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DNA microarray analysis of neonatal mouse lung connects regulation of KDR with dexamethasone-induced inhibition of alveolar formation

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Clerch, Linda Biadasz, Alex S. Baras, Gloria DeCarlo Massaro, Eric P. Hoffman, and Donald Massaro. DNA microarray analysis of neonatal mouse lung connects regulation of KDR with dexamethasone-induced inhibition of alveolar formation. *Am J Physiol Lung Cell Mol Physiol* 286: L411–L419, 2004. First published November 7, 2003; 10.1152/ajplung.00306.2003.—Treatment of newborn mice with dexamethasone (Dex) inhibits the subdivision of lung saccules to form alveoli; treatment with all-*trans* retinoic acid (RA) prevents this inhibition of septation. To better understand the early molecular signals responsible for the effects of Dex and RA, Affymetrix gene profiling was done on RNA isolated from 4-day-old mice after treatment with *1*) diluent, *2*) RA (1 mg/kg), *3*) Dex (0.7 μg/pup), or *4*) RA + Dex. Each sample was assayed in duplicate on U74Av2 GeneChips. Data were analyzed with Affymetrix suite 5.0, corrected for saturation, and evaluated with GeneSpring 5.1 software. Stringent filtering of data by the global error model and condition-to-condition comparisons was used to identify 46 genes demonstrating significantly different expression between the lungs of Dex- and RA + Dex-treated mice. A query of the gene ontology database revealed that the major biological processes affected by treatment with Dex and RA were cell growth/maintenance and cellular communication. On the basis of microarray data analysis, we hypothesize that Dex-induced inhibition of septation is associated with a block in angiogenesis due to downregulation of the kinase domain receptor (KDR), also known as VEGF receptor-2 and fetal liver kinase, and that the downregulation of KDR is prevented by treatment with RA.

lung development; corticosteroid; retinoic acid; angiogenesis; kinase domain receptor

IN HUMANS, THE DEVELOPMENT of a mature lung structure for efficient gas exchange occurs late in gestation and continues during the early childhood years (19, 41, 42). Premature infants, in particular those who acquire bronchopulmonary dysplasia (BPD), have impaired lung development (16). The overall aim of this study was to use a newborn animal model to understand factors that regulate the formation of normal, impaired, and restored lung structure to gain insight into potential therapeutic intervention because the prevalence of BPD continues to rise and is a major cause of newborn lung disease (40).

In rats and mice, pulmonary alveoli are formed after birth partly by subdivision of saccules present at birth in a process termed septation (9, 10). Therefore, the newborn of these species are useful in the study of factors regulating alveolarization. In rats, corticosteroid treatment impairs lung septation that occurs in the early postnatal period (6, 25). Moreover, there appears to be a critical period for septation to occur, and, once impaired by treatment with dexamethasone (Dex) during the postnatal period, septation does not occur after treatment is stopped (tested up to age 95 days in the rat) (21, 25, 36). However, treatment with all-*trans* retinoic acid (RA) prevents and reverses the effect of Dex on lung structure in both rats and mice (23, 27, 28). The present study was exploratory, with the goal of understanding the molecular basis for Dex-induced inhibition of the formation of alveoli and the ability of RA to prevent the inhibition of septation. High-density Affymetrix oligonucleotide arrays were used to identify genes in lung that are early responders to treatment with Dex and RA. Microarray data were generated to profile gene expression in four groups of 4-day-old mice: diluent-treated control, RA-treated, Dex-treated, and RA plus Dex-treated. All primary array data are posted on the web (http://microarray.cnmcresearch.org; project name: Murine Lung Septation; investigator: Dr. Linda Clerch). In addition, the data are contained in an Oracle-based public expression profiling resource that includes a novel time series query analysis tool (SGQT) (11) and in the National Center for Biotechnology Information's (NCBI) Gene Expression Omnibus (GEO) database (record GDS250). In this paper we describe unbiased analytic methods that identified genes whose expression was altered in a statistically significant manner by Dex and RA. Data analysis resulted in a hypothesis regarding a signaling pathway that may regulate postnatal lung development, that is, control of angiogenesis by the kinase domain receptor (KDR), also known as vascular endothelial growth factor receptor-2 (VEGFR-2) and fetal liver kinase (Flk-1).

EXPERIMENTAL PROCEDURES

Animals. All animal procedures adhered to the National Research Council's Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of Georgetown University. Pregnant C57BL/6J mice were purchased from Jackson Laboratory and housed in the Georgetown University animal care facility. Mice were maintained on a 12:12 h-light-dark cycle and allowed ad libitum access to water and food (Ralston Purina laboratory chow 5001). After mice were born, the litters were maintained at six pups per litter, and the day of birth was designated as postnatal *day 1*. For the morphometric study, on *day 3*, mice were injected intraperitoneally with diluent (cottonseed oil) or with RA in diluent, 1.0 μg/kg (Sigma, St. Louis, MO). On *day 4*, half of the diluent-treated

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mice and half of the RA-treated mice were treated subcutaneously with Dex, 0.5 μg/pup or 1.0 μg/pup. Thus there were four treatment groups: diluent, RA, Dex, and RA + Dex. Daily treatments were given up to and including age 14 days, and the mice were killed the next day (age 15 days). Some animals that had been given diluent or Dex from *days 4* to *14* were killed on *day 37*. We thank Matthew Hind (King's College London, UK) for his advice on the effective dosage of Dex and RA in mice. For the gene profiling study, mice were injected intraperitoneally with diluent for RA (cottonseed oil) or RA (1 mg/kg) on postnatal *days 3* and *4*. On postnatal *day 4*, the mice were concomitantly injected subcutaneously with diluent for Dex (0.075 NaCl) or Dex (0.7 μg/pup) and killed 6 h after the treatment. All mice were killed by cutting the abdominal aorta while they were anesthetized with xylazine (~10 mg/kg) and ketamine (~75 mg/kg).

