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Novel treatment avenues for uterine leiomyoma: a new implication for endothelin?

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Abstract

Cysteine-rich angiogenic inducer 61 (CYR61), an angiogenic factor whose expression is decreased in fibroids. The aim of the present study was to determine if CYR61 secretion in smooth muscle cells (SMCs) is regulated by hypoxia and through the endothelin A (ET_A) receptor. SMCs from fibroids (fSMC) and the adjacent myometrium smooth muscle cells (mSMCs) were extracted from ten women undergoing hysterectomy for uterine fibroids and cultured with or without 1.0 μM of an ET_A receptor antagonist for 24 h under either normal or hypoxic oxygen conditions. Cellular secretion of endothelin-1 (ET-1) and CYR61 were measured via enzyme linked immunosorbent assay in the cell culture media. SMCs were collected to determine cell proliferation and CYR61 protein expression via Western blot. ET-1 secretion was significantly increased in fSMC and was decreased with blockade of the ET_A receptor under both normoxia ($P=0.0004$) and hypoxia ($P=0.008$). CYR61 expression was decreased in fSMCs and significantly increased with blockade of the ET_A receptor under hypoxia ($P=0.04$). Cell proliferation decreased with ET_A blockade under normoxia ($P=0.0001$) and hypoxia ($P=0.001$). These results suggest that suppression of CYR61 secretion in fSMC is regulated by the ET-1 and that blockade with ET_A could be considered for a future treatment option.

Introduction

Uterine leiomyomas, also known as fibroids, are benign smooth muscle neoplasms that typically arise from the myometrium. Many differences have been observed between fibroids and the surrounding myometrium including differences in their vasculature. Fibroids have been shown to have a smaller vascular area and microvessel density than the surrounding myometrium [1]. Additionally, there are differences in gene expression between

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Author contribution

K.W. and B.L. designed these studies, analyzed the data, and wrote the first draft of the manuscript. V.J. recruited and consented all study participants. V.J. and K.C. performed cell culture and flow cytometry experiments. K.C., A.B., and J.R. performed all assays. All authors took part in reviewing and editing subsequent drafts of the manuscript.

Competing interests

The authors declare that there are no competing interests associated with the manuscript.

fibroid tissue and the adjacent myometrium. In particular, cysteine-rich angiogenic inducer 61 (CYR61), an angiogenesis factor that has been found to be decreased in fibroids relative to the myometrium [2,3]. Given that when secreted CYR61 can function in a paracrine/autocrine manner, this protein functions as a messenger amongst several cell types, including SMCs and can influence different biological functions within its local environment [4–6]. In breast, colon rectal and ovarian cancer, CYR61 is increased and has been implicated in contributing to tumor progression, and is often linked to a poor prognosis [7]. However, in some other types of cancer, such as endometrial and lung, CYR61 expression is decreased [8,9].

While the etiology of fibroids remains unknown, hypoxia is believed to play an important role in the development of fibroids. There have been several studies that have shown fibroids to be hypoxic compared with the surrounding myometrium [10,11]. We have recently shown hypoxia increased fibroid secretion of endothelin-1 (ET-1), a powerful vasoconstrictor and increased circulating levels of ET-1 in the fibroid patient. CYR61 has been reported to decrease smooth muscle cell (SMC) vasoconstriction, which is one of the functions of ET-1, suggesting that the decrease in CYR61 reported in women with uterine fibroids might coincide with the increase in ET-1 [12]. ET-1 has been suggested to have a role in the development of fibroids through both activation of the endothelin A (ET_A) receptor and chronic periods of hypoxia [13–15]. Similar to CYR61, ET-1 and hypoxia both have roles in angiogenesis and cell proliferation [16–18]. Importantly, ET-1 has been linked to various female cancers and ET-1 acting via the ET_A receptor, and has been implicated in the development of cancers such as cell proliferation, migration, invasion, transition, and angiogenesis.

Given the importance of ET-1 and CYR61 in contributing to angiogenesis, cell migration and extracellular matrix development, all of which is dysregulated in fibroids, we conducted the current study examining the impact of ET_A receptor blockade on CYR61 production in the presence of hypoxia and their influence on fibroid SMC proliferation.

Experimental methods

Sample collection

Fibroids and adjacent myometrium tissue were collected from ten women undergoing hysterectomies for complications associated with fibroids at the University of Mississippi Medical Center. The Institutional Regulation Board approved the sample collection with patient informed consent prior to hysterectomy. All of the tissue was excised from the uterus while in the operating room and placed in sterile media containing 5% penicillin/streptomycin (P/S; Invitrogen) before being brought to the laboratory.

