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REPORTS OF ORIGINAL INVESTIGATIONS

Cerebral cortical gene expression in acutely anemic rats: a microarray analysis

L'expression génique dans le cortex cérébral de rats anémiques aigus: une analyse par microarray

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Abstract

Purpose *Hemodilution in perioperative patients has been associated with neurological morbidity and increased mortality by undefined mechanisms. This study assesses whether hemodilutional anemia up-regulated inflammatory cerebral gene expression (microarray) to help define the mechanism.*

Methods *Hemodilution was performed in anesthetized rats by exchanging 50% of the estimated blood volume (30 mL kg^{-1}) with pentastarch. Two groups of control animals were utilized, i.e., a non-anesthetized control (Normal Control) and an anesthetized control group (Anesthesia Control). Blood pressure, hemoglobin concentration, and arterial blood gas analysis were performed before and after hemodilution. Cerebral cortex was harvested from isoflurane-anesthetized rats ($n = 6$) after 6 and 24 hr of recovery and was used to perform complimentary DNA (cDNA) microarray analyses. Pro-inflammatory chemokine and cytokine protein levels were also measured.*

Results *Microarray analysis demonstrated up-regulation of 72 and 27 genes (6 and 24 hr, respectively) in anemic cerebral cortex. These genes were involved in a number of biological functions, including (1) inflammatory responses; (2) angiogenesis; (3) vascular homeostasis; (4) cellular biology; and (5) apoptosis. Chemokine ribonucleic acid (RNA) expression (CXCL-1, -10, and -11) was highest in anemic brain tissue ($P < 0.0125$ for each). Protein measurements demonstrated a significant increase in interleukin-6, tumor necrosis factor α, and monocyte chemoattractant protein-1 ($P < 0.05$ for each).*

Conclusion *This study utilizes microarray technology to elucidate changes in cerebral cortical gene expression in response to acute hemodilution. These findings demonstrate an increase in pro-inflammatory chemokines (RNA, protein) and cytokines (protein). An improved understanding of the inflammatory response to anemia may help to minimize associated morbidity and mortality.*

Résumé

Objectif *L'hémodilution en périopératoire chez les patients a été associée à une morbidité neurologique et une mortalité accrue en raison de mécanismes indéterminés. Cette étude examine si l'anémie par hémodilution régule à la hausse l'expression génique cérébrale inflammatoire (microarray) afin d'aider à définir ce mécanisme.*

Méthode *Une hémodilution a été réalisée chez des rats anesthésiés en échangeant 50 % du volume sanguin estimé (30 mL·kg^{-1}) avec du pentastarch. Deux groupes d'animaux témoins ont été utilisés, soit un groupe témoin non anesthésié (témoin normal) et un groupe témoin anesthésié (témoin anesthésié). La pression artérielle, la concentration d'hémoglobine et une gazométrie sanguine artérielle*

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ont été réalisées avant et après hémodilution. Le cortex cérébral a été récolté chez les rats anesthésiés à l'isoflurane (n = 6) après 6 et 24 h de récupération et a été utilisé pour réaliser des analyses par microarray d'ADN complémentaire (ADNc). Les niveaux des protéines pro-inflammatoires chimiokine et cytokine ont également été mesurés.

Résultats *L'analyse par microarray a démontré une régulation à la hausse de 72 et 27 gènes (6 et 24 h, respectivement) dans le cortex cérébral anémique. Ces gènes étaient impliqués dans plusieurs fonctions biologiques, notamment 1) les réactions inflammatoires; 2) l'angiogenèse; 3) l'homéostasie vasculaire; 4) la biologie cellulaire; et 5) l'apoptose. L'expression de l'acide ribonucléique (ARN) des chimiokines (CXCL-1, 10 et 11) était la plus élevée dans le tissu cérébral anémique (P < 0,0125 pour chacune). Les mesures des protéines ont démontré une augmentation significative de l'interleukine-6, du facteur onconécrosant α, et de la protéine MCP1 (P < 0,05 pour chacun).*

Conclusion *Cette étude utilise la technique du microarray pour expliquer les changements dans l'expression génique du cortex cérébral en réaction à une hémodilution aiguë. Ces résultats démontrent une augmentation des chimiokines (ARN, protéines) et des cytokines (protéines) pro-inflammatoires. Une meilleure compréhension de la réaction inflammatoire à l'anémie pourrait nous aider à minimiser la morbidité et la mortalité associées.*

