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A new hypothesis about the origin of uterine fibroids based on gene expression profiling with microarrays

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Abstract

This article will discuss some recent insights based on our microarray studies that have emphasized the role the extracellular matrix, transforming growth factor beta, and collagen structure in fibroid formation. These studies led to appreciation of molecular similarities between fibroids and keloids. Collectively, these observations suggest a model of fibroid development based on an abnormal response to tissue repair, resulting in disordered healing and formation of an altered extracellular matrix.

Keywords

Leiomyoma; Fibroids; Keloid; Microarray; Extracellular matrix; Collagen

Fibroids are benign growths, and based on cytogenetic studies the tumors within a single uterus are clonal, each arising from a different myometrial cell.¹ Epidemiologic, clinical, and experimental data suggest sex steroids promote growth of the tumors.² Increased parity may reduce the incidence of the problem, possibly caused by exposure to progesterone.³ A genetic predisposition to the condition appears to be present, because a familial association has been shown, and rare genetic conditions, such as hereditary leiomyomatosis and renal cell cancer (HLRCC) feature fibroid development.⁴ However, with the exception of a guinea pig model,⁵ the Eker rat,⁶ and a recently reported transgenic mouse,⁷ there are few model systems and the origin of these common tumors remains unknown (for review, see Walker and Stewart).⁸

Although the origin of fibroids remains unknown, as health care providers for women, gynecologists must be keenly interested in defining the cause because such an understanding often leads to successful treatment. As we consider what might cause fibroids, there are some puzzling questions to be addressed such as: Why are fibroids so common? As a

neoplasm in prevalence and expense they eclipse all others,⁸ but because genomic instability is a hallmark of neoplasia, why do only 40% of fibroids exhibit genomic instability? Also, why are there differences in the prevalence of the disease in black women? Fibroids are 3 times more likely to affect women of African-American ethnicity.^{9,10} This last point is illustrated in work by Dr Myers (Figure 1). The increased incidence can be seen in hysterectomy rates, which are 3-fold higher in black women, with life-time risk of hysterectomy approaching 22%. It is logical to assume that a comprehensive explanation for fibroid development must provide an answer to the question: Why is there such a difference in the racial prevalence and incidence of the disease?

Given the apparent clonal nature of fibroids, our group reasoned that the myofibroblast cells comprising fibroids may provide clues to fibroid development. Myofibroblasts are cells of an intermediate phenotype, not quite normal uterine muscle, but neither are they differentiated fibroblasts.¹¹ Myofibroblasts secrete collagens and other components of the extracellular matrix, but inappropriate function of myofibroblasts has been shown to cause fibrosis.¹² For this reason, our laboratories have focused on gene profiling experiments of these cells.^{13,14} Gene profiling, or microarray experiments, enables normal myometrium to be compared with fibroid tumors. The microarray takes advantage of robotics and the information from the human genome project. Few assumptions are required and with high-density genome wide chips available, the approach is almost devoid of inherent bias. Fibroids are particularly well suited to this approach, as the tumors are clonal and normal myometrium from the same patient is available as a control. Understanding the expression pattern would then allow more complex and specific hypotheses to be generated. We¹³⁻¹⁵ and others¹⁶⁻²¹ have used this potentially powerful approach to study uterine fibroids.