Morphometry. After the mice were anesthetized, cold 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, was infused into the trachea at a transpulmonary pressure of 20 cmH_2O; the trachea was then ligated, the lungs were removed from the thorax, and fixation was continued for 2 h at 0–4°C. Lung volume was measured by volume displacement (38). The lungs were cut into ~1-cm^3 blocks and selected for study by a systematic sampling technique (13). Tissue blocks were washed in cacodylate buffer, followed by postfixation for 1 h in cold 2% osmium tetroxide in 0.1 M sodium cacodylate buffer, dehydrated, and embedded in epoxy resin (26). Sections of lung were cut at ~ 0.8-μm thickness and examined under a light microscope. A multipurpose test system (29) was used to determine mean chord length (L_m), surface area (S_a), and the surface-to-volume ratio (S/V).

Affymetrix GeneChip hybridizations. Lungs were removed and immediately frozen and stored in liquid nitrogen. RNA was isolated with TRIzol reagent (Invitrogen) and RNeasy columns (Qiagen). We prepared individual cDNAs using the SuperScript Choice System (Invitrogen) and subsequently used them to generate biotin-labeled cRNA by in vitro transcription with the BioArray labeling kit (Enzo). Biotin-labeled cRNA was hybridized on duplicate Affymetrix MG-U74Av2 GeneChips, which were subsequently processed on an Affymetrix Fluidics Station 400. Fluorescent images were measured with a Hewlett Packard G2500A Gene Array Scanner. Data were analyzed with Affymetrix suite 5.0, corrected for saturation, and evaluated with GeneSpring 5.0 software. The primary gene expression data can be found on the web at the NCBI's GEO database (record GDS250) and at http://microarray.cnmcresearch.org.

Western analysis. Lung tissue from individual mice was homogenized at 4°C in lysis buffer containing 50 mM Tris (pH 7.4), 4 mM KCl, 1% Nonidet P-40 (vol/vol), 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 0.2 U/ml aprotinin, 1 μg/ml pepstatin, 1 mM Na_3VO_4, and 1 mM NaF. The homogenates were centrifuged at 10,000 *g* at 4°C for 1 h. The protein concentration was measured spectrophotometrically with Coomassie Plus Protein Assay Reagent (Pierce) with bovine serum albumin as a standard. Lung protein extracts (15 μg) were separated by 7% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose Hybond ECL membranes (Amersham). After incubation overnight at 4°C with 5% nonfat milk in Tris-buffered saline-Tween [TBS-T; 0.1% Tween 20, 20 mM Tris (pH 7.6), and 137 mM NaCl], the membranes were incubated with polyclonal rabbit anti-KDR (Upstate Biotechnology) at a dilution of 1:1,000 in TBS-T containing 0.5% nonfat milk. This antibody detects the M_r ~230-kDa protein that was visualized using goat anti-rabbit secondary antibody (Bio-Rad) at a dilution of 1:5,000 in TBS-T containing 0.5% nonfat milk for 1 h at

