A high-throughput study of gene expression in preterm labor with a subtractive microarray approach

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OBJECTIVE: We propose that elucidation of the pathophysiology of preterm labor can be achieved with genome-scale analyses of differential gene expression.

STUDY DESIGN: CD-1 mice on day 14.5 of a 19- to 20-day gestation were assigned to one of 4 treatment groups modeling different clinical conditions (n = 5 per group): group A, infection with labor (intrauterine injection of 10^{10} heat-killed *Escherichia coli*, which causes delivery within an average of 20 hours); group B, infection without labor (intrauterine injection of 10^{7} heat-killed *E coli*, which leads to normal delivery at term); group C, labor without infection (ovariectomy, which causes delivery within an average of 27 hours); and group D, no infection and no labor (intrauterine injection of vehicle). Total pooled myometrial RNA was prepared 3.5 hours after surgery for groups A, B, and D and 5 hours after surgery for group C. The relative expression of 4963 genes was assayed in these pools by using DNA microarrays. Transcripts specifically involved in infection-induced labor were identified by subtracting from the list of differentially regulated genes in group A those with common expression in groups B and C.

RESULTS: In group A 68 differentially expressed transcripts (≥2-fold upregulation or downregulation) were identified. Among these are 39 characterized genes. Fourteen (45%) are involved in inflammatory responses, 7 (18%) are involved in growth-differentiation-oncogenesis, and 3 (8%) are involved in apoptosis. Subtraction identified 13 gene products most likely to be important for bacterially induced labor, as opposed to labor without infection or bacterial exposure without labor.

CONCLUSION: This study demonstrates the potential of the subtractive DNA microarray technique to identify transcripts important specifically for bacterially induced preterm labor. (Am J Obstet Gynecol 2001;185:716-24.)

Key words: Gene expression, microarray, preterm labor

Traditional research methodologies for studying preterm labor have focused on testing the functions of selected postulated critical factors. This paradigm has proven inadequate to model the complex interactions of fetal and maternal systems required for parturition. We propose that the best approach to understanding the physiology and pathophysiology of parturition is one that has the power to undertake an analysis of fetomaternal gene expression on a global scale. In this article we describe such a strategy using the technique of cDNA microarray hybridization. DNA microarray technology[1-3] was developed to allow the simultaneous comparison of RNA expression for thousands of individual genes in 2 source tissues. Microarrays have been used to monitor changes in gene expression profiles in cancer,[3, 4] development,[5] various metabolic states,[6] and growth conditions,[2, 7] as well as for other uses. We focus specifically on the problem of infection-induced preterm labor, which may account for up to 30% of cases of prematurity,[8] using a previously described and well-characterized murine model of bacterially induced preterm labor.[9-11] We test the feasibility of enriching the list of candidate genes important specifically for infection-induced preterm labor by eliminating from further analysis those genes that are related to infection or labor only. This is accomplished by using a novel subtractive strategy.

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Material and methods

Mice. All procedures involving animals were approved by the Institutional Animal Care and Use Committee of Columbia University. CD-1 mice were purchased from Charles River Laboratories (Wilmington, Mass). A previ-

ously published murine model of infection-induced preterm delivery was used.[9-11] Briefly, female mice 5 to 14 weeks of age were mated with fertile males. Copulation was verified by the presence of a vaginal plug. On the morning of day 14.5 after plugging (approximately 75% of the typical 19- to 20-day gestation), animals were randomly allocated to treatment groups designed to model 4 clinical conditions (n = 5 per group):

A. Infection and labor: Intrauterine inoculation with 1.4×10^{10} heat-killed *Escherichia coli* (prepared as previously described[11]).

B. Infection without labor: Intrauterine inoculation with 1.4×10^{7} heat-killed *E coli*.

C. Labor without infection: Bilateral ovariectomy.

D. No infection and no labor: Intrauterine injection of sterile medium.

Surgical procedures. After induction of anesthesia with 0.017 mL/g body weight of 2.5% tribromoethyl alcohol and 2.5% tert-amyl alcohol in phosphate-buffered saline, a 1.5-cm midline incision was made in the lower abdomen. The mouse uterus is a bicornuate structure joined at the cervix. Intrauterine inocula suspended in a 100-μL volume were injected into the midsection of the right uterine horn at a site between 2 adjacent fetuses. Ovariectomies were performed bilaterally through a midline abdominal incision by placing a hemostat across the vascular supply to the ovary for 1 minute, followed by excision with a sterile scalpel. Abdominal incisions were closed in 2 layers by using interrupted sutures through the peritoneum and staples at the skin. Animals recovered individually in clean cages.