We used oligonucleotide-based chips, specifically Affymetrix HG-U133 A&B chips (Affymetrix, Santa Clara, CA), that contain products from up to 33,000 genes. Fifteen micrograms of total RNA from matched samples from myometrium and leiomyoma were used to generate biotin-labeled complementary RNA (cRNA). For this platform, each sample is prepared separately and hybridized to the chip, then the matched chips are compared by using a computer for the data that have been digitalized. Arrays were analyzed on a Hewlett-Packard Genearray scanner (Hewlett Packard, Palo Alto, CA) using the GeneChip software (Affymetrix). The GeneChip software assigned intensity files for each transcript based on the signal intensity across the 11 pairs of 25 mer oligonucleotide probes of perfect match (PM) or mismatch (MM) sequences. A 1-sided Wilcoxon signed rank test was used to assign a detection *P*-value. After background subtraction based on 1-step Tukey's biweight estimate of transcript expression, global scaling (using 500 as target intensity) was used to normalize and control for any differences in probe intensities. Candidate genes were eliminated if their signal intensity was below 250 U, based on a scatter plot. For pair wise comparison for differences in expression, a Wilcoxon signed rank test generated *P*-value "change calls" of fold changes in transcript expression of either up/increase (+) or down/decrease (-). We used a cutoff of more than 2.0-fold for further investigation of gene expression. The data presented in this article were based on 4 pair wise experimental-control comparisons with an average-fold change across the experiments. In addition, differences in gene expression were confirmed by using other approaches, such as

reverse transcription polymerase chain reaction (RT-PCR), real-time PCR, and for some gene products confirmed the differences in protein expression by immunohistochemistry.

Our first observation was that there were some differences between arrays from different core facilities and different Affymetrix platforms. For instance, using HG-U133 chips and a different core, we found differences between our collaborator and our results. Furthermore, if we simply performed an array on 1 sample, and repeated the array a second time, we observed differences in levels of gene expression.²² This is not too surprising given the vast number of genes sampled. We interpret the differences to be largely because of variation in procedure, subtle differences in hybridization, RNA handling and probe preparation, and data management. To address this concern (gene-specific reproducibility) we repeated the microarray experiments across several specimens and focused our attention on genes identified to be differentially regulated across the experiments.¹⁴ Stated differently, a single experimental comparison is not as meaningful as repeated observations across several experiments. However experiments are performed, at the single gene level of accuracy, microarray results must be confirmed using an ancillary approach to quantify amounts of RNA present, especially in the instance of uterine fibroids.

The second somewhat surprising result was that genes involved in sex steroid action were not featured as differentially expressed genes. For instance, estrogen receptor (ER) alpha, ER- β , progesterone receptor, and nuclear cofactors such as steroid receptor cofactor 1 (SRC-1) and p300/CBP were not different in fibroids compared with adjacent myometrium. We had suspected that such genes might be differentially expressed because estrogen had been shown to be a promoter of fibroid disease. This finding was supported by other investigators performing microarray experiments, and other core facilities.²³

What was featured prominently on the arrays were genes involved in formation of the extracellular matrix (ECM), the collagens, proteoglycans, and elastin that make up the connective tissue between cells. In fact, roughly 30% of underexpressed genes and 20% of overexpressed genes encoded ECM proteins or were closely involved in the synthesis or secretion of ECM, for an average of approximately 25% of the entire differentially expressed gene group. A few critical genes involved in ECM formation are shown in the Table, selected based on their known role in formation of ECM and because levels of expression have been confirmed by using RT-PCR. ECM accumulation is a feature of fibroids, and accumulation of ECM represents an imbalance between synthesis and dissolution. In particular, increases in large proteoglycans such as versican have been associated with increased transforming growth factor beta (TGF- β) signaling, a growth factor that works at the cell surface.

Disordered TGF- β signaling was suspected based on prior reports,^{7,24} but the degree to which ECM genes were dysregulated in leiomyoma was unexpected. A significant body of evidence supports a key role for TGF- β in ECM accumulation in many pathologic conditions. TGF- β is a potent promoter of connective tissue formation. TGF- β has pleiotropic effects, but has been shown to play critical roles in pathologic conditions involving fibrosis. As shown in Table, array experiments suggested that TGF- β 3 was increased 3-fold in fibroid relative to myometrium, and we confirmed this elevation using

