Dysregulation of IGF-I signaling in uterine leiomyoma

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Abstract

IGF-I expression has been observed in human uterine leiomyomas. To examine whether autocrine IGF-I signaling plays a role in the growth of these tumors, we used an animal model of uterine leiomyoma (the Eker rat) to investigate regulation of *IGF-I* and the IGF-I receptor (*IGF-IR*) expression in tumors and normal myometrium. During the normal estrous cycle, myometrial *IGF-I* expression peaked on the day of proestrus when the rate of proliferation in this tissue is greatest. In leiomyomas, the expression of *IGF-I* was increased 7·5-fold compared with the age-matched normal tissue. The level of *IGF-IR* mRNA in both tumor and non-tumor tissues was found to inversely correlate with that of *IGF-I*. Changes observed in IGF-I signaling components correlated with the activation state of the signal-transducing protein insulin receptor substrate-1 (IRS-1). During diestrus and proestrus when *IGF-I* levels were increasing, tyrosine phosphorylation of IRS-1 was increased up to 5·7-fold in the normal myometrium relative to estrus, when *IGF-I* levels were the lowest. Additionally, IRS-1 phosphorylation was 4-fold greater in leiomyomas relative to age-matched normal myometrium. Autocrine stimulation of the IGF-IR may, therefore, play a role in regulating the normal growth of the myometrium, and dysregulation of IGF-I signaling could contribute to the neoplastic growth of uterine leiomyomas.

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Introduction

Steroid hormones produced by the ovaries are thought to modulate the growth of uterine leiomyomas (fibroids); however, a mechanistic understanding of the effects of estradiol (E_2) and progesterone (Pg) on these tumors remains elusive. The characterization of a rodent model for this disease, the Eker rat, now offers an opportunity for more in-depth mechanistic studies into this aspect of leiomyoma biology. Like human fibroids, Eker leiomyomas are hormonally responsive tumors. The growth of cell lines derived from primary Eker tumors can be modulated by E_2 and anti-estrogens in culture and in nude mice (Howe *et al.* 1995, Fuchs-Young *et al.* 1996). The development of leiomyomas in adult females can be prevented by ovariectomy at a young age (Walker *et al.* 2000), demonstrating a complete dependence on ovarian hormones for tumorigenesis. Additionally, a 4 month course of anti-estrogen treatment can reduce tumor incidence by almost 50% (Walker *et al.* 2000). Therefore, the Eker rat provides a model which appears to mimic the human disease and which is useful to further investigate factors controlling the development and growth of uterine fibroids.

To better understand the hormone-responsive nature of fibroids, we previously characterized the proliferative and apoptotic responses of leiomyomas and normal myometrial cells in the Eker rat to endogenously produced ovarian hormones (Burroughs *et al.* 2000). The response of myometrial cells to hormones changed throughout the lifetime of the animal and during tumorigenesis. The normal myometrium of 2- to 4-month-old Eker females proliferated when serum E_2 levels were high and underwent apoptosis when ovarian hormones were low (Burroughs *et al.* 2000). With age, these responses to changing hormone levels were dampened, but cell number was maintained by attenuation of both proliferative and apoptotic rates. In leiomyomas, tissue growth resulted from aberrant cell proliferation and the inability of cells to undergo apoptosis. High levels of E_2 were no longer required to stimulate cell proliferation, and tumor cells were refractory to the induction of programmed cell death during periods of low ovarian hormone secretion. These data suggested that alterations in replication and survival signaling were driving the inappropriate response of leiomyoma cells to the ovarian hormone milieu and the disruption of tissue homeostasis.

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A potential mediator of the effects of steroid hormones on uterine fibroids is insulin-like growth factor-I (IGF-I). IGF-I is an ubiquitous growth promoter which acts via endocrine, paracrine and autocrine pathways to stimulate normal and neoplastic growth by regulating the rates of both proliferation and apoptosis in target cells (Werner & LeRoith 1997). In the normal myometrium and other uterine tissues, both *IGF-I* and the IGF-I receptor (*IGF-IR*) are co-expressed (Ghahary & Murphy 1989, Ghahary *et al.* 1990). Ovarian hormones regulate the synthesis of IGF-I in uterine cells, where E_2 has been shown to stimulate *IGF-I* transcription and tyrosine phosphorylation of the IGF-IR and its downstream signaling partner the insulin receptor substrate-1 (IRS-1) (Murphy *et al.* 1987, Ghahary *et al.* 1990, Richards *et al.* 1996). Partially mimicking the effect of E_2 on the uterus, administration of recombinant IGF-I to ovariectomized rats has been shown to stimulate an increase in uterine wet weight (Sahlin *et al.* 1994). Autocrine and/or paracrine IGF-I signaling could, therefore, be important mediators of normal uterine growth regulation by ovarian steroid hormones, and alterations in this signaling could represent a possible pathway for stimulation of neoplastic growth in this organ.

To date, signaling via the IGF-IR, and its relationship to *IGF-I* and *IGF-IR* expression in uterine leiomyomas have not been examined, warranting experimental studies to clarify the role of IGF-I signaling in these tumors. Two studies reported that fibroids bind more IGF-I than normal myometrium, suggesting that overexpression of *IGF-IR* may be a common event in tumorigenesis (Tommola *et al.* 1989, Chandrasekhar *et al.* 1992). However, the regulation of *IGF-I* expression in leiomyomas is less clear, particularly as *IGF-I* expression by leiomyomas may vary with hormonal milieu. Two studies in which menstrual cycle was not considered when uterine tissue samples were harvested reported conflicting data regarding *IGF-I* levels. Boehm *et al.* (1990) reported *IGF-I* mRNAs to be higher in leiomyomas than normal tissue, whereas a study by Gloudemans *et al.* (1990) concluded that no difference existed. Investigations in which tissue samples were grouped according to menstrual cycle reported that leiomyomas contained higher levels of *IGF-I* message than like-staged myometrium, but these data either failed to reach statistical significance (2-fold) or were marginal (1·1- to 1·4-fold) (Vollenhoven *et al.* 1993, Englund *et al.* 2000). In a study by Giudice *et al.* (1993), *IGF-I* was expressed by follicular phase leiomyoma and normal myometrium, but small sample size and lack of quantitation made correlation of changes in *IGF-I* levels with phase of menstrual cycle problematic. Therefore, data concerning *IGF-I* expression in human leiomyoma have remained equivocal.