Introduction

Acute anemia has been associated with cognitive and neurophysiologic dysfunction in healthy volunteers[1,2] and increased neurological injury and mortality in perioperative patients by currently undefined mechanisms.[3–6] One of the most common causes of acute anemia is acute blood loss and fluid resuscitation (hemodilution). Therefore, we have adopted a model of acute hemodilutional anemia to study the cerebral gene responses. Experimental evidence suggests that hemodilution may augment cerebral injury by limiting cerebral tissue oxygen delivery.[7–12] However, the mechanism by which cerebral injury occurs in anemic patients has not been clearly defined.

Our research has demonstrated that acute hemodilutional anemia leads to the up-regulation of a number of proteins in the brain, including hypoxia inducible factor-1α (HIF-1α) and neuronal and inducible nitric oxide synthase (nNOS and iNOS, respectively).[8,12] However, the role of these or other proteins in the pathophysiology of anemia-induced brain injury has not been defined.

Previous studies have identified that acute hemodilution can increase the migration of leukocytes into the central nervous system.[9] In addition, our published experimental study demonstrated an increase in cerebral cortical iNOS expression, which is suggestive of a pro-inflammatory response.[8] Interestingly, this increase in iNOS expression was not associated with an increase in circulating cytokines, as plasma tumor necrosis factor (TNF)α, interleukin (IL)-6, and IL-1β levels did not differ between the control and anemic groups.[8] To assess whether the increase in cerebral cortical iNOS expression was the result of a local tissue inflammatory response, we performed a microarray analysis to test the hypothesis that acute inflammation could contribute to cerebral injury associated with acute hemodilutional anemia.

In addition, the discovery of other novel genes that are up-regulated by acute hemodilution may provide important insights into adaptive and maladaptive mechanisms activated in the cerebral cortex in response to anemia. These data may provide clues to potential target genes for preventing cerebral injury and optimizing the treatment of anemic patients.

Materials and methods

Animal model

All animal protocols were approved by the Animal Care and Use Committee at St. Michael's Hospital in accordance with the requirements of Canadian Animal Care. Anesthesia was induced in male Sprague–Dawley rats (Charles River, St. Constant, QC, Canada) with 3–4% isoflurane in 100% oxygen in an induction chamber and maintained with 1–2% isoflurane in 50% oxygen (Abbott, St.Laurent, QC, Canada). Following intubation, ventilation was maintained with a pressure-controlled ventilator (Kent Scientific, Litchfield, CT, USA). Incision sites were infiltrated with 2% lidocaine prior to cannulation of the tail artery and vein (24 G BD Angiocath™, BD Medical Systems, Oakville, ON, Canada). Vascular access was utilized to measure mean arterial blood pressure (MAP), arterial blood gases (ABGs), and hemoglobin concentration by co-oximetry (Radiometer ALB 500 and OSM 3 London Scientific, London, ON, Canada) and to perform acute hemodilution. Ventilation was adjusted to achieve normocapnia and normoxia as determined by blood gas analysis. A heating pad and heating lamp were used to maintain rectal temperature near 37°C. Data were recorded using a computerized data acquisition system (DASYLab 5.6, Kent Scientific, Litchfield, CT, USA).

After establishing baseline ABGs and hemoglobin concentrations, acute hemodilutional anemia was induced by simultaneously exchanging 30 mL · kg^{-1} of arterial blood (50% of the estimated blood volume) withdrawn from the tail artery with an equivalent volume of pentastarch (Pentaspan, Bristol-Myers Squibb, Montreal, QC, Canada)