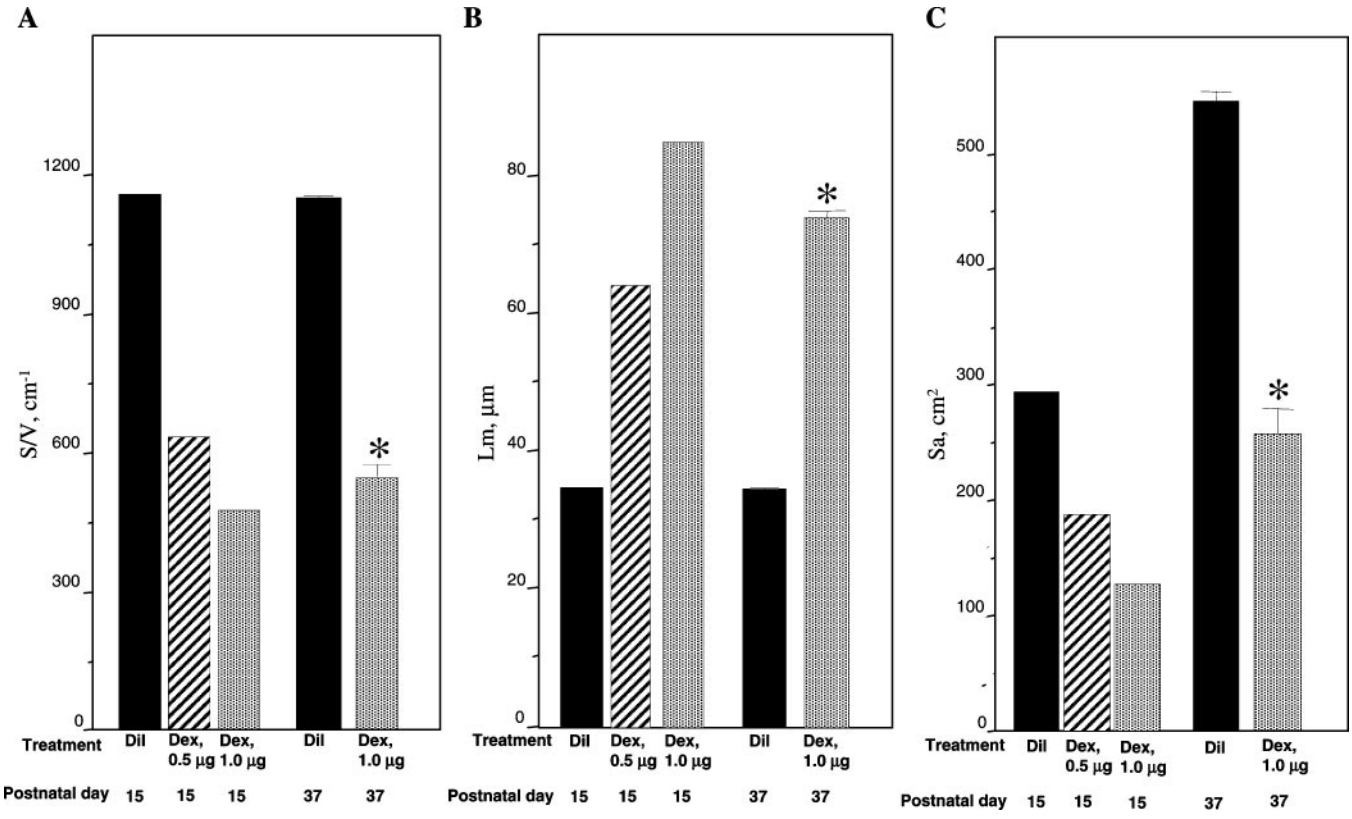

Fig. 1. Morphometry data. *A*: surface-to-volume ratio (S/V); *B*: mean chord length of gas-exchange air spaces (L_m); *C*: surface area (S_a). The data at *day 15* represent of the average of 2 trials in which mice were injected with oil or retinoic acid (RA, 1.0 μg/kg) daily from *days 3* to *14* and with saline [diluent (Dil)] or dexamethasone (Dex, 0.5 or 1.0 μg/pup) daily from *day 4* to *14*; mice were killed on *day 15*. The data at *day 37* are given as means ± SE where $n = 3$ for each group. Mice were injected with Dil or Dex (1.0 μg/pup) daily from *day 4* to *14* and killed on *day 37*. *$P < 0.001$ compared with *day 37* Dil.

room temperature followed by detection with an enhanced chemiluminescence kit (Amersham). The KDR-specific protein bands were quantified by laser densitometry (Molecular Dynamics) with Image Quant Software; data were expressed as relative densitometry units.

Statistical analysis. Analysis of between-group measurements was made by using an unpaired two-way Student's *t*-test. A value of $P < 0.05$ was considered significant.

RESULTS

Dex inhibits septation in mice. Before proceeding with gene profiling studies in the mouse, we confirmed that Dex blocked septation in C57BL/6J neonatal mice. In prior studies in the rat, we used serial sectioning to distinguish between alveoli and alveolar ducts and showed Dex treatment impaired septation (26–28). In this paper, we measured L_m, S_a, and S/V, because, although these measures do not distinguish between alveoli and alveolar ducts, they were sufficiently sensitive to achieve our goal of demonstrating that Dex also impairs alveolus formation in mice. That it did so is clear from the lower S_a in lungs of Dex-treated mice in the absence of an effect of Dex on lung volume. Figure 1 shows that, at postnatal *day 15*, L_m was higher and S_a and S/V were lower in Dex-treated mice than in diluent-treated mice in a dose-response manner. The effects of Dex were not reversed after Dex treatment was stopped from *days 15* to *37*.

Expression profiling data analysis. The experimental design for the gene array study is shown in Fig. 2. Lungs were harvested 6 h after treatment on postnatal *day 4* because the goal was to look for early changes in gene expression that might be responsible for the effect of Dex and RA plus Dex. To minimize interanimal variability, lungs of each animal were divided in half, and a mixture of right and left lungs was pooled to generate total RNA for duplicate GeneChips in each group. This strategy was used to control for both tissue and experimental variability including variables in RNA isolation and processing. Data were analyzed and visualized with GeneSpring 5.1 software. The global error model of GeneSpring was used to address the variability of a gene within a replicate pair compared with all other genes interrogated by the MG-U74Av2 Affymetrix GeneChip (7, 32, 37) and to correct for the problem that lower-range values from GeneChip measurements are currently less reliable than higher-range values of expression. Expression profiles were normalized to the control (diluent) condition, and statistical significance was determined by condition-to-condition comparisons requiring a significance level of 90% or greater. All calculations were based on the log of the normalized values to ensure that downregulated and upregulated patterns of expression were given equal weight, that is, log(2/1) is equal to −log(1/2).

Figure 3 shows the filtering protocol used in this study. Data analysis revealed significant variation in 499 genes between diluent and Dex treatments and in 394 genes between the Dex and combination RA-Dex treatments ($P < 0.1$). By calculating the intersection of these two gene lists, we found that 85 genes had a significant difference with 99% confidence [1.0 − (0.1·0.1)]. The 85 genes present in the intersection of these two lists of genes were screened further based on the specific pattern of expression exhibited. Of the 85 genes, 74 were upregulated by Dex, and, of these, the expression of 35 was lowered by combination RA-Dex treatment. Of the 85 genes at the intersection of significant genes, 11 were downregulated by