Specimen collection and processing. Mice were euthanized by means of carbon dioxide inhalation 3.5 hours after surgery for groups A, B, and D (intrauterine injections) and 5 hours after surgery for group C (ovariectomy). These time points were considered comparable in proportion to the average time to delivery (20 vs 27 hours for intrauterine *E coli* administration and ovariectomy, respectively). Immediately on death, the abdomen was opened. The injected uterine horn (or the right horn for ovariectomized mice) was cut away from its mesometrium and then incised longitudinally along the antimesenteric border. The gestational sacs were shelled out, and the uterus was washed in ice-cold phosphate-buffered saline. The decidua at each implantation site was removed by sharp dissection, leaving behind the myometrium.

Total RNA was extracted from the washed myometrial specimens by using the guanidine isothiocyanate method (TRIzol Reagent, GIBCO BRL). Each RNA preparation was quantified spectrophotometrically, and quality was assessed by running 10 μg of the extract on a formaldehyde agarose gel. Twenty micrograms of total RNA were then pooled from each of the 5 animals in each treatment group and purified by passage over a silica gel membrane system (RNeasy, QIAGEN).

Probe preparation. cDNA was synthesized directly from 100 μg of pooled total RNA by using reverse transcriptase and oligo-dT primers, incorporating either Cy3 (green) or Cy5 (red) dUTP fluorescent tags directly into the probe. Unincorporated nucleotides were removed by means of centrifugation in a spin column (MicroSpin G-50, Amersham Pharmacia Biotech, Inc). For every reaction, 2 probes were hybridized simultaneously to one microarray. In each case an experimental treatment (groups A, B, or C) was compared with the control treatment (group D).

Microarray techniques. The microarray technique allows users to estimate, for each of the spotted genes, differential expression of RNA between 2 sample tissues. An overview is presented in Fig 1. Fluorescently labeled probes (see above) are hybridized to microarrays spotted with target cDNAs prepared from existing gene libraries. The different emission wavelengths of the 2 fluorophores allow intensity measurements to be determined separately for each probe. The ratio of these intensities represents the ratio of mRNA present in the source tissues.

The microarrays used in these experiments were spotted and scanned in the Department of Molecular Genetics of the Albert Einstein College of Medicine, New York. Each array is composed of 8956 sequence-verified cDNA clones (corresponding to 4963 unique mouse genes) acquired from Genome Systems. Approximately 1600 of these cDNAs represent characterized genes, 1200 are annotated expressed sequence tags (ESTs; ie, DNAs for which only partial information other than sequence is known), and the remainder are nonannotated ESTs.

The combined labeled probe pools (see above) were heated to 80°C and then hybridized to the microarray overnight at 50°C in a humidified chamber in a mixture of formamide, Denhardt's solution, sodium dodecyl sulfate, saline-sodium phosphate-EDTA buffer, and blocking reagents (poly-dA, tRNA, and mouse Cot1 DNA). Excess unbound probe was washed away at high stringency (0.2× SSC). A dual-color laser scanner was used to generate two 16-bit color TIFF images of each array (one image per wavelength). For each hybridization, one of the treatment groups was compared with the control group (ie, group A vs D, group B vs D, or group C vs D). Each of these comparisons was repeated once with reversal of fluorophore labeling between the 2 source tissues for a total of 6 hybridizations in the study. Fluorophore reversal was done to control for possible differences in labeling efficiency or fluorescence intensity of the probes.

Data analysis. By using the ScanAlyze software developed by Michael Eisen (http://rana.Stanford.edu/software/), a grid was drawn over the image of the array, and pixel intensity minus local background was measured for each spot. The data were exported to the Microsoft