infused via the tail vein ($n = 6$ rats per time period) (Anemia Group). Volume exchange was performed over 10 min using a programmable "push–pull" pump as previously described (PHD 2000, Harvard Apparatus, Saint-Laurent, QC, Canada).[10] This model of acute hemodilution approximates clinical conditions of hemodilution that commonly occur during cardiopulmonary bypass or fluid resuscitation with crystalloid and/or colloid solution prior to utilization of blood transfusion. Following completion of volume exchange, all parameters were recorded for an additional 20 min. The rats were then recovered until they were breathing room air spontaneously. Two groups of control animals were utilized. One group was briefly anesthetized while spontaneously breathing 2% isoflurane in 50% oxygen prior to blood sampling by aortic puncture (room air), decapitation, and brain harvest (Normal Control). The second group served as a procedural control similar to the hemodilution group with respect to all interventions, including anesthetic and cannulation technique, but did not undergo hemodilution (Anesthesia Control). The two different groups of control anesthetized and hemodiluted rats were recovered for either 6 or 24 hr prior to brain harvest. At 6 or 24 hr, the rats were anesthetized with 2% isoflurane in 50% oxygen and then decapitated. The brains were harvested and segments of cerebral cortex were snap frozen in liquid nitrogen (−80°C) within 4 min. RNA extraction and microarray analysis were performed as outlined below.

Microarray analysis

High-density cDNA microarrays (GeneChip® Rat Genome 230 2.0 Array, of 31,000 well-substantiated rat genes, Affymetrix®, Santa Clara, CA, USA) were used to examine genes whose expressions are regulated following acute hemodilution. To define the transcriptomes of anemia, RNA samples were prepared from the cerebral cortex of rats 6 and 24 hr after hemodilution. The samples were compared with RNA from animals that had previously received sham treatment consisting of anesthesia and surgical procedures without hemodilution (Anesthesia Control) and from animals immediately killed under brief general anesthesia (Normal Control). The cerebral cortex from two rats for each experimental condition were pooled (three pools per condition) to minimize potential variation among animals. Predefined criteria were set to increase the level of confidence in the analysis of the microarray data.[11] These criteria were as follows: (1) filtering genes of interest using a confidence interval of 0.0125; (2) correcting the one-way analysis of variance (ANOVA) using the Benjamini and Hochberg method to control the false discovery rate; and (3) presenting only those genes with a two-fold change from Normal Controls.

As outlined below, samples of RNA were prepared for microarray analysis, which was performed by the Microarray Facility at the Centre for Applied Genomics, Hospital for Sick Children (Toronto, ON, Canada).

RNA preparation

Total RNA was isolated from the snap-frozen cerebral cortex tissue using QIAzol Lysis reagent (Qiagen, Mississauga, ON, Canada) according to the manufacturer's protocol. After isolation, the RNA was purified using the RNeasy total RNA isolation kit (Qiagen). RNA samples were quantified spectrophotometrically, and samples with a ratio A260/A280 from 1.8 to 2.1 were analyzed for quality using a bioanalyser (Agilent Technologies, Palo Alto, CA, USA). Equal amounts of total RNA preparation from two individual animals in each group (Anemia 6 and 24 hr, Anesthesia Control 6 and 24 hr, and Normal Control 0 hr) were pooled ($n = 3$ arrays, $n = 6$ rats per group and time) and used as a starting material for each microarray template reaction.

Synthesis of complimentary DNA (cDNA)

The Affymetrix® protocol for GeneChip® one-cycle cDNA synthesis was used. Briefly, the reaction was primed by annealing a T7-oligo (dT) primer coupled to a T7 RNA polymerase promoter to the RNA sample. RNA was reverse transcribed with Superscript II™ reverse transcriptase. Second-strand synthesis was carried out immediately in the presence of *Escherichia coli* DNA polymerase I with RNAse H, incubated in the presence of T4 DNA polymerase, and terminated with ethylenediamine tetraacetic acid (EDTA). The samples were cleaned (Qiagen cDNA clean-up column).

Synthesis of biotin-labeled complementary RNA (cRNA)

Complementary RNA was generated in an in vitro transcription reaction by using T7 RNA polymerase and biotinylated ribonucleotide analog (IVT labelling kit, Affymetrix®). The in vitro transcription samples were cleaned (Qiagen RNA clean-up column) and eluted. The quality was confirmed spectrophotometrically (260/280).

Fragmentation and hybridization to species microarray

Twenty microgram of each resulting biotinylated cRNA were fragmented into strands of 35–200 bases in length according to the manufacturer's protocol (Affymetrix GeneChip® analysis technical manual) and added to the hybridization buffer. Eukaryotic hybridization controls (BioB, BioC, BioD, and cre) and control oligonucleotides

were added to the cocktail. The hybridization cocktail was denatured (99°C for 5 min). The Affymetrix® chip arrays (rat 230 2.0 array) were equilibrated to room temperature. Sample cocktails were introduced into the chamber of the preconditioned chips and incubated (45°C for 16 hr). The chips were washed, stained, and scanned using a fluidic station 400 and an Affymetrix GeneChip® Scanner 3000.