Primary culture of uterine SMCs

SMCs from fibroid or myometrium tissue were isolated as previously described [19]. Briefly, 1 mm³ pieces of tissue were minced, placed into 2 ml of PBS containing 4% collagenase II (Invitrogen), and digested for 4 h at 37°C in a hot water bath. Ten milliliter of media (Dulbecco's Modified Eagle Medium (DMEM; Invitrogen), 10% FBS (5% P/S) was

added to the solution followed by centrifugation at 1000 *g* for 5 min. The pellet was resuspended in 10 ml of media and filtered. The filtrate was plated in 10-cm tissue culture dishes and incubated at 5% CO₂ at 37°C in a humidified atmosphere until cells attached. Media was replaced every 2 days until a monolayer of SMCs was established. Confluent monolayers were dissociated with trypsin (Sigma) and split into fresh complete media. All experiments were conducted between passages 2 and 4.

To verify the purity of cultures, a single cell suspension of cells from either the myometrium or fibroid was stained for α -actin. Briefly, cells were rinsed with warm PBS and centrifuged at 400 *g* for 5 min. The cell pellet was re-suspended in 1 ml of ice cold PBS with 10% FBS containing 1% sodium azide and stained for 30 min at 4°C with FITC-conjugated anti-human α -actin (Abcam, Cambridge, MA) or isotype control to evaluate non-specific binding. Cells were analyzed using a Beckman Coulter Gallios Flow Cytometer. The analyses were performed using Kaluza Software. Cultures that did not have <90% α -actin purity were not used in the current analysis.

Effect of hypoxia and the ET_A receptor on ET-1 and CYR61 secretion and proliferation in SMCs

SMCs were seeded at 25,000 cells/well in 2.5 ml of media and incubated under hypoxic (1% O₂) or normoxic (6% O₂) conditions in a humidified atmosphere at 37°C for a period of 24 h utilizing a ProOx P110 oxygen-controlled hypoxia chamber (BioSpherix) as previously described [15]. To determine the role of the endothelin activation, SMCs were also seeded at 25,000 cells/well in 2.5 ml of media with 1.0 μ M ABT-627 (ET_A antagonist used to determine if ET-1 secretion is due to the stimulation of this receptor) and incubated under hypoxic or normoxic conditions for 24 h. At the end of the 24-h incubation, cell culture media was collected, and the cells were harvested for cell counts and lysed for protein analysis.

Measurement of ET-1 and CYR61 in cell culture media

ET-1 and CYR61 concentrations were determined in the collected medium and using the ET-1 Quantikine ELISA and the CYR61 Quantikine ELISA (RnD Systems and BosterBio). All samples were assayed in duplicate according to the manufacturer's recommended plate layout. Total protein was measured in the cell culture media, using a BCA Protein Quantitation Kit from Pierce.

CYR61 protein expression via Western blot analysis

SMCs were lysed with radioimmunoassay precipitation buffer in the presence of protease inhibitors (Santa Cruz Biotechnology). Solubilized protein (30 μ g) was loaded on a Mini-protean TGX gel (Bio-Rad) and underwent electrophoresis at 240V for 40 min using the Mini-PROTEAN Tetra Cell (Bio-Rad), transferred to a nitrocellulose membrane via a Bio-Rad Transblot Turbo, which was blocked with 5% milk buffer for 1 h at room temperature, rinsed with TBS plus 0.5% tween 20 and incubated with 1:750 of CYR61 (Santa Cruz Biotechnology) overnight in 5% milk buffer followed by 1:2500 of goat anti-mouse HRP (Santa Cruz Biotechnology) in 5% milk buffer the following day. Membranes were developed with Clarity ECL (Bio-Rad) and stained with Ponceau (Sigma) to visualize total

protein. The membrane was scanned using Chemidoc™ XRS+system (Bio-Rad), and the bands were analyzed using ImageJ software [20]. SMCs from one participant were used per membrane with a total of three participants being assayed.

Statistical analysis

All data are expressed as mean $\pm$ S.E.M. Student's *t* test (paired and unpaired) analysis was used to assess statistical differences between normoxic compared with hypoxic and control compared with ET_A antagonist groups. Values of $P < 0.05$ are considered statistically significant.

Results

Hypoxia significantly increases ET-1 secretion in SMCs

SMCs derived from fibroids (fSMC) secreted significantly more ET-1 compared with mSMCs under normoxic conditions ($P = 0.0004$; Figure 1A). Blockade of the ET_A receptor significantly decreased ET-1 secretion in fSMCs compared with untreated fSMCs ($P = 0.007$; Figure 1A) but did not have a statistically significant effect on mSMCs ($P = 0.62$). There was no statistically significant difference in ET-1 secretion between mSMCs and fSMCs treated with the ET_A receptor ($P = 0.84$). While untreated fSMCs secreted significantly more ET-1 than untreated mSMCs under hypoxic conditions ($P = 0.001$; Figure 1B), blockade of the ET_A receptor significantly decreased ET-1 secretion in mSMCs ($P = 0.02$) and fSMCs ($P = 0.008$) relative to untreated controls. In response to hypoxia, fSMCs cultured with the ET_A receptor antagonist secreted significantly more ET-1 compared with mSMCs cultured under the same conditions ($P = 0.04$, Figure 1B).