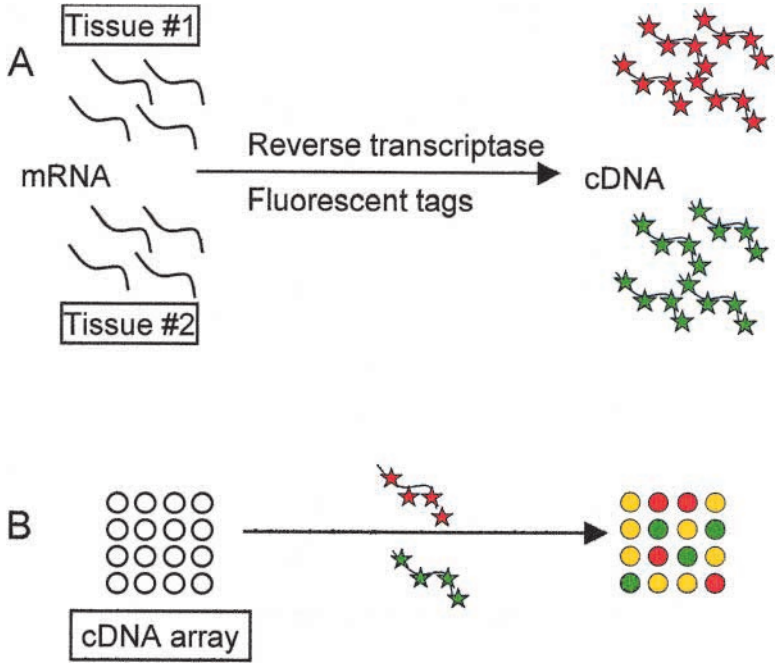

Fig 1. Overview of the microarray technique. See "Methods" section for details. **A**, Synthesis of fluorescently labeled cDNAs from pooled total RNAs extracted from each of 2 tissues. **B**, Hybridization of labeled cDNA probes to a cDNA microarray. In this depiction *red spots* represent genes whose expression is higher in tissue 1, *green spots* represent genes whose expression is higher in tissue 2, and *yellow spots* have approximately equal levels of expression in the 3 tissues.

Office suite, allowing the user to match each spot on the grid with its corresponding cDNA, clone ID number, and GenBank accession number (http://www.ncbi.nlm.nih.gov).

To correct for overall differences between the Cy3 and Cy5 channels in labeling intensity and RNA concentration unrelated to the biologic processes under consideration, the data were normalized after background subtraction. An array-specific correction factor was calculated as the average signal in the control channel divided by the average signal in the experimental channel. All ratios derived from each array were multiplied by this factor, thereby setting the mean ratio to a value of 1.0. Only spots where the signal was at least 50% above local background were considered for the initial analysis. This criterion was relaxed for the subtractive analysis (see below). Spots obscured by artifact (eg, dust or smearing) were eliminated. For each spot, the ratio of the adjusted intensity in the experimental groups (A, B, or C) to that of the control group (group D) corresponds to the differential expression (ie, fold upregulation or downregulation) in the 2 samples. Changes greater than 2-fold (ie, ratios >2 or <0.5) were considered meaningful.

The overall reproducibility of the method was good, with correlations of 0.92, 0.85, and 0.99 for the 3 pairs of replicates for genes judged to be expressed in both replicates by the criteria described above. To assist in judging the quality of each data point, we used a confidence measure described by Hughes et al,[12] which takes into account the absolute difference in intensity between the 2 channels and estimates errors in fluorescence measurement and background subtraction. For the purposes of the analysis presented in this article, a confidence measure of 1.0 or greater was considered a reliable indication of change in gene expression.

Subtraction procedure. To identify genes with expression patterns unique to bacterially induced labor (as opposed to bacterial exposure without labor or labor not associated with bacteria), we used the following subtraction procedure. Starting with the list of genes identified in the high-dose *E coli* group, genes were eliminated if they had similar expression (ie, greater than a 2-fold change in the same direction) in one of the replicates in either of the other 2 treatment groups. For the purposes of the subtraction, we did not require the signal in the latter 2 groups to be above 50% over background but only for the confidence score to be over 1.0. This was done to eliminate from future consideration as many potential false-positive results as possible. More or less stringent inclusion and exclusion criteria could be established, depending on the desired characteristics of the output.

Relative quantitative polymerase chain reaction. A relative quantitative polymerase chain reaction (PCR) assay was used to verify microarray results independently. This

Table I. Primers used for the relative quantitative PCR assay

Gene	*Forward primer*	*Reverse primer*
18S RNA	GGACCAGAGCGAAAGCATTTGCC	TCAATCTCGGGTGGCTGAACGC
Glyceraldehyde-3-phosphate dehydrogenase	GGGGCTGGCATTGCTCTCAA	CAGTGTTGGGGGCCGAGTTG
Calgranulin A	TGGAGAAGGCCTTGAGCAACCTC	GGCTGTCTTTGTGAGATGCCACACC
Granzyme F	CCCCACTCCCGCCCTTACAT	GGGTGGGGAATGGCTTTTGC
EST #7520 (GenBank accession No. AA038253)	CGGTGACCCGAAGCTGCCCCTA	CCGGTCTTGCAGCCCCTCCCTA
I-κBα	TGACGCAGACCTGCACACCCCAGCA	CAAGGTGGAGGGCTGTCCGGCCATT
Apoptosis inhibitor 1	TGGCCTGCTTTGCGTGCGATGG	ACGGGCTGCGTGTGTCTGCATGCTC
IL-1α	CCAGCCCGTGTTGCTGAAGG	TGCCAGGTGCACCCGACTTT