To examine whether autocrine IGF-I signaling is involved in regulating the growth of the normal myometrium and primary leiomyomas, expression levels of *IGF-I* and the *IGF-IR* were determined in Eker rat tumor and non-tumor tissues and tyrosine phosphorylation of IRS-1 examined to determine whether changes in the expression of *IGF-I* and *IGF-IR* correlated with changes in intracellular signaling from the IGF-IR. We report that expression levels of *IGF-I* correlate with cell proliferation in normal myometrial cells and that dysregulation of *IGF-I* expression in leiomyomas results in hyper-activation of IGF-I signaling, providing a possible explanation for the changes in cell growth kinetics and in cellular responses to ovarian hormones that occur in these tumors.

Materials and Methods

Animals

Female rats, genotyped as to carrier status with respect to the Eker mutation in the Tsc-2 tumor suppressor gene, were maintained on a 14 h light:10 h darkness cycle with food and water freely available. Daily vaginal smears were performed to determine the regularity of reproductive cycling and were used to kill animals representing each day of the estrous cycle. Non-carrier isogenic Eker females between the ages of 2 and 9 months were used to determine the status of IGF-I signaling components in the normal myometrium, and tumor-bearing carrier females between the ages of 12 and 16 months were used for analyses concerning leiomyomas. All animals were killed by CO_2 asphyxiation between 1200 and 1600 h, at which time ovaries and vaginas were fixed in 10% neutral-buffered formalin. Myometrial tissue was obtained by opening the uterus, scraping attached endometrium from the muscle layer using a sterile scalpel, and rinsing the myometrium in sterile PBS. We have shown this method to completely remove endometrial tissue from the myometrium as determined by histological examination. Samples for RNA and protein analysis were snap frozen in liquid nitrogen and stored at -70 °C. The reproductive stage of each animal at the time of killing was determined by vaginal and ovarian histology (Lu *et al.* 1979, Yuan & Carlson 1987). Normal myometrium from each age group and reproductive stage was pooled prior to RNA or protein extraction, and each group represented tissue samples from at least three individual rats, with the exception of the 2- to 4-month-old diestrous myometrium, which contained two. All leiomyoma samples were analyzed individually. The handling of rats was according to National Institutes of Health (NIH) guidelines. Animals were housed in Association for the Accreditation of Laboratory Animal Care-accredited facilities, and all protocols involving the use of these animals were approved by the Institutional Animal Care and Use Committee.

Quantitative reverse transcription PCR (RT-PCR)

The expression levels of *IGF-I* and *IGF-IR*, as well as glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*),

were measured using a quantitative RT-PCR technique. A competitor template was generated for each gene using the method of Celi *et al.* (1993) from rat cDNA for all three mRNAs. The primer set for each gene included upstream, internal downstream (ID), and external downstream (ED) primers. The sequences for these primers were: 5′-ACAGGCTATGGCTCCAGCAT-3′ *IGF-I* (upstream); 5′-TCCTTCTCCTTTGCAGCTTCCTTG GGCATGTCAGTGTGG-3′ *IGF-I* (ID); 5′-TCCTTC TCCTTTGCAGCTTC-3′ *IGF-I* (ED); 5′-GTCACG GAATACGACGGGCA-3′ *IGF-IR* (upstream); 5′-AAG TTACCATTGGGCAGAGTGAGGAGGAGTTTGAT GCCGA-3′ *IGF-IR* (ID); 5′-AAGTTACCATTGGGCA TAGT-3′ *IGF-IR* (ED); 5′-AAACCCATCACCATCT TCCAG-3′ *GAPDH* (upstream); 5′-AGGGGCCATCC ACAGTCTTCTTCCACGATGCCAAAGTTGTCA-3′ *GAPDH* (ID); and 5′-AGGGGCCATCCACAGTCT TCT-3′ *GAPDH* (ED). Each competitor template was amplified, purified by electrophoresis, quantitated by spectrophotometry, serially diluted in water, and stored at −70 °C. Generated competitors were identical to the portion of each gene to be amplified by the primers chosen with the exception that each competitor lacked an internal sequence of 30–40 bp. The similarity of internal sequence and identity at priming sites ensured that amplification of both endogenous and competitor targets could be performed using a single primer pair (upstream and ED) and proceeded with similar efficiencies.

Total RNA was isolated from tissue as previously described (Chirgwin *et al.* 1979, Chomczynski & Sacchi 1987). RT of 10 μg total RNA was performed for 1 h at 37 °C in a 100 μl reaction containing 50 mM KCl, 20 mM Tris–HCl (pH 8·4), 2·5 mM $MgCl_2$, 0·4 mM dNTPs, 5 pmol/μl random hexamer primers, 400 U RNase inhibitor (Promega, Madison, WI, USA), and 1000 U M-MLV reverse transcriptase (Gibco BRL, Gaithersburg, MD, USA). Quantitation of genes for each sample was done in duplicate from separate RT reactions and proceeded by amplifying cDNA in each of six reactions. To 3 μl cDNA, the following were added to each 25 μl reaction: 2·5 μl 10 × PCR buffer (Perkin Elmer, Branchburg, NJ, USA), 1·25 μl 4 mM dNTPs, 1·25 μl of both upstream and ED primers at 10 pmol/μl, 5 μl competitor template, and 2·5 U Taq polymerase (Perkin Elmer). These six reactions consisted of five different competitor concentrations and a '0 competitor' control. Amplification was performed for 26 cycles (95 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s) with a final extension at 72 °C for 5 min. PCR products were electorphoresed on 6% TBE/urea gels (Novex, San Diego, CA, USA) and stained with ethidium bromide. The density of bands was then determined using a Molecular Dynamics Fluorimager (Sunnyvale, CA, USA) and ImageQuant software. Linear regression was used to calculate a line with at least $r^2 \geq 0{\cdot}95$ from the plot of the ratio of band densities (competitor/endogenous) versus the known competitor concentration. The concentration of endogenous target in each reaction was assumed to be equal to the concentration of competitor template that corresponded with a ratio of band densities equal to 1 on the equation generated by linear regression. *GAPDH* determinations were run simultaneously with *IGF-I* and *IGF-IR* for all samples. To do this, *GAPDH* reactions were performed using 3 μl per reaction of a 1:300 dilution from cDNA due to the much higher levels of expression and the need for linear amplification with 26 cycles. Data are expressed as the ratio of *IGF-I* or *IGF-IR* message levels to *GAPDH* levels at a 1:300 dilution.