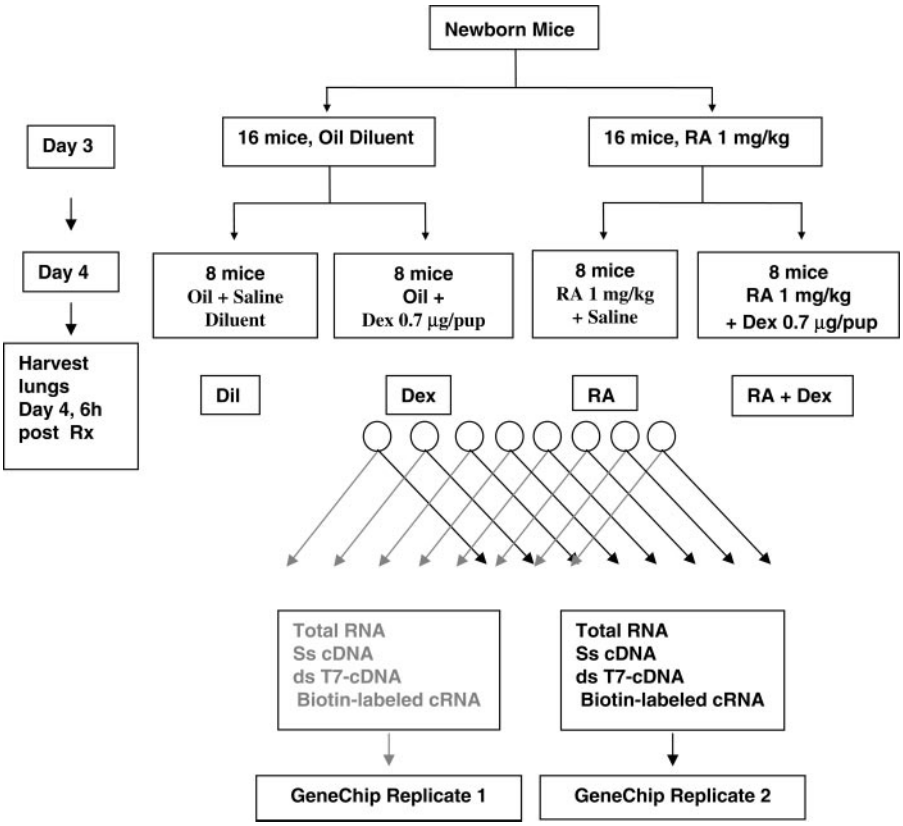

Fig. 2. Experimental design for gene profiling. Rx, treatment; Ss, single strand; ds, double strand.

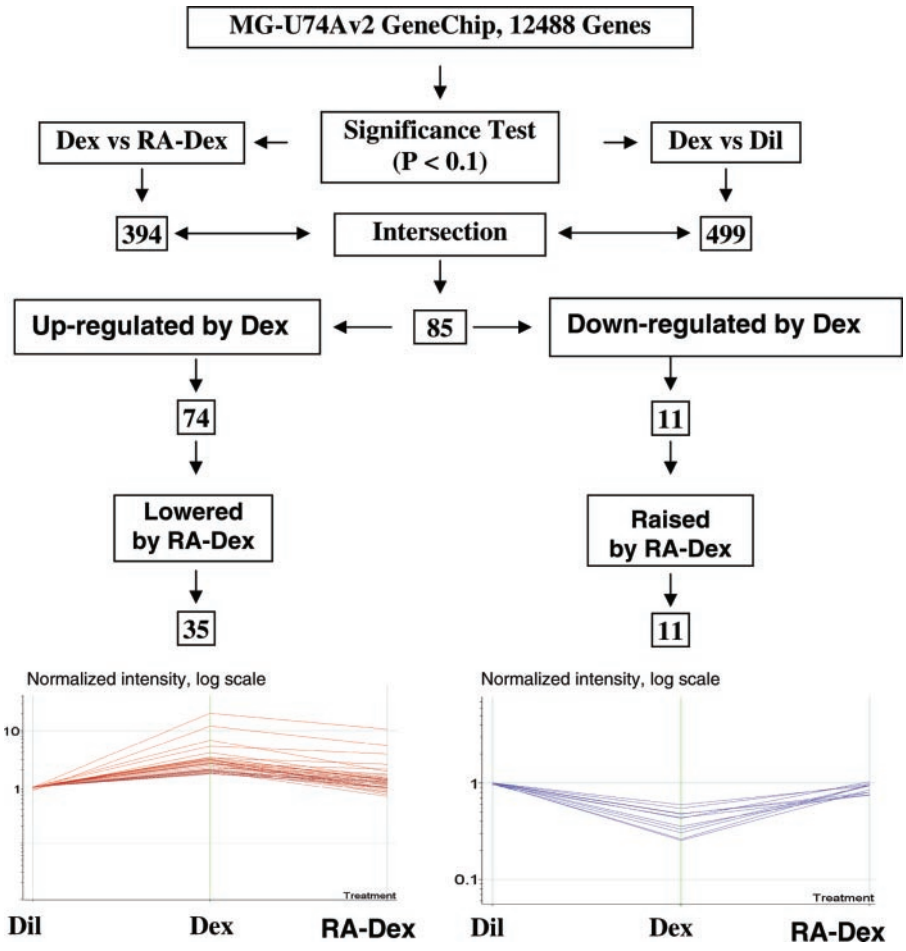

Fig. 3. Microarray data analysis: filtering to identify significantly altered gene expression. Gene expression was analyzed with the global error model and a series of condition-to-condition comparisons requiring a significance level of 90% or greater (alpha set at 0.10). Expression profiles were normalized to the control (Dil) condition. The data were analyzed for significant variation between Dex and Dil (499 genes) and between Dex and RA-Dex (394 genes). The intersection of these 2 lists of genes (85 genes) was filtered to select genes whose expression was upregulated (74 genes) or downregulated (11 genes) by Dex and further filtered to select genes whose alteration by Dex was abrogated by combination treatment with RA + Dex (46 genes). Panels at *bottom*: expression pattern of the 35 genes upregulated by Dex (red) and the 11 genes downregulated by Dex (blue).