Table II. Treatment of mice

Group	*Labor model*	*Treatment*	*No.*	*Average time to delivery*
A	Infection and labor	IU injection of 10^{10} killed *E coli*	5	20 h[11]
B	Infection without labor	IU injection of 10^{7} killed *E coli*	5	4-5 d[11] (healthy litters at term)
C	Labor without infection	Ovariectomy	5	27 h*
D	No labor and no infection	IU injection of sterile medium	5	4-5 d[11] (healthy litters at term)

All procedures were performed in CD-1 mice on the morning of day 14.5 of pregnancy. Time to delivery of groups A, B, and D have been published previously, with groups B and D delivering healthy litters at term.
IU, Intrauterine.
*Time to delivery after ovariectomy (group C) was determined experimentally in 11 animals. The mean was 27 hours, with 10 of 11 being delivered 22 to 27 hours and the eleventh 50 hours after surgery.

published method can detect differential RNA expression in the range of 0.2- to 10-fold.[13] By using the cDNA pools generated for the microarray analysis, individual genes of interest were amplified over a range of cycles in the same tubes as 18S RNA, which was amplified for 18 cycles. The method relies on the fact that the number of cDNA copies of the gene of interest in the starting sample determines the number of cycles required for the quantity of its amplification product to equal that of the 18S RNA amplification product (the threshold value).

Primers (Table I) were selected with the program Primer3 (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi/). The known sequence of the spotted clone (or a longer sequence if available in GenBank) was used to select paired primers of 65% to 75% GC content and a melting temperature of 65°C to 75°C, generating a PCR product of 150 to 250 nucleotides in length. Optimal cycling conditions were determined empirically.

The amplification products were run through an ethidium bromide–stained agarose gel, which was then scanned with a digital camera. The band intensities for the mRNA of interest were normalized to the 18S RNA band by simple division and were plotted as a function of cycle number. An exponential regression equation was fitted to this plot and was used to calculate the relative differences in abundance of the mRNA of interest between the test samples (ie, group A, B, or C tissues) and the control group (group D). This value is approximately 2^n, where n is the difference between the test samples and the control in the number of cycles required to reach the threshold. Values greater than 1 indicate repression in the test versus the control sample (ie, a greater number of cycles required to reach the threshold); values less than 1 indicate induction.

Results

Table II summarizes the treatments and their effects on the duration of pregnancy for the 4 intervention groups. These treatments were designed to model 3 different clinical conditions: group A, preterm labor induced by intrauterine infection; group B, preterm intrauterine infection without labor; and group C, preterm labor without infection. The fourth group (group D) is a control group treated with laparotomy and intrauterine injection of sterile medium alone.

Fig 2 is an artificially colored overlay of the images obtained at the 2 probe emission wavelengths in a microarray analysis comparing group A mice (modeling infection and labor) with group D mice (no infection and no labor). The color of each spot in Fig 2 corresponds to the ratio of the intensity of the fluorescent emissions at each of the 2 wavelengths and therefore to the quantity of mRNA present in the original tissues. Comparison of Fig 2, *B* and *C*, demonstrates that reversal of labeling with the 2 fluorophores yields comparable results, with corresponding inversion of color.

The initial analysis was performed for the comparison of group A (high-dose bacteria) with group D (control). By using a cut-off point of 2-fold upregulation or

