textit{S100A11} in human myometrium and leiomyoma. (A) \textit{S100A11} mRNA expression in leiomyoma and myometrial tissue. Real-time PCR was performed on a Perkin–Elmer/Applied Biosystems 7000 Prism instrument using cDNA reverse-transcribed from RNA of human myometrium and leiomyoma. \(\beta\)-Actin was used as an internal reference control. \textit{S100A11} expression was calibrated relative to \(\beta\)-actin expression. Leiomyoma showed stronger \textit{S100A11} expression than myometrium in 23 of 28 samples. (B) Statistical analysis. The Wilcoxon signed ranks test revealed that \textit{S100A11} mRNA expression in leiomyoma (L) was significantly stronger compared with that in matched myometrium (M). Values are the mean \(\pm\) SE. EP = pre-surgical estrogen and progestogen administration; Mens = menstruation phase.

Figure 4. \textit{S100A11} expression in the human leiomyosarcoma tissues and cell line. (A) Immunohistochemistry in leiomyosarcoma tissue. \textit{S100A11} expression was detected predominantly in the cytoplasm in both leiomyosarcoma and leiomyoma. Inset: higher magnification of \textit{S100A11} expression. (Magnification: \(\times\)500.) (B) Immunohistochemistry in SKN cell line. \textit{S100A11} expression was detected (Magnification: \(\times\)200.) predominantly in the cytoplasm. There was abundant expression of \textit{S100A11} protein in SKN; leiomyosarcoma cell line. (Magnification: \(\times\)200) (C) Real-time PCR analysis of \textit{S100A11} mRNA expression. Real-time PCR analysis demonstrated the abundance of \textit{S100A11} mRNA expression in leiomyosarcoma (LMS) and the SKN cell line, compared with the expression in leiomyoma (L). A representative experiment is shown of five experiments with similar results. The result of the real-time PCR analysis was consistent with the immunohistochemistry.
the paired t-test, and this significant decrease was confirmed statistically significant (Figure 6C).

Discussion

We first screened the transcriptional expression of all S100 members in human uterine leiomyosarcoma, leiomyoma and myometrium by RT–PCR and summarized the spectrum of their expression. Expression of S100A1, A4, A6, A7, A10 and A11 was found in leiomyosarcoma, leiomyoma and myometrium by RT–PCR. Among these S100 family members, S100A11 showed especially distinct expression in leiomyoma. Therefore, our study was focused on S100A11 among these family members. S100A11 protein was actually expressed more strongly in leiomyoma than in myometrium, as shown by immunohistochemistry with S100A11 antibody. The mRNA expression showed the same tendency, as demonstrated by the quantitative RT–PCR.

S100A11 (previously named S100C or calgizzarin) is a novel and relatively unknown member of the large family of S100 proteins, and exhibits several unique properties. S100A11, first purified and partially characterized from porcine heart (Naka et al., 1994) and chicken gizzard (Allen et al., 1996), was subsequently found to be highly expressed in colorectal cancer (Tanaka et al., 1995). The low expression in normal colon tissue suggested a role in cell transformation. This would be consistent with the localization of the S100A11 gene on human chromosome 1q21 (Moog-Lutz et al., 1995), a region frequently amplified in tumour tissues (Inada et al., 1999).

S100A11 was found to regulate the cytoskeletal function via Ca²⁺-dependent interaction with annexin I (Naka et al., 1994; Mailliard et al., 1996), targeting S100A11 to early endosomes (Seemann et al., 1997). S100A11 was shown to be most abundant in the placenta, expressed in significant amounts in human heart, lung, kidney, uterus, bladder, prostate and skeletal muscle, and poorly expressed in other tissues (Inada et al., 1999).