Dex, and the expression of all 11 was raised by RA-Dex treatment. Thus the expression of 46 (35 + 11) genes was significantly different among control, Dex, and RA-Dex samples. These genes are the likely candidates to be involved in Dex-induced inhibition of septation that is prevented by treatment with RA. Figure 4 shows a hierarchical dendrogram representing the expression profiles of the 46 candidate genes. Although we did not filter using the RA-only condition, we show this parameter in Fig. 4 because knowledge of gene expression under RA treatment alone provides additional information on which to formulate hypotheses and prioritize genes for further study. Put differently, statistically significant data were identified by condition-to-condition comparisons of diluent, Dex, and RA-Dex, because our main objective was to identify genes involved in Dex-induced inhibition of septation by taking advantage of our biological knowledge that RA prevents this effect. In this way, the effect of Dex on septation might be detached from the general effect of Dex on overall gene expression. However, prior studies also show RA alone induces alveolar formation (28); therefore, in mice treated with RA alone, genes whose expression either did not change or changed in an opposite manner to Dex will be of special interest.

Expression data analysis: clustering by k-means. The expression profiles of the 46 genes that passed the highest selection criteria were clustered into eight groups by the standard k-means algorithm provided in GeneSpring (Fig. 5). In addition to Dex and RA-Dex, the RA-alone expression data were included in this clustering. The list of genes in each set and their probe set number, accession number, description, and gene ontology are given in supplementary material. Although the decision to cluster for eight k-means was arrived at empirically, the eight sets revealed distinct patterns of expression. The genes in *sets 1* and *2* meet the criteria that their expression was downregulated by Dex, rectified by RA-Dex, and was either not different (*set 1*) or slightly elevated (*set 2*) by RA alone, as such these genes would be of special interest. The genes in *sets 1* and *2* are likely to be positive regulators of septation whose expression was diminished by Dex in parallel with Dex-induced inhibition of septation. Conversely, genes upregulated by Dex (*sets 3–8*) are likely to be negative regulators of septation. The genes whose expression was elevated by Dex segregate into six patterns. *Sets 5* and *7* represent genes that were either decreased (*set 5*) or remained close to control (*set 7*) during treatment with RA alone and whose expression, although somewhat lowered by treatment with RA plus Dex compared with Dex, still remained above control values. *Sets 6* and *8* represent genes that remained elevated in lungs of mice treated with RA alone. *Sets 3* and *4* represent genes that were slightly decreased (*set 3*) or slightly elevated (*set 4*) by RA alone, as such these genes would be of special interest.

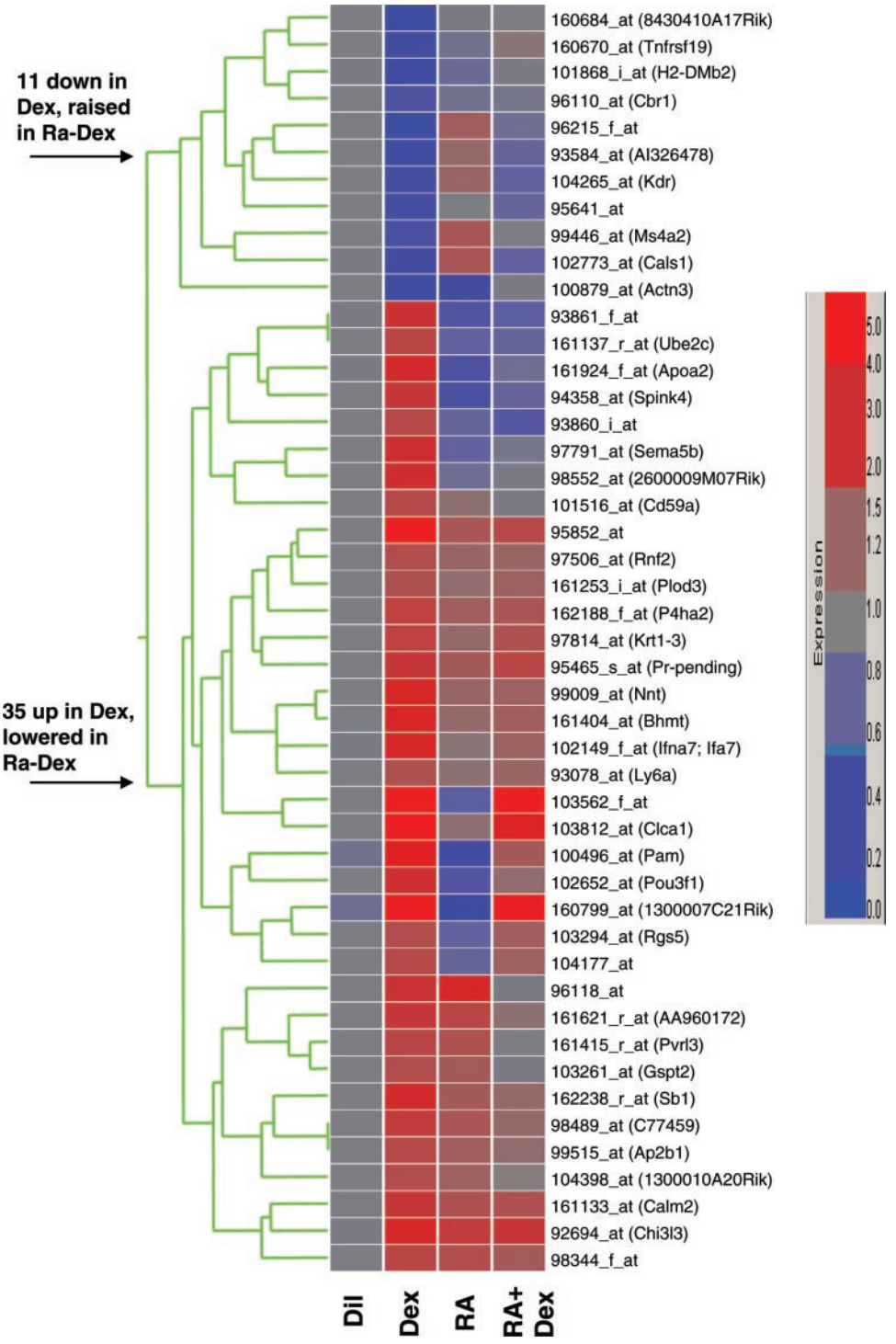

Fig. 4. Hierarchical dendrogram representing the expression profiles of significantly altered genes. Hierarchical clusters were obtained after filtering as described in Fig. 3. The final 46 genes were clustered by GeneSpring into 2 specific branches on the dendrogram representing genes up- or down-regulated by Dex. The Affymetrix probe set number and gene name is given on the right of the dendrogram. The color bar shows the gene expression level (red, high; blue, low).