Protein analysis

After analysis of the microarray data at 6 hr, maximal responses to inflammatory chemokines were identified. Hemodilution or anesthesia control was performed on additional groups of rats ($n = 6$ for each). Brain tissue was harvested to assess changes in chemokine and cytokine protein levels using the Procarta cytokine kit (PC4110, Panomics Inc., Fremont, CA, USA) and Luminex 100 detection unit.

Statistical analysis

Physiological data

Statistical analysis was performed using SigmaStat (V2.03S, Systat Software Inc., Pint Richmond CA, USA). Data were assessed by a one or two-way ANOVA. For two-way ANOVA, significant time, group, and interaction effects were identified. When a significant F value was observed, *post hoc* analysis was performed using a Tukey test. Data are reported as mean ± SD. $P < 0.05$ was assessed as significant.

Microarray data

The Affymetrix GeneChip® system was used to convert intensity data into quantitative estimates of gene expression for each probe set (which includes 16 probe pairs consisting of a perfect match oligonucleotide and a mismatch oligonucleotide that contains a one-base mismatch at a central position). A probability statistic was generated for each probe set to define the genes that were present, marginal, or absent after background subtraction. Global scaling expression data were analyzed using GeneSpring software (Silicon Genetics, Redwood City, CA, USA). The data for both treatments (Anemia and Anesthesia Control) and the two times (6 and 24 hr) were normalized to Normal Control (0 hr). The data were filtered to decrease the number of irrelevant unchanging genes.[11] One filter was designed to identify genes that are present and/or marginal in at least 2–3 arrays for each treatment and time. The second filter was designed to identify genes that were significantly up- or down-regulated compared with Normal Control by using a confidence interval of 0.0125 for at least one condition for each time point. After filtering, one-way ANOVA with Benjamini and Hochberg correction was performed and twofold up- or down-regulated genes in each time point for both treatments were used for ontology analysis and pathway construction.

Chemokine and cytokine protein data

Cerebral cortical chemokine and cytokine levels were assessed by one-way ANOVA.

Results

Physiological data

The MAP of the anesthesia control and anemia groups was not significantly different either before (69 ± 10 *vs* 65 ± 7 mmHg, respectively) or after hemodilution (72 ± 8 *vs* 75 ± 4 mmHg, respectively). Baseline hemoglobin values did not differ between anesthesia controls and rats about to undergo hemodilution (120–130 $g \cdot L^{-1}$) (Table 1). Following hemodilution, there was a significant reduction in the hemoglobin concentration to values approximating 60 $g \cdot L^{-1}$ (Table 1). No differences in any other co-oximetry or ABG measurement were observed between the anesthetized controls and the hemodilution group.

Microarray data

Microarray analyses demonstrated that 18,000 out of 31,000 genes passed the first filter step for both time points. After the second filter, 435 and 217 genes met the second criterion at 6 and 24 hr, respectively. At 6 hr, 131 of these genes demonstrated differential regulation for both conditions (Anemia and Anesthesia Control) when compared with the cerebral cortex from the non-intervention controls. At 24 hr, only 33 genes showed differential regulation in both groups. None of the genes reported demonstrated greater than twofold down-regulation at either 6 or 24 hr.

Gene ontology, a method of classifying expressed genes by their reported or suggested molecular function, was utilized to group genes according to function. This was achieved by a multimodal approach utilizing information from the program, GeneSifter™ (www.genesifter.net), the universal protein resource (www.expasy.uniprot.org), and a literature review. Some genes may fall into more than one functional category. Utilizing these categories helps to assess the large amount of data acquired (Tables 2 and 3). Pathway construction based on gene expression data was performed using Pathway Architect (www.stratagene.com). In the anemia group, 89% and 90% of the defined genes showed at least a twofold change in expression at 6 and 24 hr, respectively. In the anesthesia group, 72% and 46% of the defined genes showed at least a two-fold change in expression at 6 and 24 hr, respectively. Of these genes up-regulated