IGF-I promoter usage

A multiplex RT-PCR method was used to determine the promoter usage for initiation of *IGF-I* transcription in tissues. Primers were designed to amplify two fragments of 390 and 361 bp based on the inclusion of exon 1 or exon 2 respectively into mature mRNAs. The set of primers consisted of an upstream primer positioned in exon 1 (5′-ATGGGGAAAATCAGCAGT-3′), an upstream primer positioned in exon 2 (5′-CGACCCGGGACG TACCAA-3′), and a common downstream primer positioned in exon 3 (5′-GGGCATGTCAGTGTGGCG-3′). cDNA was generated from 10 μg total RNA using the method described above in a 20 μl reaction and then amplified by PCR. Each 50 μl PCR reaction contained 10 μl cDNA, 5 μl 10 × PCR buffer, 2·5 μl 4 mM dNTPs, 2·5 μl of each of the three primers at 10 pmol/μl, and 5 U Taq polymerase. Amplification was carried out for 35 cycles (95 °C for 30 s, 50 °C for 30 s, 72 °C for 30 s) with a final extension at 72 °C for 5 min. Products were resolved on a 3% NuSieve (FMC, Rockland, ME, USA) agarose gel and visualized by ethidium bromide staining.

IRS-1 and phosphotyrosine Western blotting

All protein lysates were prepared in a solution of 150 mM NaCl, 1% NP-40, 0·5% Triton X-100, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 50 μM sodium molybdate, 1 mM sodium vanadate, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 4 μg/ml 4-amidinophenylmethanesulfonyl fluoride, and 50 mM Tris–HCl (pH 7·5). Two milligrams of protein were immunoprecipitated in the above buffer containing 0·1% BSA with 4 μg rabbit polyclonal antibody directed against IRS-1 (Upstate Biotechnology, Inc., Lake Placid, NY, USA) or normal rabbit IgG as a control, and antigen–antibody complexes were retrieved with 40 μl of a Protein G Plus-Agarose bead slurry (Oncogene Research Products, Cambridge, MA, USA). Proteins remaining attached to beads were liberated, denatured, and reduced in a 2 × Laemmeli buffer by heating for 5 min at 95 °C. The resulting supernatant was divided in half and electrophoresed on a 4–12% gradient polyacrylamide Tris–glycine gel (Novex). Proteins were

transferred to nitrocellulose and probed for IRS-1 using the same antibody as for immunoprecipitation in a solution of 3% non-fat dry milk and 1% BSA in Tris-buffered saline (TBS) for phosphotyrosine-containing proteins using a monoclonal anti-phosphotyrosine antibody (clone PY99; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in a solution of 5% non-fat dry milk and 1% BSA in TBS. Secondary antibodies conjugated to horseradish peroxidase were purchased from Zymed Laboratories, Inc. (South San Francisco, CA, USA). Washes were performed in TBS containing 0·1% Tween 20. Labeled peptides were visualized with an efficient chemiluminescence system and quantitated by densitometry.

Statistics

Comparisons between measured values were performed using one-way ANOVA followed by Fisher's least significant difference test except for the following, in which Student's one-sided *t*-test was used: all comparisons between the 2- to 4-month-old and 6- to 9-month-old myometrium and the comparison between tumor IRS-1 tyrosine phosphorylation levels. The correlation coefficient (*r*) between leiomyoma *IGF-I/GAPDH* and IRS-1 tyrosine phosphorylation values was generated and tested using routine methods.

Results

IGF-I and IGF-IR expression in the normal myometrium

To determine how expression of *IGF-I* and *IGF-IR* genes are regulated in the normal myometrium, we first examined 2- to 4-month-old Eker rats which displayed regular estrous cycles. The concentration of *IGF-I* mRNA in myometrial cells varied during the estrous cycle with the greatest level of expression occurring on the day of proestrus (Fig. 1). *IGF-I* levels fell 4·5-fold following proestrus to their lowest level on the day of estrus ($P=0·05$). The concentration of growth factor message then rose slowly through metestrus and diestrus in the latter part of the cycle. Regulation of the *IGF-IR* gene also differed by estrous stage, and *IGF-IR* mRNA levels tended to vary inversely with *IGF-I* message. The lowest *IGF-IR* expression occurred in proestrus when *IGF-I* message was maximal, then rose almost 4-fold with decreased ligand synthesis in estrus and metestrus.

We previously observed that the proliferative and apoptotic responses of the myometrium that occur during ovarian cycling were dampened in 6- to 9-month-old females compared with 2- to 4-month-old animals, despite a lack of difference in the reproductive cycles and hormone levels of the two age groups (Burroughs *et al.* 2000). To investigate whether differences in the expression of *IGF-I* or its receptor with age could mediate the decreased

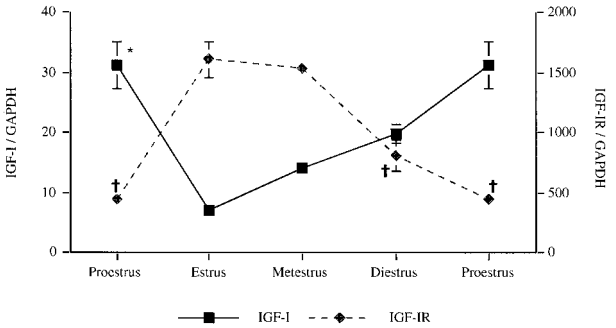

Figure 1 The expression of *IGF-I* and the *IGF-IR* was determined in the 2- to 4-month-old myometrium by quantitative RT-PCR. Values were normalized according to *GAPDH* expression and plotted as a function of the estrous cycle (means ± S.E.M.). $^{*}P \leq 0·05$ versus estrus and metestrus. $^{\dagger}P \leq 0·05$ versus estrus and metestrus.