Expression data analysis: gene ontology. To assess the biological process or function of the 46 genes that showed a significant pattern of expression, we queried the gene ontology database created by the Gene Ontology (GO) Consortium (2) and provided on the analysis site of Affymetrix NetAffX (22). The results of this analysis are shown in Fig. 6 and Table 1. Of the 35 genes upregulated by Dex and lowered by treatment with RA, 19 have annotation in GO biological processes. On the first branch of GO, 13 of the 19 genes are involved in cell growth and/or maintenance, five have a role in cellular communication, and two are annotated in developmental processes. Of the 11 genes down-regulated by Dex and raised by treatment with RA, seven are annotated in GO biological processes. On the first branch of GO, six of the seven are involved in cell growth and/or maintenance and four are involved in cellular communication. For the annotated genes, the GO biological process, accession number, name, and fold change in Dex and RA-Dex compared with diluent are provided in Table 1. Of note, several genes are found in both cell growth/mainte-

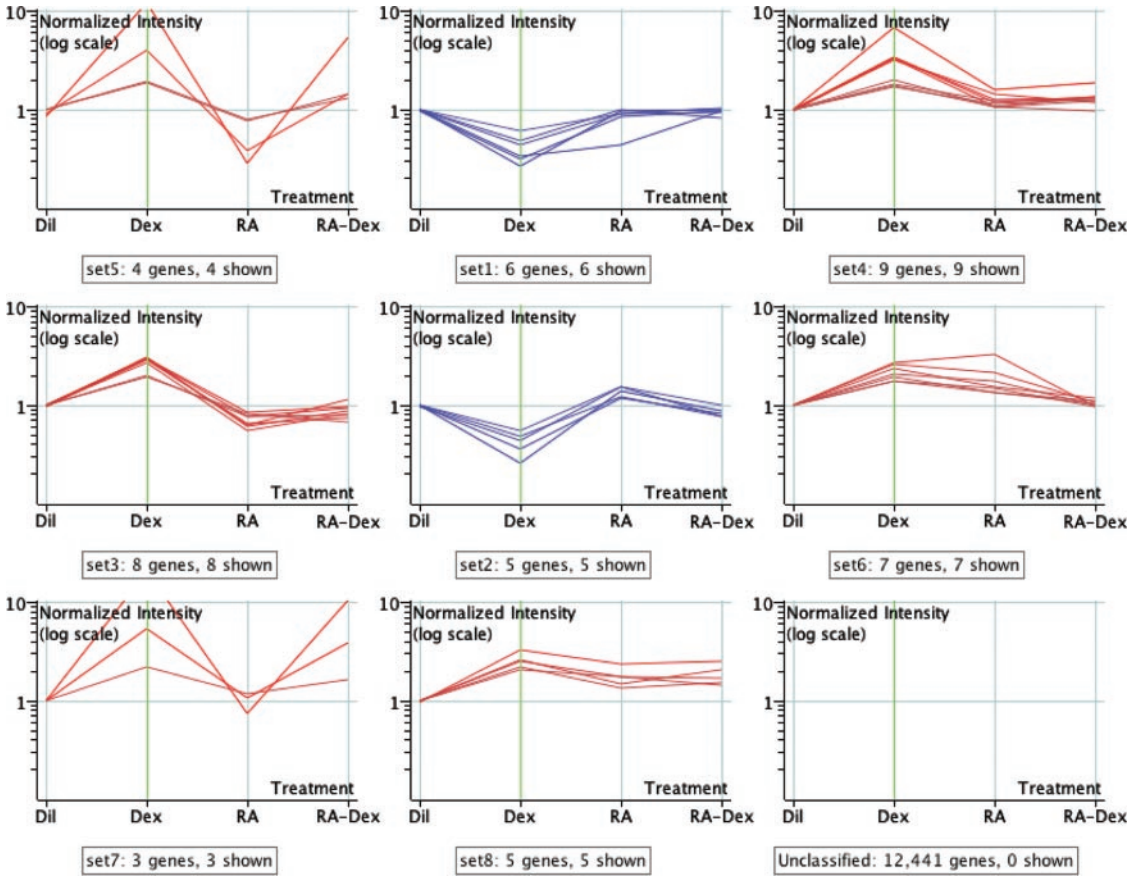

Fig. 5. k-Means clustering. k-Means analysis of the final 46 filtered genes into 8 classes is represented. The expression profile for each gene is colored by its value in the Dex condition with red indicating upregulation and blue indicating downregulation compared with control. The details of each cluster are given in supplementary material.

nance and cell communication as the genes involved in these GO processes are expected to interact in vivo.

Flk-1/KDR expression. From the analysis of the array data, KDR expression became of special interest. KDR is present in k-means cluster *set 2* (Fig. 5, supplementary material); its RNA expression was decreased 2.1-fold by Dex compared with control, slightly elevated (1.2-fold) by RA, and decreased 1.3-fold in lung treated with RA plus Dex. To test whether the array data were manifest in protein expression, we measured KDR protein concentration by Western analysis. The protein concentration in lungs from Dex-treated mice was decreased 1.8-fold compared with control ($P < 0.03$, $n = 3$ in each group). The RNA data obtained by microarray and the protein data obtained by Western analysis are remarkably concordant (Fig. 7).