Table 1 Arterial blood gas and co-oximetry data

Time (hr)	Description	pH	P_aCO_2 (mmHg)	P_aO_2 (mmHg)	Hb ($g \cdot L^{-1}$)	% Saturation	O_2 Content ($mmol \cdot L^{-1}$)
Normal control ($n = 6$)							
0	Baseline (F_IO_2; 0.21)	7.38 ± 0.02	38.1 ± 4.5	95.5 ± 29.1	137 ± 11	83 ± 11	6.9 ± 1.1
Anesthesia control ($n = 6$)							
6	Baseline (F_IO_2; 0.50)	7.36 ± 0.07	41.6 ± 10.5	183.5 ± 33.4	122 ± 10	100 ± 0.1	6.8 ± 0.7
	Post-baseline (F_IO_2; 0.50)	7.36 ± 0.06	40.4 ± 6.8	160.8 ± 36.5	110 ± 9	100 ± 0.1	6.7 ± 0.6
24	Baseline (F_IO_2; 0.50)	7.37 ± 0.12	48.7 ± 18.1	229.4 ± 46.4	131 ± 8	100 ± 0.1	8.1 ± 0.5
	Post-baseline (F_IO_2; 0.50)	7.36 ± 0.05	41.3 ± 9.1	174.9 ± 42.0	123 ± 11	100 ± 0.1	7.5 ± 0.6
Hemodilution ($n = 6$)							
6	Baseline (F_IO_2; 0.50)	7.38 ± 0.17	44.3 ± 22.5	163.9 ± 29.1	125 ± 15	100.0 ± 0.1	7.7 ± 1.0
	Hemodilution (F_IO_2; 0.50)	7.37 ± 0.09	44.9 ± 13.9	151.1 ± 31.1	61 ± 18*	100.0 ± 0.1	3.6 ± 1.1*
24	Baseline (F_IO_2; 0.50)	7.37 ± 0.11	45.4 ± 11.3	186.7 ± 51.5	134 ± 14	100 ± 0.1	8.2 ± 1.0
	Hemodilution (F_IO_2; 0.50)	7.33 ± 0.09	46.7 ± 14.5	184.4 ± 17.4	61 ± 9*	100 ± 0.1	3.7 ± 0.6*

* $P < 0.001$, relative to baseline

by anemia, 72 and 27 genes had a known function at 6 and 24 hr, respectively. For the anesthesia controls, 58 and 14 genes had known functions at 6 and 24 hr, respectively.

After 6 hr of anemia, up-regulation of cerebral cortical gene expression occurred in a number of important biological areas: (1) immune and inflammatory response genes (26% of all filtered genes); (2) regulation of transcription/translation and protein biology (10%); (3) apoptosis (9%); (4) angiogenesis (7%); (5) cellular biology (7%); (6) signal transduction (6%); (7) vascular homeostasis (5%); and (8) ion transport (3.7%). After 24 hr of anemia, changes in gene expression still occurred in: (1) immune and inflammatory pathways (32% of all filtered genes); (2) cellular biology (24%); (3) vascular biology (12%); (4) protein biology (8%); and (5) angiogenesis, metabolism, ion transport, and signal transduction (4%, respectively) (Fig. 1). There was no evidence of up-regulation of genes involved in apoptosis and regulation of transcription/translation after 24 hr of anemia (Fig. 1). A particular common pattern of gene expression occurred when comparing the gene responses with the Anemia and Anesthesia Controls. However, the number of genes specifically up-regulated by anemia was larger at 6 hr in the following areas: (1) immune and inflammatory response; (2) vascular homeostasis; (3) cellular biology; and (4) signal transduction (Fig. 1, Upper panels) and larger at 24 hr in the following areas: (1) immune and inflammatory response; (2) angiogenesis; and (3) ion transport (Fig. 1, Upper and middle panels).