myometrial response to ovarian hormone signaling, the message levels of both genes were determined in the myometrium of 6- to 9-month-old Eker females. In these animals, expression of *IGF-I* varied less than 2-fold during the estrous cycle, and on the day of proestrus when *IGF-I* expression was maximal in the 2- to 4-month-old proliferative myometrium, the concentration of *IGF-I* message in the 6- to 9-month-old animals was reduced by 52% ($P=0·05$; Fig. 2). Differences were not observed on other days of the cycle (data not shown). Similar to its ligand, *IGF-IR* levels in older animals were no longer altered by ovarian cycling, and receptor expression was reduced 28% in proestrus compared with the 2- to 4-month-old myometrium. Therefore, changes in both *IGF-I* and *IGF-IR* mRNA levels in response to ovarian cycling were dampened in the 6- to 9-month-old myometrium.

Dysregulated IGF-I expression in uterine leiomyoma

The frequency of uterine leiomyomas in female Eker rats increases with age and in 16-month-old animals tumors occur in approximately 65% of gene carriers. We and others previously reported that aging rats, including the Long–Evans strain on which the Eker mutation exists, no longer exhibit normal reproductive cycles and arrest in stages of reproductive senescence in which hormone levels remain static over prolonged periods of time (Lu *et al.* 1979, Burroughs *et al.* 2000). These stages are termed pseudopregnant (PP), persistent estrus (PE) and atrophic (AT), as defined by hormone levels and vaginal and ovarian histology (Lu *et al.* 1979). Therefore, uterine leiomyomas and age-matched normal myometrium from 12- to 16-month-old Eker females were grouped according to each animal's stage of reproductive senescence.

In aged normal myometrium, expression of *IGF-I* remained static throughout all three stages of reproductive

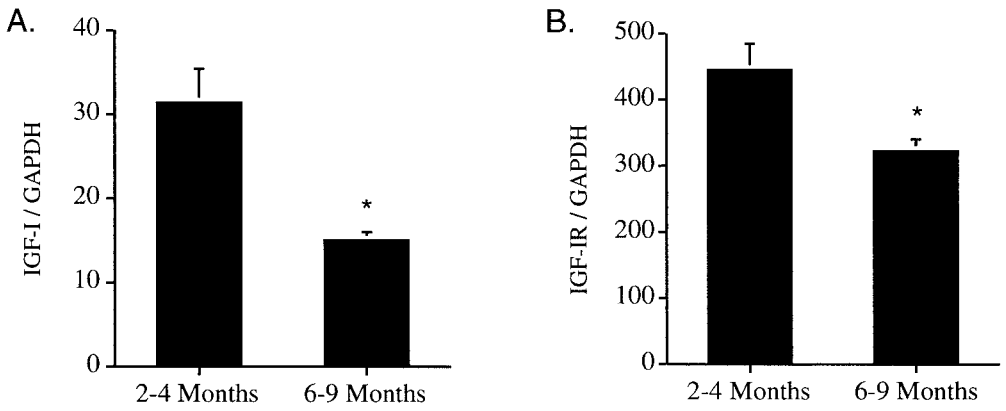

Figure 2 The expression of *IGF-I* (A) and the *IGF-IR* (B) transcripts on the day of proestrus was compared between the 2- to 4-month-old and 6- to 9-month-old myometrium (expressed as the mean ± S.E.M.). *$P \leq 0{\cdot}05$ versus 2- to 4-month-old myometrium.

senescence and was similar to low levels found on the day of estrus in 2- to 4-month-old cycling animals, consistent with the depressed levels of circulating estrogen (<10 pg/ml) present in these animals. Expression of *IGF-I* in leiomyomas was elevated 7·5-fold relative to age-matched normal tissue. However, unlike the myometrium, differences were observed in tumor *IGF-I* levels between stages of reproductive senescence (Fig. 3A). Elevated *IGF-I* expression in PE and AT tumors was significantly different from myometrium. Additionally, leiomyomas from PE animals had significantly greater amounts of *IGF-I* mRNA than tumors from PP or AT animals ($P=0{\cdot}05$). In PE, there was a 200 and 70% increase in *IGF-I* expression relative to PP and AT respectively.

As was seen in the 2- to 4-month-old normal myometrium, *IGF-IR* message levels in tumors were inversely related to *IGF-I* levels (Fig. 3B). Elevated expression of *IGF-I* in leiomyomas was associated with significantly lower concentrations of receptor mRNA compared with the age-matched myometrium ($P=0{\cdot}05$). No differences were noted in *IGF-IR* expression between tumors from the three stages of reproductive senescence.

Promoter usage for initiation of IGF-I transcription

Transcription of the *IGF-I* gene in the rat and human can be initiated by two separate promoters, giving rise to mature transcripts containing either exon 1 or exon 2 in a mutually exclusive manner (LeRoith *et al.* 1992). The