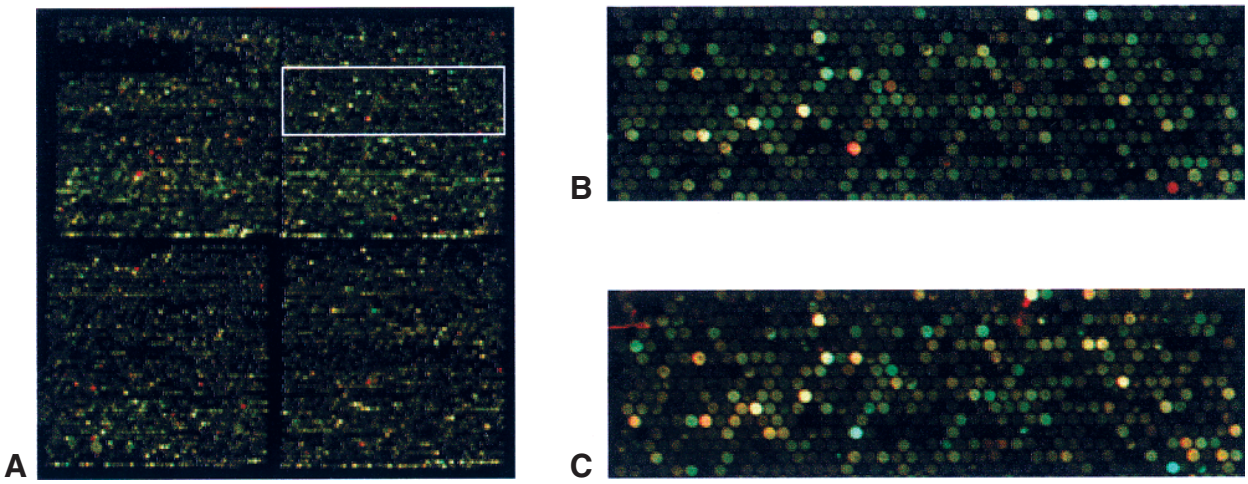

Fig 2. Sample DNA microarrays. Fluorescently labeled cDNA was synthesized from total RNA pooled from mice inoculated with 10^{10} killed *E coli* (group A) or with sterile media (group D, n = 5 per group). The cDNAs were hybridized simultaneously to an 8956-member cDNA grid and scanned at 2 wavelengths. **A**, The entire array, with cDNA from group A mice labeled with Cy5-dUTP (red) and group D mice labeled with Cy3-dUTP (green). *Red spots* represent transcripts upregulated in group D compared with those in group A. *Green spots* represent downregulated transcripts. *Yellow spots* represent transcripts with approximately equal levels of expression in the 2 source tissues. **B**, Enlargement of the boxed area indicated in **A**. **C**, Identical region as in **B** from a separate array hybridized with the same cDNAs but with fluorescent tags reversed.

downregulation (ie, an expression ratio >2 or <0.5 in both repetitions of this comparison), 68 differentially regulated genes were identified (1.3% of the 4963 unique genes in the array). Of these, 62 (91%) were upregulated, and 6 (9%) were downregulated. Forty-three percent of the differentially regulated genes were ESTs, for which no additional information is known other than sequence and, in some cases, sequence homology to other ESTs. Table III provides a complete list of the 39 characterized genes that were differentially expressed by intrauterine inoculation with high-dose killed *E coli*, along with the change in their expression levels in each of the 3 treatment groups. Among these 39 genes, 14 (45%) had functions in inflammatory processes, including signal transduction, cytokine activation, acute-phase responses, and anti-inflammatory responses. An additional 7 (18%) had functions in growth and differentiation or oncogenesis. Three (8%) are involved in apoptosis (one gene, caspase 11, has roles both in apoptosis and inflammation). Of the 68 genes identified as changed in the high-dose group, 10 were represented on the array by 2 different clones. The correlation for these pairs was greater than 0.8 for both high-dose arrays.

High-throughput methodologies typically generate large numbers of candidate genes, from which it becomes difficult to select the most promising for follow-up analysis. We wished to pare the list of 68 candidates identified by our analysis for group A to a subset most likely to be important specifically for bacterially induced labor, as opposed to bacterial exposure without labor or labor without bacterial exposure. This was done by subtracting from the list of differentially expressed genes identified for group A those similarly regulated in either group B (low-dose intrauterine bacteria) or group C (ovariectomy). Similar regulation was defined as a change of 2-fold or greater in either group B or group C in the same direction as in group A. Eleven of the 68 differentially regulated genes in group A produced signals below the confidence level for detection in the analysis for groups B and C, and thus no comment can be made about their specificity for bacterially induced labor. Among the remaining 57 transcripts, 77% were eliminated by the subtraction, leaving only 13 candidates for further analysis. Among these 13 candidates, 7 are ESTs, and the remaining 6 have been characterized to some extent. These 6 genes are listed in Table IV. Thus from the approximately 5000 genes represented in the microarray, a subset of only 13 (<0.3%) were identified as most likely to be uniquely involved in bacterially induced labor.

Relative quantitative reverse transcription PCR (RT-PCR) was performed for selected transcripts to validate the results of the microarray analysis with an independent method, comparing the pooled cDNA from each of the 3 treatment groups (groups A-C) with that of the control group (group D). The output of the RT-PCR analysis, like that of the microarray, is a number representing the fold difference in the quantity of mRNA between 2 sample pools.