CYR61 secretion is increased in response to blockade of the ET_A receptor

fSMCs secreted significantly less CYR61 compared with mSMCs in the presence of hypoxia ($P = 0.02$, Figure 1C). Under normoxic conditions blockade of the ET_A receptor did not significantly increase CYR61 expression in mSMCs ($P = 0.820$) or fSMCs ($P = 0.35$; Figure 1C) compared with the untreated cultures. Under hypoxic conditions fSMCs still secreted significantly less CYR61 compared with mSMCs ($P = 0.04$; Figure 1D). Blockade of the ET_A receptor did not lead to significant changes in CYR61 secretion in mSMCs ($P = 0.882$); however, it did significantly increase CYR61 secretion in fSMCs compared with untreated fSMCs ($P = 0.04$, Figure 1D).

There was no statistically significant difference in protein expression of CYR61 measured via Western blot ($P = 0.157$, Figure 2A,B).

Hypoxia induced SMC proliferation is decreased with blockade of the ET_A receptor

Cell proliferation in fSMCs was significantly increased compared with mSMCs under both normoxic ($P = 0.005$) and hypoxic conditions ($P = 0.0001$; Figure 2C) by 1.7- and 2.2-fold respectively. However, hypoxia did lead to a significant 2.2-fold increase in mSMC proliferation compared with normoxic conditions ($P = 0.0004$); with similar results occurring in fSMCs which were increased 2.8-fold compared with normoxic fSMCs ($P < 0.0001$; Figure 2C). Blockade of the ET_A receptor did not lead to a significant decrease in mSMC

proliferation under normoxia ($P=0.12$); however, mSMC proliferation was significantly increased in the absence of ETA blockade under hypoxia ($P=0.0006$; Figure 2C). Cell proliferation in fSMCs was decreased in the ET_A treated groups under both normoxic ($P=0.0001$) and hypoxic oxygen conditions ($P=0.001$, Figure 2C).

Discussion

The CCN (Cyr61, CTGF, Nov) family encodes secreted proteins that modulate the actions of growth factors, extracellular matrix, and affect cell migration [9,21]. CYR61 induces angiogenesis and chemotaxis of microvascular endothelial cells *in vitro* and neovascularization *in vivo* [4,5]. CYR61 has been reported to be decreased in fibroids compared with the adjacent myometrium [2,3,22]. Given the known associations between hypoxia and ET-1 secretion and angiogenesis, CYR61 and fibroids, the potential interactions through the ET_A receptor were investigated.

In the present study, there was a decrease in CYR61 secretion from fSMC compared with mSMC under normoxic conditions. Previous studies by Weston et al. [2] confirm that CYR61 expression in fSMC is decreased compared with mSMC. Hypoxia, a known inducer of angiogenesis, had no significant effect on CYR61 in mSMC or fSMC. These results are contrary to what has been reported in other cell types. Studies in human umbilical vein endothelial cells and in nasal polyp fibroblasts have reported that CYR61 mRNA and protein expression to be increased in the presence of hypoxia [23,24]. It is possible that prolonged periods of hypoxia, such as that proposed to occur in fibroids, lead to a decrease in CYR61 over time. Indeed, a study by Chen et al. [25], found that CYR61 expression in trophoblasts cells was increased due to hypoxia; however, after 24 h of exposure to hypoxia CYR61, mRNA expression had significantly decreased.

The vasoconstrictive peptide ET-1 is induced by hypoxia in fibroids, via the ET_A receptor which is overexpressed in fibroids, and induces proliferation of leiomyoma cells [2,15,26]. To determine if hypoxia stimulated ET-1 release from SMCs via the ET_A receptor, we used an ET_A receptor antagonist in the cell culture medium as a reduction in ET-1 secretion from SMCs would indicate that ET-1 secretion is being regulated through the ET_A receptor [27,28]. Similar to what we previously reported, ET-1 secretion from fSMCs was significantly increased compared with mSMCs under both normoxia and hypoxia. In addition, blockade of the ET_A receptor significantly decreased secretion of ET-1 from fSMCs as well as hypoxic mSMCs. Interestingly, ET_A blockade had no effect of CYR61 secretion in mSMCs under either normoxic or hypoxic oxygen conditions. However, blockade of the ET_A receptor under hypoxic conditions, significantly increased CYR61 secretion from fSMCs relative to untreated hypoxic fSMCs, suggesting that suppression of CYR61 secretion in fSMC is linked to the ET_A receptor. In addition, ET_A blockade significantly suppressed hypoxia stimulated fSMC proliferation. Contrary to what we found in the current study, ET-1, through the ET_A receptor, has been found to induce expression of CYR61 via a cyclo-oxygenase-2 (COX-2)/PGI₂-independent pathway in human proximal artery SMCs [29]. Our results suggest that ET-1 may have a suppressive effect on CYR61 secretion; however, we did not add ET-1 to our cultures to determine its ability to induce CYR61. More importantly, the hypoxia induced increase in cell proliferation was decreased