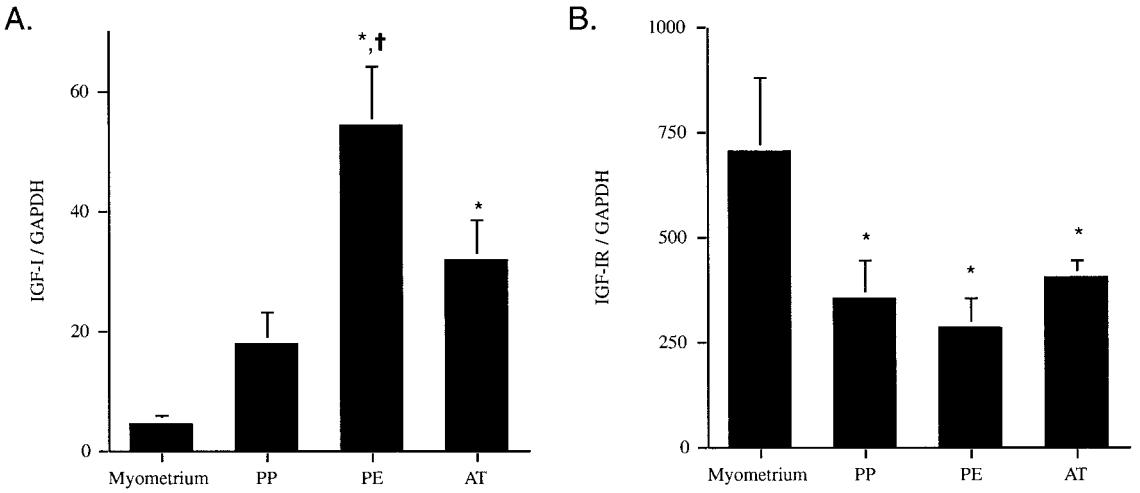

Figure 3 (A) The expression of *IGF-I* by leiomyomas and age-matched myometrium was determined by quantitative RT-PCR and plotted as the mean ± S.E.M. Shown are tumors separated by stage of reproductive senescence (PP, $n=5$; PE, $n=6$; AT, $n=5$) and myometrium with the three stages combined. *$P \leq 0{\cdot}05$ versus myometrium. †$P=0{\cdot}05$ versus PP and AT tumors. (B) *IGF-IR* gene expression was determined and plotted as the mean ± S.E.M. for leiomyomas separated by stage of reproductive senescence and age-matched myometrium (three stages combined). *$P \leq 0{\cdot}05$ versus myometrium.

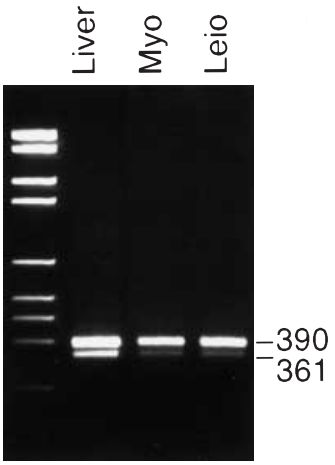

Figure 4 The promoter usage for *IGF-I* transcription initiation was determined in myometrial (Myo) and leiomyoma (Leio) tissues using a multiplex RT-PCR approach. Liver was used as a positive control for transcripts generated from promoter 1 (390 bp) and promoter 2 (361 bp).

regulation of gene transcription offered by the two promoters is quite different and promoter usage could potentially contribute to alterations in *IGF-I* expression in leiomyomas. To determine the pattern of promoter usage in myometrium and leiomyomas, we employed a multiplex RT-PCR approach to specifically amplify transcripts containing either exon 1 or exon 2. Using liver as a positive control, we could detect the presence of both transcripts (Fig. 4), whereas in both normal myometrium and leiomyomas, while both transcripts could be detected, exon 1-containing transcripts predominated (Fig. 4) and no differences were noted in the ratio of exon 1 to exon 2 products in either of these tissues during the estrous cycle (data not shown). Thus, promoter usage appeared to be similar despite the observed changes in *IGF-I* expression.

Hyper-activation of IGF-IR signaling in uterine leiomyoma

Tyrosine phosphorylation of IRS-1 was examined by immunoprecipitation and Western blotting to determine if changes in the expression of *IGF-I* and *IGF-IR* in tissues at the message level impacted signaling from the receptor. Binding of ligand to the IGF-IR leads to receptor autophosphorylation and subsequent phosphorylation of IRS-1 on multiple tyrosine residues by the receptor kinase domain (Feltz *et al.* 1988, Lamphere & Lienhard 1992). We verified using a cell line derived from an Eker leiomyoma that IGF-I stimulation leads to tyrosine phosphorylation of IRS-1 (data not shown). Subsequently, differential phosphorylation of IRS-1 was detected in the 2- to 4-month-old myometrium at times of high and low E_2 during the estrous cycle (Fig. 5A). Basal activation occurred on the day of estrus, and values at other stages were expressed relative to this day. The levels of IRS-1 tyrosine phosphorylation were significantly greater during times of increasing (diestrus) and maximal (proestrus) circulating E_2 levels than on the day of estrus ($P=0{\cdot}05$) when E_2 levels are at their nadir. IRS-1 phosphorylation increased to 2·9-fold on metestrus before reaching its maximum of 5·7-fold in diestrus. Phosphotyrosine content of IRS-1 then decreased slightly on the day of proestrus to 4·0-fold relative to the level in estrus. Importantly, tyrosine phosphorylation of IRS-1 was reduced from $4{\cdot}0 \pm 0{\cdot}6$ (mean ± S.E.M.) fold induction in the 2- to 4-month-old myometrium to $2{\cdot}5 \pm 0{\cdot}2$ in the 6- to 9-month-old tissue during proestrus ($P=0{\cdot}05$), a reduction of 38%. These results suggest changes in levels of IGF-IR signaling consistent with the observed changes in expression of *IGF-I* in the myometrium during the estrous cycle and aging.

The phosphotyrosine content of IRS-1 in tumors was compared with age-matched 12- to 16-month-old myometrium. On average, the level of IRS-1 phosphorylation in leiomyomas was 4-fold higher than myometrium (Fig. 6A, Table 1). Leiomyomas also contained 2·3-fold (range 0·7–3·9) more IRS-1 protein than normal myometrium, raising the possibility that increased levels of phosphorylated IRS-1 in tumors was the result of both increased IRS-1 protein and increased autocrine production of IGF-I. Therefore, we determined the amount of IRS-1 phosphorylation in tumors relative to the amount of IRS-1 protein. After normalizing for IRS-1 expression, phosphorylation of IRS-1 was found to be elevated 1·8-fold (range 0·4–3·5) (Table 1) in tumors relative to normal tissue. Therefore, in some tumors the increase in phosphorylated IRS-1 was a result of increased IRS-1 levels, whereas in others increased phosphorylation *per se* was responsible for this elevation. In addition, we asked if phosphorylation of IRS-1 correlated with the expression of *IGF-I* in normal myometrium and tumors. Figure 6B shows a positive correlation existed between *IGF-I* expression levels and relative IRS-1 phosphorylation ($r=0{\cdot}72$, $P=0{\cdot}05$). Finally, greater amounts of phosphorylated IRS-1 were found in PE leiomyomas compared with AT ($P=0{\cdot}05$), consistent with higher *IGF-I* message levels observed in PE tumors. Taken together, these data suggest that increased expression of *IGF-I* by tumors increased signaling from the IGF-IR relative to normal myometrium.