RT-PCR. This was not unexpected, because earlier studies by Arici and Sozen,²⁵ Lee and Nowak,²⁶ and Luo et al²³ had also reported an elevation in TGF- β 3 in fibroids. TGF- β action is regulated in part by other proteins in the ECM, such as decorin, a 100-kDa small leucine-rich proteoglycan in the ECM that binds TGF- β with a KD = 10^{-8} mol/L. Decorin exists as several transcripts; however, based on the array results, levels of transcripts A, B, and C of decorin were not altered in fibroids.¹⁵

TGF- β is known to affect transcription of genes encoding collagen chains. We therefore examined expression of collagen genes. Consistent with activation of TGF- β signaling in leiomyoma cells, we noted that many genes encoding collagen chains were increased, and a few were decreased. For instance, the array suggested that collagen messenger RNAs (mRNAs) were increased in many instances, consistent with tissues undergoing repair. This finding led us to examine the ultrastructural organization of ECM in fibroids (Figure 2) and notice that collagen fibrils were randomly oriented in fibroids.²⁷ These findings are of significance because they indicate that the ECM of fibroids is not only excessive in amount, but it is abnormally formed, and the components of such critical structural elements, such as the orientation of the fibrils and their length are altered in fibroids. It is reasonable to conclude that formation of abnormal oriented fibrils may be, at least in part, a result of abnormal regulation of genes encoding the synthesis and secretion of collagen. The reason this is significant is that abnormally formed ECM may not be degraded as would a normally formed ECM.

Interestingly, an abnormality in collagen fiber architecture had been reported in the dermatopontin knockout mouse. Dermatopontin is an ECM protein known to bind TGF- β , and the collagen-binding protein, decorin. When we compared the lists of differentially expressed genes between our collaborator, and published lists using similar methodology, several genes were consistently identified,²⁸ fewer than one might expect. Transcripts encoding dermatopontin were commonly noted to be underexpressed among the lists. We confirmed the reduction in dermatopontin mRNA and protein levels.¹⁵ This, coupled with the electron microscopy finding of abnormal collagen formation, led us to focus further attention on dermatopontin. Dermatopontin is a 22-kDa extracellular protein that binds to the protein decorin with an affinity constant of 100 nmol/L. Binding of decorin to dermatopontin has been shown to alter TGF- β function. Thus, both dermatopontin and decorin may affect signaling of TGF- β . Perhaps most intriguing was the fact that dermatopontin been studied in skin and reductions in dermatopontin were associated with hypertrophic scar.²⁹ This was interesting, because keloid is a form of hypertrophic scar is increased 3-fold in black women,³⁰ similar to the increased incidence of fibroid disease.

We had begun with the prevalent view that fibroids might be linked to sex steroid action, but microarray results led us to the conclusion that fibroids possessed gene features that resembled keloids. Keloids are distinguished from hypertrophic scar, as in repair the lesion extends beyond the borders of the initial injury. Keloids-scars thus represent the far spectrum of hypertrophic scarring. We were intrigued to find that the ultrastructure of keloids resembled fibroids. Keloid formation is understood to be a disorder of wound healing. Normally, wounds go through 3 stages of repair during tissue remodeling (Figure 3): inflammation, proliferation leading to differentiation of cells to a myofibroblast phenotype.

The remodeling stage ultimately leads to the loss of the differentiated myofibroblasts by programmed cell death. During wound healing, collagens and other components of the ECM are secreted by cells in the wound. Corresponding to each stage of repair, the cells in the wound undergo changes in their state of differentiation. It is currently believed that cells in a keloid scar do not progress past the intermediate stage. On the basis of microarray results, cells in fibroids also do not appear to progress past the proliferation stage and myofibroblasts fail to undergo apoptosis (Figure 3).