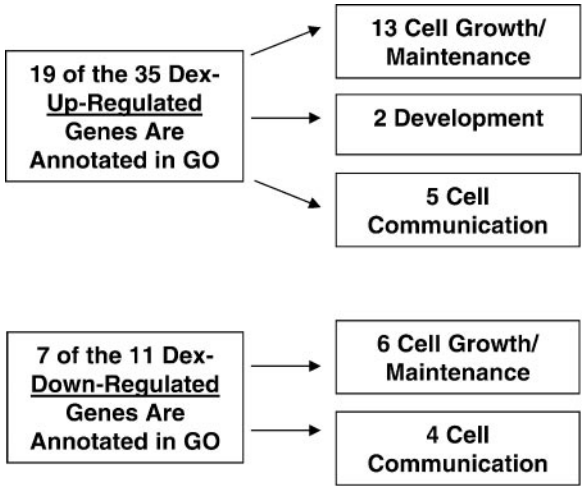

Fig. 6. Gene ontology (GO) biological processes. A diagram of the first GO branch as determined by NetAffX (21) is shown.

DISCUSSION

The inhibitory effect of Dex on alveolar formation and the regenerative effect of RA in rats and mice have been known for some time (6, 23, 26–28, 36). However, the mechanism(s) responsible for the effects of these agents is unclear. Gene profiling was a logical approach to begin a search for pathways involved in these processes. We chose to study expression in newborn mice because more known genes are present on mouse than on rat GeneChips. Our data indicate that, like the rat, postnatal mouse alveolar septation was inhibited by Dex and the inhibition of septation did not resolve after treatment with Dex was stopped (Fig. 1). These findings, together with those of Maden and Hind (23), who showed RA regenerated the gas-exchange surface in adult mouse lung in which alveologenesis had been blocked by treatment with Dex, support the use of the mouse model to study mechanisms of alveolar formation. Although taking place at different times of devel-

Table 1. *GO biological processes of genes altered by Dex and rectified by RA*

Accession No.	Gene Name	Fold Change Dex vs. Dil	Fold Change RA-Dex vs. Dil
GO: cell communication			
M13710	Ifa7, interferon-α, gene 7	3.3	1.3
AV122577	Calm2, calmodulin 2	2.6	1.7
AV318045	Pvrl3, poliovirus receptor-related 3	2.1	−1.0
U67188	Rgs5, regulator of G protein signaling 5	1.8	1.4
X04653	Ly6a, lymphocyte antigen 6	1.7	1.2
X90807	H2-DMb2, histocompatibility 2, class II, locus Mb2	−3.2	1.0
V00821	immunoglobulin μfragment	−2.8	−1.2
X70842	Kdr, kinase insert domain protein receptor	−2.1	−1.3
M62541	Ms4a, membrane-spanning 4-domains, subfamily A	−1.8	−1.0
GO: cell growth/maintenance			
AF047838	Clca1, chloride channel calcium-activated 1	5.3	3.8
U79523	Pam, peptidylglycine α-amidating monooxygenase	4.1	1.5
Z49204	Nnt, nicotinamide nucleotide transhydrogenase	3.3	1.3
M94584	Chi313, chitinase 3-like 3	3.2	2.5
AV038316	Apoa2, apolipoprotein A-II	3.0	−1.2
X56959	Pou3f1, POU domain, class 3, transcription factor 1	3.0	1.1
Y11505	Spink4, serine protease inhibitor	2.7	−1.2
AI849587	protein distantly related to the γ-subunit family	2.6	2.0
AV122577	Calm2, calmodulin 2	2.6	1.7
X75650	Krt1-3 keratin complex 1, acidic, gene 3	2.2	1.6
AV370017	P4ha2, procollagen-pro, 2-oxoglutarate 4-dioxygenase	2.2	1.5
AV117844	Ube2c, ubiquitin-conjugation enzyme E2C	2.0	−1.3
AB003503	Gspt2, guanine nucleotide regulatory protein	1.8	−1.0
AF093775	Actn3, actinin α 3	−3.0	−1.1
V00821	immunoglobulin μ-fragment	−2.8	−1.2
X61397	Cals1, carbonic anhydrase-like	−2.3	−1.3
AW122863	Tnfrsf19, tumor necrosis factor receptor family, 19	−2.3	1.0
X70842	Kdr, kinase domain receptor	−2.1	−1.3
U31966	Cbr1, carbonyl reductase	−1.7	−1.1
GO: development			
X97818	Sema5b, semaphorin G	2.9	−1.1
AI043016	Rnf2, ring finger protein 2	1.8	1.2

GO, gene ontology; Dex, dexamethasone; RA, retinoic acid; Dil, diluent.

opment, the process of increasing lung surface area by septation of saccules occurs across species (9, 10, 19, 41, 42); thus, it is our hope that the molecular mechanisms revealed in mice will translate to humans.

The microarray study was intended to serve as a first exploration of molecular changes that occur at an early time point after treatment. The strategy was to use stringent filtering criteria to identify genes that were significantly altered by Dex treatment and whose altered expression (up or down) was rectified by concomitant treatment with RA. Forty-six genes met these criteria; of these 46, 26 genes have been annotated by the Gene Ontology Consortium and fall mainly into the biological processes of cell communication and growth/maintenance. It is reasonable that these particular processes would be affected at an early time after treatment, considering the effect of Dex to inhibit septation and of RA to abrogate the inhibition. In addition to alteration in expression by Dex and rescue by RA, interest would be heightened for genes whose expression in mice treated with RA alone did not change or changed in an opposite direction compared with Dex alone. In this manner, the focus would be on candidate genes involved in signaling septation based on the established effects of Dex, RA-Dex, and RA on lung morphology. By this approach, KDR appears to be such a gene. In validation of the array data, KDR RNA and protein concentration were concordant (Fig. 7).