Discussion

This study examined the regulation of autocrine IGF-I signaling in uterine leiomyomas and the normal myometrium in an experimental animal tumor model to determine whether this signaling system could contribute to the dysregulated growth kinetics, altered steroid-responsiveness and apoptotic defect observed in these tumors. In the normal tissue of 2- to 4-month-old females,

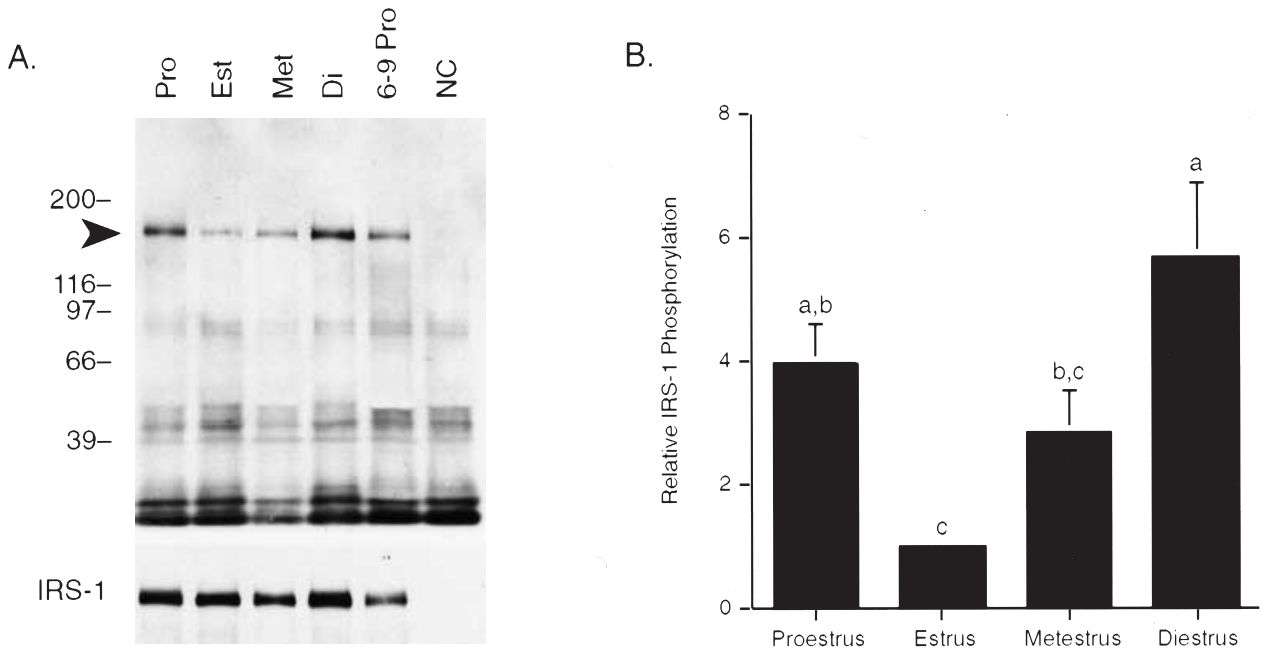

Figure 5 (A) The tyrosine phosphorylation status of IRS-1 in the 2- to 4-month-old and 6- to 9-month-old myometrium was determined by immunoprecipitation for IRS-1 followed by Western blotting for tyrosine-phosphorylated proteins. A negative control (NC) representing the diestrus myometrium immunoprecipitated with normal rat IgG was also processed. The band representing IRS-1 is denoted by an arrow. The lower part of this panel shows an identical blot run in parallel and probed with the anti-IRS-1 antibody used for immunoprecipitation. Pro=proestrus, Est=estrus, Met=metestrus and Di=diestrus. (B) Densitometric analysis of the above blot is represented in graphical form. Basal phosphorylation of IRS-1 was seen on the day of estrus, and values for other stages were expressed relative to this day (means ± S.E.M.). a ≠ b ≠ c, $P=0{\cdot}05$.

IGF-I expression levels were controlled by ovarian cycling, varying more than 4-fold during the estrous cycle, and were greatest on the day of proestrus when E_2 levels peaked. *IGF-IR* expression in myometrial cells was inversely related to that of *IGF-I*. With age, *IGF-I* expression was significantly decreased in the myometrium, which we have previously shown to become proliferatively quiescent (Burroughs *et al.* 2000). In leiomyomas, dysregulated expression of *IGF-I* resulted in message levels that were 7·5-fold greater than those seen in age-matched normal myometrium. Increased *IGF-I* mRNA in tumors was not associated with changes in promoter usage for gene transcription. Tyrosine phosphorylation of IRS-1 was highest in the myometrium when *IGF-I* expression was high, and increased *IGF-I* expression in leiomyomas correlated with increased phosphorylation of IRS-1. These data suggest that overproduction of IGF-I by these tumors resulted in activation of signaling pathways emanating from the IGF-IR.

Expression of *IGF-I* in the uterus is regulated by steroid hormones produced by the ovaries. In the rat, *IGF-I* message levels vary in the whole uterus during the estrous cycle, and direct E_2 administration to ovariectomized females elicits an increase in *IGF-I* transcripts in the myometrium as determined by *in situ* hybridization (Ghahary *et al.* 1990, Sahlin *et al.* 1994). In the current study, quantitative RT-PCR was used to show that the *IGF-I* gene was specifically regulated in the normal myometrium by normal ovarian cycling. The peak of *IGF-I* expression was observed on the day of proestrus when E_2 levels have been shown to be maximal (Burroughs *et al.* 2000). In addition, the extent of tyrosine phosphorylation of IRS-1 was elevated on the day of proestrus. These data, in conjunction with previous studies in which exogenous hormones were administered to spayed animals, indicate that IGF-I is probably an important mediator of steroid hormone signaling in the myometrium of the intact, cycling rodent.