Table III. Characterized genes differentially expressed in group A mice (10^{10} *E coli*)

Gene	*Genbank accession No.*	*Fold upregulation or downregulation*			*Comments*
		10^{10} E coli *IU*	*10^{7}* E coli *IU*	*Ovariectomy*	
Inflammatory response factors and signal transduction					
Small inducible cytokine A4	AA178155	↑14X	↑12X	↔	Adhesion, chemotaxis
CD14 antigen	AA396117	↑6X	↑8X	↓2X	LPS receptor
Thymidylate kinase homologue	AA172346	↑6X	↑5.5X	↔	Induced by LPS
TNF-induced protein 2(B94)	AA014136, AA537672	↑5X	↑6X	↔	Possible role in myelopoiesis; induced by LPS, IL-1, TNF
Pentaxin-related gene	W42321	↑4.5X	↔	↑*	Induced by LPS, IL-1, TNF
Secretory leukocyte protease inhibitor	AA200339	↑4X	↑3X	↔	Induced by LPS, IL-10, IL-6; inhibits prostaglandin synthase 2; anti-inflammatory
NF-κB P100 subunit (IκBα)	AA060802	↑4X	↑4.5X	↔	I-κB–like subunit cleaved to p52; induced by LPS, IL-1, TNF
Caspase 11 (ICH-3)	AA175651	↑3.5X	↔	↑2X	Required for IL-1β–converting enzyme activation; induced by LPS
Cytokine-inducible SH2-containing protein	W36940	↑3.5X	↔	↔	JAK-STAT signal transduction; ?prolactin signaling
Monocyte chemotactic protein 5 (small inducible cytokine A12)	AA172456, AA286393	↑3X	↔	↑2.5X	Chemokine; induced by LPS
Zinc finger protein 36 (tristetrapolin)	AA200282	↑3X	↔	↔	Induced by TNF-α
Lipocalin 2 (24p3)	AA087193	↑3X	↑2.5X	↔	Antimicrobial defense; expression regulated by ovarian steroids
Calgranulin A	AA230451	↑2.5X	↑3X	↑4X	Inflammatory dermatoses; Ca^{++}-binding protein
IL-1 receptor, type I	AA177717	↑2.5X	↔	↑2X	Cytokine receptor
Prostaglandin EP1 receptor	AA396341	↑*	↑*	↔	Induces smooth muscle contractions
Granzyme F	AA023418	↓3X	↓2X	↔	Serine protease in lymphoid granules
Growth-differentiation-oncogenesis					
B-cell leukemia/lymphoma 3 (Bc13)	AA266002	↑5.5X	↑6.5X	↔	Oncogene
Leukemia inhibitory factor receptor (LIFR)	AA207338	↑4.5X	↑3X	↔	Growth factor receptor
Trop 2	W64550, W34921	↑3.5X	↑2X	↔	Trophoblast antigen; tumor marker
Myeloid differentiation primary response protein (MYD116)	AA050417	↑3X	↔	↔	Differentiation
Myelocytomatosis (Myc)	AA009268	↑3X	↑2.5X	↔	Oncogene
TGIF protein	AA260654, AA038657	↑2.5X	↑3X	↔	Homeobox protein; transcriptional repressor
Delta-like 1 homologue	AA015264	↑*	↔*	↔*	Notch ligand
Apoptosis					
Apoptosis inhibitor 1	AA197349	↑4.5X	↑2.5X	↔	Induced by TNF
DAP kinase–related protein 1	W82116	↑4X	↑3.5X	↔	?Mediates TNF-induced apoptosis
Fas-binding protein (Daxx)	AA184772	↑2.5X	↔*	↔*	Binds to Fas death domain
Miscellaneous					
Interferon-induced guanylate binding protein 2 (GBP2)	AA153021	↑7X	↑2.5X	↑2.5X	GTPase
Histocompatibility 2, complement component factor B	W18121, AA209640	↑6.5X	↑11X	↔	
Myxovirus (influenza virus) resistance 2	AA145795	↑6.5X	↑2.5X	↔	Interferon-responsive element
Pyruvate dehydrogenase E1α subunit	AA423301	↑4X	↑2.5X	↔	Metabolism
3' repair exonuclease (TREX1)	AA197643	↑4X	↔	↑*	DNA exonuclease
Guanylate binding protein 3 (GBP3)	AA240404	↑4X	↑2.5X	↔	
Protein tyrosine phosphatase, nonreceptor type 12	AA175740	↑3X	↔	↔	?Differentiation
Guanine nucleotide dissociation stimulator RALGDSA	W48230	↑3X	↑3X	↔	GTPase
Carbon catabolite repression 4 homologue (Ccr4)	AA051133	↑3X	↔	↔	Repressor of transcription
Serine/threonine kinase 2	AA268478	↑3X	↑3.5X	↔	?Signal transduction
CGMP-inhibited 3'-5' cyclic phosphodiesterase	AA144377	↑3X	↑4.5X	↓*	
Intestinal trefoil factor 3	AA273366	↑2.5X	↑2X	↔	Intestinal mucin protein; ?immune modulator
Interferon-inducible gene 203	AA174447	↑2.5X	↑3X	↑2X	Nuclear protein