Specific changes in individual genes are outlined in Tables 2 and 3. Different genes were defined for each functional group in anemic and anesthesia controls. When compared with controls at 6 hr, the most striking fold change differences observed among the gene profile in response to anemia were: (1) immune and inflammatory responses (chemokine [C–X–C motif] ligands 1, 10, and 11 [CXCL-1,10,11]; guanylate nucleotide binding protein 2; and interferon-induced protein with tetratricopeptide repeats 2: 66.0-, 35.0-, 23.1-, 16.6-, and 11.9-fold, respectively); (2) vascular homeostasis (prostaglandin E synthase: 64.5-fold); (3) apoptosis (cyclin-dependent kinase inhibitor 1A and lipocalin 2: 63.1- and 36.1-fold, respectively); and (4) cellular biology (uridine phosphorylase 1, S100 calcium binding protein A9, and metallothionein 1a: 41.9-, 18.8-, and 14.1-fold, respectively). Of these genes, only S100 calcium binding protein A9 remained significantly elevated at 24 hr in both conditions (lower for Anemia [18.5-fold] than for Anesthesia Control [26-fold]). In contrast, S100 calcium binding protein A8 was only up-regulated at 24-hr and at the same level in both conditions (25-fold). Additional cerebral cortical genes that were increased after acute anemia included programmed cell death 1 ligand 2 (6.9-fold at 6 hr), CCAAT/enhancer binding protein (C/EBP), beta (3.8-fold at 6 hr), zinc finger protein 36 (3.1-fold at 6 hr), angiopoeitin 2 (2.6- and 2.8-fold at 6 and 24 hr), thrombospondin 1 (2.9-fold at 6 hr), fibrinogen-like 2 (7.3-fold at 6 hr), albumin (2.1-fold at 6 hr), von Willebrand factor (3.0-fold at 24 hr), annexin A2 (2.1-fold at 24 hr), vimentin (2.5-fold at 24 hr), and palmedelphin (2.1-fold at 24 hr). A number of genes increased in response to anesthesia and surgical stress (Anesthesia Control) at 6 and 24 hr (Tables 2 and 3). Pathway Architect was used to demonstrate the relationship between up-regulated genes after acute anemia. A specific network between several up-regulated genes from a number of different categories was assessed with respect to their relationship with three hypoxia-regulated molecules previously demonstrated to be involved in the cerebral response to acute anemia (hypoxia inducible factor-1α (HIF-1α), erythropoietin (EPO), and vascular endothelial growth factor (VEGF)[12] (Fig. 2).

Table 2 Genes showing an increased expression with anemia and anesthesia at 6 hr

Gene no.	Anemia[a]	Anesthesia[a]	Gene product	Function—description
Immune & inflammatory response				
NM_030845	66.0	0.6	Chemokine (C–X–C motif) ligand 1	Cytokine activity/growth factor activity
U22520	35.0	7.3	Chemokine (C–X–C motif) ligand 10	Cytokine activity/signal transduction
BF281987	23.1	8.2	Chemokine (C–X–C motif) ligand 11	Chemokine receptor binding
AI044222	8.9	2.8	Chemokine (C–X–C motif) ligand 9	Cytokine activity
NM_133624	16.6	8.6	Guanylate nucleotide binding protein 2	GTP binding
AW521319	6.9	3.8	Programmed cell death 1 ligand 2	T cells proliferation and IFNγ production
AA955213	3.9	1.9	Similar to interferon-inducible GTPase	Cytokine and chemokine mediated signaling pathway
BE118697	11.9	5.2	Interferon-induced protein with tetratricopeptide repeats 2	
AW525366	5.9	3.4	Interferon gamma induced GTPase	GTPase activity
BI285494	4.0	2.6	Interferon induced transmembrane protein 3	
BF284262	2.4	1.6	Interferon consensus sequence binding protein 1	Myeloid cell differentiation/Regulation of transcription, DNA-dependent
BF411036	6.0	3.5	Interferon regulatory factor 7	Negative regulation of transcription from RNA polymerase II promoter
NM_012591	2.4	1.2	Interferon regulatory factor 1	Cell proliferation/regulate growth-inhibitory interferon genes
NM_017020	2.4	1.9	Interleukin 6 receptor	Signal transduction/hematopoietin/interferon-class (D200-domain) cytokine receptor activity
AI178808	2.0	2.2	Interleukin 2 receptor, gamma	Jak-STAT signaling pathway
AW434057	2.1	1.6	Complement component 1, q subcomponent, beta polypeptide	Complement activation
NM_053843	2.0	1.1	Fc receptor, IgG, low affinity III	Positive regulation of tumor necrosis factor-alpha biosynthesis
NM_024125	3.8	2.0	CCAAT/enhancer binding protein (C/EBP), beta	Acute-phase response/regulation of interleukin-6 biosynthesis
AB025017	3.1	1.2	Zinc finger protein 36	Negative regulation of inflammatory response/ mRNA catabolism
AI639117	2.5	1.5	B-factor, properdin	Complement activation, proteolysis
BI285863	2.1	1.6	Signal transducer and activator of transcription 3	Acute-phase response/cytokine and chemokine mediated signaling pathway/JAK-STAT cascade
Angiogenesis				
BI275292	2.6	3.0	Angiopoietin 2	Signal transduction/Vascular endothelial growth factor receptor binding
AI406660	2.9	1.9	Thrombospondin 1	Anti angiogenic molecule/central nervous system synaptogenesis
NM_024400	3.1	2.3	A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 1	Negative regulation of angiogenesis/integrin-mediated signaling pathway
NM_012935	2.4	2.2	Crystallin, alpha B	Response to stress
NM_023090	2.0	2.3	Endothelial PAS domain protein 1	Response to hypoxia
AA818262	1.9	2.0	Angiopoietin-like protein 4	Negative regulation of lipoprotein lipase activity & apoptosis
Vascular homeostasis				
AB048730	64.5	32.6	Prostaglandin E synthase	Prostaglandin metabolism/signal transduction
BG663284	7.3	4.0	Fibrinogen-like 2	Cytolysis
BI296054	2.3	1.4	Selectin, platelet	Inflammatory response/cell adhesion
NM_134326	2.1	1.4	Albumin	Body fluid osmoregulation