There are some differences in the physiology of the murine and human reproductive cycle, and the biology of leiomyoma in the two species reflect these differences in physiology. For example, in leiomyomas cell proliferation and apoptosis vary as a function of the menstrual/estrous cycle. In humans, although the data are limited, leiomyomas appear to be most proliferative during the luteal phase of the cycle (Kawaguchi *et al.* 1989). Subsequent reports have tended to support this initial finding (Nisolle *et al.* 1999, Wu & Somlo 2000), although in one of these studies the difference between proliferation occurring in the two phases did not reach statistical significance (Nisolle *et al.* 1999). In the rat, cell proliferation in the myometrium was maximal during proestrus, when hor-

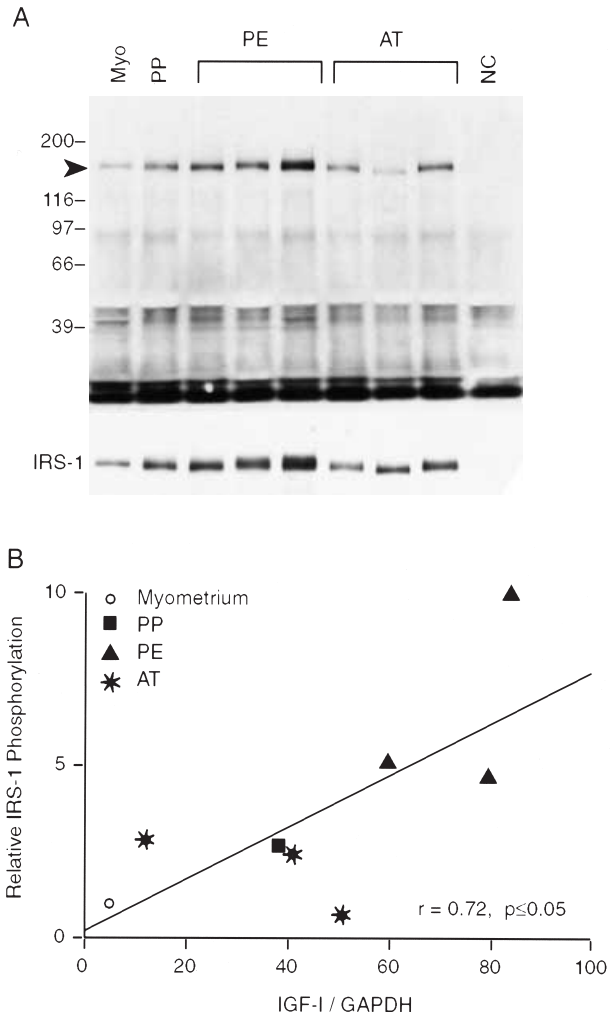

Figure 6 (A) The tyrosine phosphorylation status of IRS-1 in leiomyomas separated by stage of reproductive senescence and age-matched myometrium (three stages combined) was analyzed by immunoprecipitation for IRS-1 followed by Western blotting for tyrosine- phosphorylated proteins. A negative control (NC) representing a PE tumor lysate immunoprecipitated with normal rat IgG was also processed. The band representing IRS-1 is denoted by an arrow. The lower part of this panel shows an identical blot run in parallel and probed with the anti-IRS-1 antibody used for immunoprecipitation. Statistical analysis using Fisher's least significant difference test indicated that normalized phosphotyrosine levels were significantly different ($P \leq 0{\cdot}05$) from myometrium for five out of seven tumors analyzed (Table 1). (B) Mean values of IRS-1 phosphorylation were plotted versus the expression of *IGF-I* for individual leiomyomas and age-matched myometrium. A positive correlation was observed between *IGF-I*/*GAPDH* values for each sample and IRS-1 activation ($r = 0{\cdot}72$, $P \leq 0{\cdot}05$).

Table 1 Phosphotyrosine (PY) content of IRS-1 in tumours compared with age-matched 12- to 16-month-old myometrium

	Fold change in normalized PY*	Fold change in normalized PY/IRS-1*
Tumour		
PP (TP-8)	2·71	1·22
AT (KBV-6)	2·43	3·49
AT (TP-10)	0·66	0·41
AT (Veh-7)	2·84	1·65
PE (TP-12)	5·00	2·21
PE (TP-7)	4·64	1·46
PE (Veh-23)	9·96	2·55

*Duplicate determinations relative to age-matched normal myometrium.

mone levels correspond to the high estrogen and Pg levels that occur during the luteal phase in women (Burroughs *et al.* 2000). Thus, while proliferation is maximal in different phases of the menstrual/estrous cycle in the two species, luteal phase and proestrus respectively, in fact proliferation occurs under a similar set of hormonal conditions, i.e. when both Pg and estrogen levels are elevated. Conversely, in the normal rat myometrium, apoptosis is maximal during estrus, when steroid hormones are approaching their nadir and leiomyomas in the Eker rat model have been demonstrated to have a defective apoptotic program relative to age-matched normal myometrium (Burroughs *et al.* 2000). Human data on apoptotic rates in leiomyoma are extremely limited, although there is some suggestion of such a defect from clinical data as well (Huang *et al.* 1997, Matsuo *et al.* 1997).