A complete list of the characterized genes differentially expressed (>2-fold upregulation or downregulation in duplicate assays) in myometrium 3.5 hours after intrauterine injection of 10^{10} *E coli* (group A). The expression of the same genes in groups B and C (10^{7} bacteria and ovariectomy) is also provided for comparison. Values are rounded to the nearest 0.5 and are averages of 2 repeat assays, where available. In cases in which confidence scores were less than 1 in one of the repeat assays, only the other ratio was used. In cases in which neither of the 2 repeats had confidence scores above 1, the best estimate was used.
IU, Intrauterine; ↔, less than 2-fold change; *LPS*, lipopolysaccharide.
*Fold induction or repression was indeterminable because of overall low signal intensities.

We selected 7 transcripts for comparison of the microarray and RT-PCR methods of determining differential expression (Table V). There is nearly complete corroboration of microarray results by using the RT-PCR method. Only one minor discrepancy (near the 2-fold cut-off point) occurred for granzyme F. The magnitude of upregulation or downregulation, as calculated by using the 2 methods, was similar, with the RT-PCR method tending to yield higher values (consistent with the narrower dynamic range of fluorescence compared with PCR).

Comment

The goal of this study was to demonstrate the proof of the principle that the cDNA microarray technique can be used in a murine model with intact tissues and that the novel subtractive strategy we propose is a useful method of enriching the yield of candidate genes. We believe that this approach holds enormous potential for overcoming some of the limitations of single-factor analysis to study complex processes, such as parturition. The present studies were performed by using microarrays with approximately 5000 spotted genes. In the near future, human and mouse arrays will be available, representing practically all of the estimated 30,000 mammalian genes. This wealth of data has the potential to allow the construction of comprehensive pathways of the molecular and biochemical alterations leading to preterm labor of varying causes. Similar pathways for other processes have already been developed in lower organisms and in cell cultures, yielding novel insights into the response of fibroblasts to serum.[2] We contend that understanding the complex physiology and pathophysiology of parturition, with its interplay of maternal and fetal signals on many levels, requires the accumulation of such data.

Several genes of interest were not represented in the array available to us. These include inflammatory cytokines (interleukin [IL] 1, IL-6, and tumor necrosis factor [TNF]), prostaglandin synthase, oxytocin receptor, and many others. However, one of the strengths of the microarray technique is that the large data set, when correlated with known pathways, allows extrapolation to functionally related genes, even if they are not physically represented in the array. For example, the association of IL-1 and TNF with bacterially induced labor (already amply demonstrated in both the murine model[9, 11] and in human beings[14-16] and properly thought of as positive controls for this analysis) could have been inferred by the upregulation of related transcripts that were spotted on the array. These include TNF-induced protein 2 (upregulated 5-fold), the NF-κB P100 subunit (induced by inflammatory cytokines and involved in their signal transduction and upregulated 4-fold), and cytokine-inducible SH2-containing protein (involved in signal transduction and upregulated 3.5-fold).

In addition to the positive controls referred to above, our data also contain many examples of correctly classified negative controls. These include genes not normally expressed in gestational tissues (eg, neural-specific factors) and genes not expected to be differentially regulated by labor or infection (eg, glyceraldehyde 3-phosphate dehydrogenase).

Table IV. Characterized genes remaining after the subtractive analysis

Inflammatory response factors and signal transduction
Caspase 11 (ICH-3)
Cytokine-inducible SH2-containing protein
Zinc finger protein 36
Growth-differentiation-oncogenesis
Myeloid differentiation primary response protein (MYD116)
Apoptosis
None
Miscellaneous
Protein tyrosine phosphatase, nonreceptor type 12
Carbon catabolite repression 4 homologue

Characterized genes from group A (high-dose bacterial inoculation) persisting after the subtraction of similarly regulated genes in groups B (low-dose bacterial inoculation) and C (ovariectomy). Compare with Table III. In addition to the genes listed above, 7 ESTs persisted after the subtraction.