Fibroids exhibit a remarkable similarity to keloids, not only in ethnic prevalence, but also in disordered appearance of ECM and dysregulation of many genes in the ECM. Given the striking similarity to keloids, our group is exploring the idea that fibroids may result, at least in part, from a similar process, perhaps in the same ethnic group because of a shared molecular predisposition. This represents a new direction in thinking about fibroids: the hypothesis that leiomyoma cells may arise from normal uterine cells that undergo altered growth and a phenotypic change (transformed into myofibroblasts) in response to disordered extracellular signals. If fibroid formation more closely resembles a disorder of healing than of oncogenesis, then based on the critical role of TGF- β and the abnormal ECM, one can envision strategies to interfere with collagen formation, perhaps with agents such as pirfenidone.

Pirfenidone has been most closely studied in animal models of pulmonary fibrosis, particularly bleomycin-induced fibrosis that is accompanied by an overexpression of the TGF- β gene in the lung, where the mechanism of action of pirfenidone appears to involve interference with TGF- β or tumor necrosis factor α (TNF- α) responsive fibrotic conditions.³¹ Pirfenidone has shown efficacy in clinical trials for a number of fibrotic diseases,³² including pulmonary fibrosis due to Hermansky-Pudlak syndrome.³³ In open-labeled studies for adenomatous polyposis-associated desmoid disease and multiple sclerosis, the 800 mg/kg dose was well tolerated in women and men for as long as 18 months.³⁴

Even more to the point, pirfenidone inhibited leiomyoma proliferation and collagen production in cultured leiomyoma cells in studies by Lee et al,³⁵ and it has previously been used in women with fibroids in a small pilot study of 8 women by Stewart et al.³⁶ In that study the dose was 400 mg/kg twice a day for 3 months. All patients completed the study with reported ultrasound-determined reductions in fibroid volume from 32% to 56%. Furthermore, the study by Davis³⁷ at NIEHS suggested that growth of fibroids beyond 5 cm was largely composed of fibrotic tissue, not cells, suggesting that interference with formation of fibrosis may be beneficial precisely for those fibroids that are of clinical relevance.

The data presented support an expanded understanding of the role of TGF- β in fibroid formation. Microarray experiments have led to the rather intriguing association between keloid and fibroid disease, and the hypothesis that abnormal fibrosis and wound repair may contribute to fibroid formation.

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Figure 1.

Cumulative incidence of hysterectomy, by race. *Y*-axis: Incidence of hysterectomy. *X*-axis: Age at time of surgery. Data are from analysis of surgical outcomes by Dr Evan Myers (modified and reprinted from with permission "Management of Uterine Fibroids," AHRQ Publication No 01-E052).

Figure 2. Electron microscopy of **A**, uterine fibroid and **B**, normal myometrium. Original magnification $\times 64,000$. Note the disordered, irregularly aligned collagen fibrils in **A**, compared with the tightly packed and closely spaced collagen fibrils in **B**, normal uterine ECM.

Figure 3.

Model of abnormal wound healing in fibroids. Tissue repair is a tightly regulated process with progressive differentiation of cells secreting the ECM, and production of an ECM that is capable of bearing stress. Collagen remodeling is a key feature of repair and TGF- β plays a critical role in production of the fibrosis associated with healing. Myofibroblasts are highly differentiated cells that undergo apoptosis at completion of the repair, but arrest in differentiation before the final stages of differentiation may result in continued secretion of collagen and excessive fibrosis.

Table

Selected genes involved in ECM formation

Gene	Microarray F/M	Multiplex RT-PCR confirmed
TGF- β 3	3.0	Yes
TGF- β 1	0.88	Yes
MMP-3	0.38	Yes*
TIMP-1	1.02	Yes [†]
Versican	5.79	Yes
COL1A1	2.61	ND
COL1A2	-1.3	Yes*
COL3A1	1.27	Yes
COL5A1	2.14	ND
COL5A2	1.51	Yes
COL6A2	-1.74	Yes
COL6A3	1.02	Yes
COL7A1	3.83	Yes

F, Fibroid; *M*, myometrium; *ND*, not done.

* Overexpression confirmed, array results discrepant.

[†] Underexpression confirmed, array results discrepant.