KDR is a receptor tyrosine kinase located on the surface of endothelial cells and is a major mediator of the mitogenic,

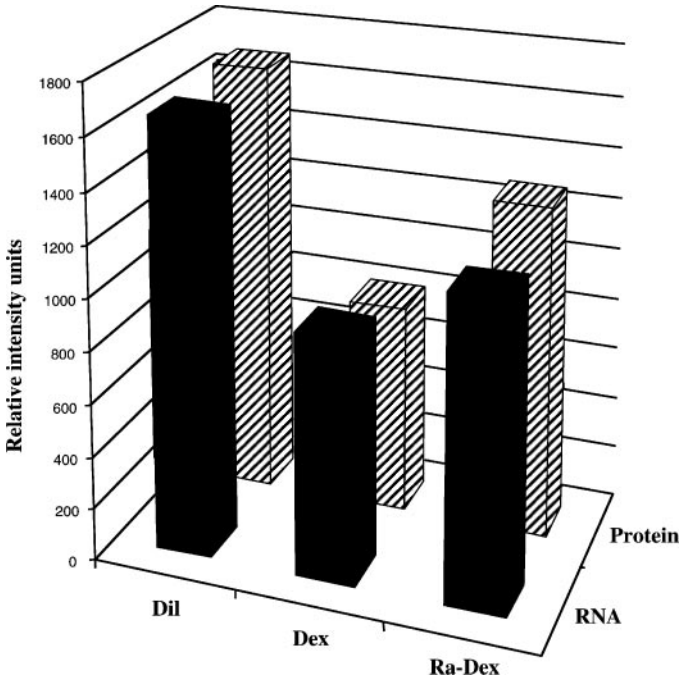

Fig. 7. Comparison of kinase domain receptor (KDR) RNA and protein expression. The graph shows the Affymetrix expression values for KDR mRNA (solid bars) and relative densitometry units for KDR protein as determined by Western analysis (hatched bars).

angiogenic, and permeability-enhancing effects of VEGF (14, 31, 33). Our data support the hypothesis that Dex inhibits septation by decreasing KDR, whereas RA abrogates this effect. This idea fits with lung KDR data in the following experimental models: *1*) During normal mouse lung development from embryonic *day 13* to adulthood, KDR and VEGF mRNAs increase in parallel (4). *2*) Decreased alveolarization and arterial density were observed on *day 14* when neonatal rats were treated from *day 1* to *13* with Su-5416, an inhibitor of KDR (18). *3*) Mild hypoxia (16% O_2) from postnatal *day 1* to *10* impaired alveolarization and decreased KDR protein in endothelial nitric oxide synthase-deficient mice (3). *4*) Independent of its ability to inhibit matrix metalloproteinases, tissue inhibitor of metalloproteinases-3 (TIMP-3) inhibits VEGF-mediated angiogenesis by blocking the binding of VEGF to KDR (35); this function may, in part, be responsible for the air space enlargement as measured by increased L_m in mice lacking TIMP-3 (20). *5*) In adult mice subjected to 66% calorie restriction for 14 days, alveolar number and alveolar surface area are significantly decreased (30); as determined by microarray analysis, KDR is decreased approximately twofold after 14 days of calorie restriction (http://microarray.cnmcresearch.org; project name: Murine Calorie Restriction; investigator: Dr. Gloria Massaro). *6*) In a study utilizing a hyperoxia protocol that inhibits alveolarization in newborn rats (24), KDR mRNA was decreased in rat lung after hyperoxic (>95% O_2) exposure from *day 4* to *14* in rats (15). This paper, together with our data, suggests that, in newborn animals, hyperoxia-arrested alveolarization and Dex inhibition of septation operate via a similar mechanism, that is, by blocking angiogenesis via downregulation of KDR. In contrast to our data, we are aware of one paper reporting Dex treatment of neonatal mice did not induce a decrease in KDR mRNA (4); in this study, mice were treated from *day 6* to *9* (vs. *day 3–4* in our study) with an ~10-fold higher dose of Dex than in our work. The difference between these findings and ours is likely due to the timing of their treatment midway into the alveolarization period rather than at the initiation of septation.

The notion that Dex is inhibiting lung formation via downregulation of KDR may have an impact on chronic lung disease (CLD), particularly in the preterm infant. In spite of advances in clinical care, including the use of artificial surfactant, BPD continues to be a major cause of pulmonary morbidity (40). Although corticosteroids are used to ameliorate the inflammation that plays a role in the pathogenesis of CLD, there are short- and long-term adverse effects that have promoted concern and controversy about their routine use (1, 17, 34, 39). The data in this paper suggest corticosteroid treatment may exacerbate the effects of preterm birth on lung development via downregulation of KDR. Although we cannot exclude the possibility that VEGF/KDR may also affect lung epithelial cell growth and differentiation (8, 12), based on the classical role of VEGF and KDR, the exacerbation most likely occurs because of decreased vasculogenesis/angiogenesis (31, 33). This notion is supported by a study on human lung specimens collected 6 h after the death of infants diagnosed with BPD and of infants dying without lung disease that showed there was a decrease in VEGF mRNA expression in the lungs of the BPD group compared with infants without lung disease (5). Future studies will examine whether the early changes in KDR are reflected in a long-term alteration in vascularization. Obtaining definitive proof of the role of KDR in modulating alveolar septation will require experiments in which we specifically target KDR expression.

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