The subtractive strategy enriched the specificity for transcripts important for bacterially induced labor by nearly 80%, as opposed to labor or bacterial exposure alone. Ultimately, 13 of the nearly 5000 screened genes were identified as having the highest likelihood of involvement in this process. Other genes emerged as important for labor in general, whether hormonally or bacterially induced (eg, calgranulin A, a calcium-binding protein).

Our analysis yielded a greater number of transcripts related to inflammation than to myometrial contractility. This is to be expected from the harvest time (3.5-5 hours after surgery), which is early enough for the development of an acute response to a bolus of bacterial organisms but probably too early for the expression of the full complement of labor-related transcripts. This pilot study was not designed to provide a comprehensive list of the important genes in infection-induced labor. Such an undertaking would require expansion of the current numbers of spotted clones, inclusion of multiple time points and tissue layers, verification in human specimens, and several years of work by the community of researchers interested in parturition.

At least 2 other gene-expression studies in pregnancy have been published. The first examined rat myometrium from day 12 of pregnancy to the first postpartum day by using the technique of differential display.[17] Over 500 differentially regulated transcripts were identified, of which 179 were characterized (157 upregulated and 22 downregulated). The second study used a cDNA array of 588 known genes to compare myometrial gene expression among 3 laboring and 3 nonlaboring women delivered at term by means of cesarean section.[18] Twelve upregulated

Table V. Expression analysis of selected myometrial genes comparing the microarray and RT-PCR techniques

	Microarray			*RT-PCR*		
Gene	*10^{10} E coli IU*	*10^{7} E coli IU*	*Ovariectomy*	*10^{10} E coli IU*	*10^{7} E coli IU*	*Ovariectomy*
GAPDH	↔	↔	↔	↔	↔	↔
Calgranulin A	↑2.5X	↑3X	↑4X	↑4X	↑4X	↑6X
Granzyme F	↓3X	↔	↔	↓4X	↔	↑2.5X
EST #7520 (GenBank accession No. AA038253)	↓3X	↔	↓2.5X	↓8X	↔	↓4.5X
IκBα	↑4X	↑4.5X	↔	↑3X	↑2.5X	↔
Apoptosis inhibitor 1	↑4.5X	↑2.5X	↔	↑9X	↑4X	↔
IL-1α*	—	—	—	↑6.5X	↑5.5X	↔

Values represent fold upregulation or downregulation compared with control for each treatment group rounded to the nearest 0.5. Differences of 2-fold or less are marked as "no change" (↔). Each value represents the average of measurements in 2 independent assays with confidence scores of greater than 1 when available. Otherwise, best measurements were used.
*IL-1α (not spotted in the microarray but known to be upregulated after bacterial inoculation by prior Northern blot and ELISA data) is included here as an independent positive control for the RT-PCR method.
IU, Intrauterine; *GAPDH,* glyceraldehyde 3-phosphate dehydrogenase.

and 9 downregulated genes were identified in laboring myometrium. Comparison between these 2 studies and our own is difficult for 2 reasons. First, only a small number of the differentially expressed genes reported in the former articles were spotted on our array. Second, our project focused on early gene expression in a preterm infection model, rather than on term labor without infection or on changes occurring over long periods of gestation. However, the availability of all 3 data sets and others yet to be published will contribute to our ability ultimately to decipher the complex process of parturition.

We chose to use mice rather than human subjects in these studies because of the impracticability of controlling for all the relevant variables in human tissues (eg, gestational age, infection status, and stage of labor). Also, our laboratory has extensive experience with murine labor models,[9-11, 19] in which a large amount of descriptive data has been accumulated that can be compared with the microarray set. These studies show that mice and human subjects share similarities in the biochemistry of infection-induced labor. We have demonstrated cytokine profiles in our mouse models mimicking those described in human subjects.[9-11] We also now know that in the mouse, inflammation-induced labor (unlike spontaneous labor) is not dependent on progesterone withdrawal (manuscript in preparation). We believe that studies such as ours in mice will generate many specific testable hypotheses for human labor.

There was excellent correlation between 2 independent methods of classifying genes into expression categories, namely microarray analysis and relative quantitative RT-PCR. The subtractive strategy we describe should help focus necessary follow-up studies on factors with the highest likelihood of importance among the hundreds or thousands of genes flagged by means of high-throughput methods.

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