Table 2 continued

Gene no.	Anemia[a]	Anesthesia[a]	Gene product	Function—description
Cellular biology				
BI292558	41.9	31.2	Uridine phosphorylase 1	Energy metabolism & nucleotide synthesis
NM_053587	18.8	12.2	S100 calcium binding protein A9 (calgranulin B)	Cell–cell signaling/Calcium ion binding/ Inflammatory response
AF411318	14.1	5.9	Metallothionein 1a	Nitric oxide mediated signal transduction
BI275741	2.0	1.7	Epithelial membrane protein 1	Cell death/growth
BE104180	2.0	1.9	SRY-box containing gene 11	Regulation of transcription, DNA-dependent
AI228623	2.1	1.9	Neuronal pentraxin II	Synaptic transmission
Apoptosis				
U24174	63.1	34.6	Cyclin-dependent kinase inhibitor 1A	Cell cycle arrest
NM_130741	36.1	22.8	Lipocalin 2	Binding
AW672589	3.6	2.4	Nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha	Cytoplasmic sequestering of NF-kappaB/ regulation of cell proliferation
AF136282	3.6	2.4	BH3 interacting domain death agonist	Involved in inducing Bax oligomerization in isolated mitochondrial outer membranes
NM_024356	4.9	3.2	GTP cyclohydrolase 1	Tetrahydrobiopterin biosynthesis/mediate cell death of striatal neuronal and non-neuronal cells
NM_019232	3.2	2.5	Serum/glucocorticoid regulated kinase	Response to stress/protein kinase activity
NM_013091	2.6	2.0	Tumor necrosis factor receptor superfamily, member 1a	Signal transduction/activation of MAPK activity
AF304333	2.0	2.2	Baculoviral IAP repeat-containing 4	Anti apoptosis/negative regulation of caspase activity
Metabolism				
AI169398	4.2	2.1	Cholesterol 25-hydroxylase	Lipid metabolism/steroid hydrolase activity
BF401689	4.4	2.4	Adenylate kinase 3	Amino acid biosynthesis/phosphotransferase activity
NM_022215	4.5	3.7	Glycerol-3-phosphate dehydrogenase 1 (soluble)	Carbohydrate metabolism/oxidoreductase activity
NM_024399	3.0	2.6	Aspartoacylase	Acetate metabolism/may play a role in central nervous system myelination
D88666	2.4	2.3	Phosphatidylserine-specific phospholipase A1	Lipid metabolism
NM_053562	3.1	2.9	Retinal pigment epithelium 65	Regulation of rhodopsin gene activity/vitamin A metabolism
Ion transport				
NM_012532	2.9	1.6	Ceruloplasmin	Ion transport/homeostasis
NM_031601	1.8	2.0	Calcium channel, voltage-dependent, T type, alpha 1G subunit	Calcium ion transport
AF313411	1.9	2.1	Putative small membrane protein NID67	Ion channel activity
Protein biology				
AW531805	7.1	3.2	Similar to this ORF—is capable of encoding 404aa, which is homologous to two human interferon-inducible proteins	Protein binding
BF397703	4.3	3.5	Similar to RIKEN cDNA 2610029K21	
BI274903	3.4	3.5	Similar to RIKEN cDNA 2310057H16	Microtubule-based movement
NM_053582	2.4	2.6	Lipocalin 7	Proteolysis/transport
BF394819	1.9	2.0	Ribulose-5-phosphate-3-epimerase (predicted)	Protein binding
BI284255	2.2	2.0	FK506 binding protein 5	Protein folding/isomerase activity
BF401793	2.5	1.5	Coatomer protein complex, subunit zeta 1 (predicted)	Protein transport
BF398127	1.7	2.1	Translocated promoter region	Protein import into nucleus