RNA interference (RNAi) provides an approach for elucidation of gene function. RNAi is a sequence-specific, post-transcriptional gene-silencing mechanism initiated by the introduction of double-stranded RNA (dsRNA) that is homologous in sequence to the silenced gene. Duplexes of 21 nt RNA with 2 nt 3' overhang, introduced by transfection into human and other mammalian cultured cells, specifically interfere with gene expression and bypass the sequence-independent response of mammalian cells to long dsRNA (Elbashir et al., 2001a). These short RNA duplexes resemble the processing products from long dsRNA, and are referred to as small interfering RNA (siRNA) (Elbashir et al., 2001b). We found that the level of apoptosis was significantly increased when S100A11 expression was inhibited by siRNA. Moreover, the level of apoptosis was decreased when S100A11 was overexpressed by transfection of its gene. These results suggest that S100A11 has an anti-apoptotic function.

One of the essential alterations in cell physiology that characterize malignant growth is the lack of apoptosis (Hanahan and Weinberg, 2000). The ability of tumour cell populations to expand in number is determined not only by the rate of cell proliferation but also by
Calcium-binding protein S100 in human uterine smooth muscle tumours

The rate of cell attrition—apoptosis—represents a major pathway of this attrition (Hanahan and Weinberg: 2000). According to our findings in this study, S100A11 expression in leiomyosarcoma was much stronger than that in leiomyoma or myometrium, and S100A11 appeared to have an anti-apoptotic function, suggesting that this gene may contribute to the malignant growth and expansion of leiomyosarcoma.

Recently, Tsibris et al. (2002) demonstrated that expression of PEP-19, along with Delta-like 1 (Dlk), myelin proteolipid protein (PLP), Frizzled-2, CD-24 signal transducer (CD-24) was up-regulated in leiomyoma compared with matched myometrium by using the microarray. PLP is one of the most abundant proteins in the central nervous system, and Tsibris et al. reported that PLP showed surprisingly high expression in human uterine leiomyoma tissue compared with normal myometrium, though myelin basic protein and other myelin-related genes had no difference of expression between leiomyoma and myometrium on the microarray. Catherino et al. (2003) reported that real-time PCR demonstrated equivalent Dlk mRNA expression between leiomyoma and myometrium, a slight Frizzled-2 overexpression, and robust CD-24 overexpression in leiomyoma. Since Dlk, Frizzled-2 and CD-24 are known to be involved in cell differentiation, they suggested that these genes might contribute to expression of a fundamental molecular phenotype that characterizes leiomyomas.

Regarding leiomyoma pathogenesis, we have the following hypothesis: leiomyomas may result from the proliferation of smooth muscle cells in myometrium that survive the repeated ischaemic reperfusion stress experienced during the menstrual cycles. In each secretory phase of the menstrual cycle, myometrial smooth muscle shows proliferative activity in preparation for pregnancy. If pregnancy does not occur, the proliferative activity of myometrial smooth muscle is interrupted at the time of menstruation. Myometrial contraction during menstruation, which results in the cessation of menstrual bleeding, induces a hypoxic state in myometrial smooth muscle cells, resulting in ischemic injury. The injured and inappropriately repaired cells might represent progenitor leiomyoma cells, as is the case for various other neoplasms (Fujii et al., 1999). Therefore, interference with appropriate apoptosis in damaged cells, caused by factors such as S100A11, may contribute to leiomyoma pathogenesis.

It is a novel and intriguing finding that the molecules that have been thought to be expressed preferentially in the nervous system are definitely expressed in human uterine leiomyoma. Furthermore, it is interesting that genes such as PEP-19 and S100A11 involved in calcium/calmodulin metabolism may participate in the pathophysiology of leiomyoma, when it is considered that the uterus is a unique organ which repeats dynamic contraction and stretching. Investigation of the molecules participating in the metabolism of calcium/calmodulin may provide new insights into the pathophysiology of human uterine smooth muscle tumours.

Acknowledgements
We are grateful to Ms Michiko Muraoka for her helpful technical support. This work was supported in part by Grants-in-Aid for Scientific Research (No. 1307047 and 13877272) from the Ministry of Education and Sports, Japan.

References


Submitted on July 9, 2004; accepted on July 24, 2004