There is some evidence from the existing clinical literature that IGF-I is regulated in an estrogen-responsive manner in human leiomyomas. Englund *et al.* (2000) reported that leiomyomas taken from premenopausal women contained more *IGF-I* mRNA than tumors taken from postmenopausal women and that IGF-I concentrations correlated with serum E_2 concentrations (Englund *et al.* 2000). Likewise, Giudice *et al.* (1993) reported that myometrium and leiomyomas taken from the follicular phase of the cycle, when E_2 levels peak, express higher levels of *IGF-I* transcripts than tumors and myometrium from the luteal phase. However, small sample size ($n = 1$ for myometrium from each phase) and lack of quantitation may have confounded this interpretation. In the present study, although basal *IGF-I* expression was elevated in leiomyomas relative to the age-matched myometrium, differences were noted in the levels of *IGF-I* transcripts among tumors between the stages of reproductive senescence. PE leiomyomas contained more *IGF-I* message than PP or AT tumors, implying the presence of a stimulus to IGF-I synthesis. Contradictions exist in the literature as to whether E_2 concentrations in PE animals are elevated (Huang *et al.* 1978, Lu *et al.* 1979, Burroughs *et al.* 2000), but the thickening and keratinization of the vaginal epithelium characteristic of PE animals implies that an estrogenic stimulus also exists during this stage of reproductive senescence.

Table 2 *IGF-I* expression, proliferation and apoptosis in leiomyomas. The rates of proliferation and apoptosis as determined by immunostaining for the presence of 5-bromo-2′-deoxyuridine (BrdU) in DNA and by TUNEL respectively are listed along with the expression of *IGF-I* for normal myometrium and leiomyomas in each stage of reproductive senescence

	***IGF-I* expression** (*IGF-I*/*GAPDH*)	**Proliferation rate*** (BrdU-positive/HPF)	**Apoptosis rate*** (TUNEL-positive/HPF)
Myometrium**	4·8	0·4	3·1
PP leiomyomas	17·9	9·1	0·4
PE leiomyomas	54·3	13·1	0·6
AT leiomyomas	32·0	7·9	0·2

*Burroughs *et al.* (2000).
**Values for the myometrium did not vary with stage of reproductive senescence, thus presented values are an average from all stages.
HPF denotes high-power field.

The estrogen-responsiveness of the *IGF-I* gene could in fact underlie some of the ambiguity regarding whether *IGF-I* expression is elevated in human leiomyomas relative to normal myometrium. As described above, surges in E_2 levels occur in both follicular and luteal phases of the reproductive cycle of women. Therefore in the absence of precise measurements of circulating steroid hormone levels, it may be difficult to accurately assess how changes in hormonal milieu might modulate changes in IGF-I levels that occur during the reproductive cycle. Andersen & Barbieri (1995) have hypothesized that human leiomyomas are hypersensitive to estrogen, pointing to data on elevated expression of several estrogen-responsive genes in leiomyomas, including *IGF-I*. Our data on increased *IGF-I* expression in the normal myometrium during proestrus, when E_2 levels peak in the rat, and elevated *IGF-I* expression in leiomyomas in aged (16-month-old) animals, where E_2 levels are low, are consistent with what is known about the estrogen-responsiveness of this gene and support the hypothesis that leiomyomas exhibit an increased sensitivity/responsiveness to E_2.

The IGF-IR is known to activate signal-transduction pathways leading to cell mitogenesis. We have previously demonstrated the existence of a mitogenic IGF-I autocrine loop in cell lines derived from Eker rat leiomyomas (Howe *et al.* 1996). These cells secrete IGF-I into growth medium, and IGF-I-neutralizing antibodies block cell proliferation, establishing the existence of an autocrine loop *in vitro*. Data taken together from this and a previous study (Burroughs *et al.* 2000) indicate maximum *IGF-I* message levels, high IRS-1 phosphorylation, and peak DNA synthesis all occur when E_2 levels peak on the day of proestrus in the normal myometrium (Burroughs *et al.* 2000). Conversely, reduced *IGF-I* expression and IRS-1 phosphorylation correlate with the decreased cell proliferation that occurs in the myometrium of 6- to 9-month-old animals. These observations suggest that IGF-I production and concomitant activation of the IGF-IR directs the level of proliferation of normal myometrial cells. Likewise, in leiomyomas, a positive correlation exists between the concentration of *IGF-I* mRNA and cell proliferation. The increased abundance of *IGF-I* message in leiomyomas relative to the myometrium is associated with high rates of proliferation. Table 2 shows how expression of *IGF-I* by tumors relates to previously determined rates of proliferation in this model (Burroughs *et al.* 2000). Even within tumors themselves, PE tumors that contained statistically greater amounts of *IGF-I* mRNA also show higher rates of DNA synthesis than PP or AT leiomyomas. These data, in conjunction with data obtained *in vitro*, support a model in which IGF-I acts as an autocrine growth factor to stimulate proliferation of both normal and neoplastic myometrial cells.

In addition to its ability to stimulate cell proliferation, IGF-I can act as a survival factor by inhibiting apoptosis. Protein kinase B/Akt, a downstream target of IGF-IR and IRS-1 signaling, has been linked to differential phosphorylation of members of the bcl-2 family of proteins, which serve to modulate apoptotic signals (Datta *et al.* 1997). In the 2- to 4-month-old myometrium, the lowest levels of *IGF-I* expression and IRS-1 phosphorylation occurred on the day of estrus, when apoptosis is maximal (Burroughs *et al.* 2000). Similarly, we previously showed that cells derived from Eker leiomyomas are refractory to the induction of apoptosis by multiple stimuli *in vitro* (Burroughs *et al.* 1997). *In vivo*, leiomyomas exhibit decreased apoptotic indices (Burroughs *et al.* 2000) relative to normal myometrium and this decrease in apoptosis may also be a consequence of increased *IGF-I* expression (Table 2). Therefore, in Eker leiomyomas overexpression of IGF-I and IGF-IR signaling as evidenced by increased IRS-1 phosphorylation may account for the resistance of tumor cells to apoptosis.

The current study provides evidence that autocrine IGF-I signaling may be an important regulator of tissue growth in the normal myometrium and that dysregulated expression of this growth factor occurs in uterine leiomyomas. Elucidation of the targets of IGF-I signaling in

fibroids and the mechanisms responsible for dysregulated signaling that occurs in these tumors may contribute significantly to our understanding of the etiology of this disease and help identify new potential avenues for developing improved therapies for this common gynecological tumor.

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