in response to blockade of the ET_A receptor, which coincides with the increase in CYR61 secretion [30,31]. Importantly, CYR61 significantly increases with ET_A receptor blockade under hypoxic conditions. These data confirm that CYR61 secretion is regulated through the ET_A receptor under normoxic and hypoxic conditions. Ultimately, these results indicate that as ET_A receptor blockade decreases fSMC secretion of ET-1 under hypoxia, there is a concurrent increase in CYR61 secretion, which is associated with decreased hypoxia stimulated fSMC proliferation. With the association of hypertension and vasoconstriction in fibroid patients, chronic administration of ET_A receptor antagonists, could lower not only blood pressure, but also hypoxia stimulate fibroid cell proliferation and could be considered a potential therapy for patients suffering from uterine fibroids.

Despite the increases in pro-angiogenic factors in fibroids, there continues to be evidence suggesting that angiogenesis is impaired. We have previously reported that hypoxia increases the secretion of anti-angiogenic factor soluble fms-like tyrosine kinase 1 (sFlt-1) along with ET-1 in fibroid tissue explants and that through blockade of the ET_A receptor both sFlt-1 and ET-1 are decreased [15]. In the current study, we demonstrate that fSMCs secrete less CYR61 and that hypoxia has no significant effect on increasing the secretion of this protein. However, when we pharmacologically block the ET_A receptor, CYR61 secretion significantly increases. These results suggest that activation of the ET_A pathway contributes to a decrease in CYR61 expression. Despite these results, studies in which CYR61 is silenced and overexpressed in SMCs need to be performed to determine the contribution of this protein to ET-1 secretion and ultimately fSMC proliferation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviation

CYR61	cysteine-rich angiogenic inducer 61
ET-1	endothelin-1
ET_A	endothelin _A
fSMC	fibroid-derived SMC
mSMC	myometrium-derived SMC

P/S	penicillin/streptomycin
sFlt-1	soluble fms-like tyrosine kinase 1
SMC	smooth muscle cell

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Clinical perspective

- CYR61 (an angiogenic factor) and ET-1 (vasoconstrictor) impact smooth muscle cell (SMC) proliferation and angiogenesis, which is dysregulated in women with uterine fibroids and within uterine fibroid tissue.
- Low oxygen conditions, hypoxia, significantly increased SMC proliferation, which was attenuated by blockade of the ET_A receptor under both hypoxic and normal oxygen conditions.
- Blockade of the ET_A receptor increased CYR61 secretion in fibroid derived SMCs, but not in myometrium derived SMCs suggesting that regulation of CYR61 in uterine fibroids might be connected to the endothelin pathway.

Figure 1. Blockade of the ET_A receptor decreases ET-1 secretion and increases CYR61 secretion in fSMC.

ET-1 and CYR61 secretion were measured in cell culture supernatants collected after a 24-h incubation without the ET_A antagonist under normoxic or hypoxic oxygen conditions. ET-1 secretion is significantly increased in fSMCs compared with untreated mSMCs and to fSMCs treated with 1.0 μ M of the ET_A receptor antagonist (A). Under hypoxic conditions, antagonism of the ET_A receptor decreased ET-1 relative to the untreated groups (B). Under normoxic conditions fSMCs secreted significantly less CYR61 compared with mSMC (C). Whereas hypoxia along with the combination of ET_A blockade significantly increased CYR61 secretion (D). *, **, *** denote significant differences between indicated groups of $P < 0.05$, $P < 0.005$, and $P < 0.0005$ respectively. indicates a statistically significant difference between paired normoxic control.

Figure 2. SMCs from three participants were individually assayed via Western blot to determine if there was a difference in CYR61 protein expression.

(A) is a representative Western blot depicting the characteristic band at 40 kDa for CYR61. There was not a significant difference in CYR61 protein expression in SMCs (B). Blockade of the ET_A receptor decreased cell proliferation while hypoxia increased cell proliferation in both mSMC and fSMCs (C). **,*** denote significant differences between indicated groups of $P<0.005$ and $P<0.0005$, respectively. indicates a statistically significant difference between paired normoxic control.