Table 2 continued

Gene no.	Anemia[a]	Anesthesia[a]	Gene product	Function—description
Signal transduction				
NM_017334	2.5	1.8	CAMP responsive element modulator	
Z23279	1.9	2.0	Adenylate cyclase activating polypeptide 1 receptor 1	Regulates neural precursor proliferation/G-protein coupled receptor protein signaling pathway
NM_019386	5.3	4.4	Transglutaminase 2, C polypeptide	G-protein coupled receptor protein signaling pathway/ apoptosis/positive regulation of cell adhesion
NM_012807	2.1	1.2	Smoothened homolog (*Drosophila*)	G-protein coupled receptor protein signaling pathway/ vasculagenesis
BG663107	3.0	1.9	A kinase (PRKA) anchor protein (gravin) 12	G-protein coupled receptor protein signaling pathway
VO5963	1.5	2.0	Protein tyrosine phosphatase, non receptor type 11	Activation of MAPK activity/DNA damage
Regulation of transcription/translation				
NM_013154	4.4	3.0	CCAAT/enhancer binding protein (C/EBP), delta (Inositol polyphosphate-5-phosphatase E)	Regulation of transcription, DNA-dependent
NM_133422	3.7	4.7	Zinc finger protein 483	Putative zinc-finger transcription factor
BE110589	3.4	2.6	Metadherin	Regulation of transcription, DNA-dependent
AI407872	2.5	2.1	Zinc finger protein 189	Regulation of transcription, DNA-dependent
AA998067	2.4	2.3	Kruppel-like factor 15	Regulation of transcription, DNA-dependent
NM_017323	1.5	2.0	Nuclear receptor subfamily 2,	Stress response pathway group C, member 2
AW523099	2.5	2.2	WD repeat domain 33 (predicted)	Post-replication repair
H31896	2.1	1.1	Neoplastic progression 3	DNA binding
Others				
NM_031697	2.4	2.0	Sialyltransferase 6 (N-acetyllacosaminide alpha 2,3-sialyltransferase)	N-acetyllactosaminide alpha-2,3-sialyltransferase activity/keratan sulfate biosynthesis
BI278962	3.4	2.3	Schlafen 5 (predicted)	Hydrolase activity
BI284420	6.1	4.5	Similar to retinoic acid-responsive protein (STRA6)	Integral to membrane/strongly expressed at the level of blood-organ barrier/involved in transport machinery
BF405705	3.2	3.1	Phosphatase and actin regulator 3	Inhibit protein phosphatase 1
AF201839	1.9	2.1	Dynamin 3	Endocytosis
NM_133393	2.0	1.9	Lunatic fringe gene homolog (Drosophila)	Regulation of Notch signaling pathway
AI137113	1.9	2.0	Similar to CGI-100-like protein	ER-Golgi intermediate compartment

$P < 0.0125$ ANOVA, Benjamini correction, threshold greater than to 2.0

[a] Fold increase compared with control

Protein data

Compared with anesthesia controls, significant increases in cerebral cortical levels of IL-6, TNFα, and monocyte chemoattractant protein (MCP)-1 were measured in anemic brain tissue ($P < 0.05$ for each, Fig. 3).

Discussion

Microarray analysis demonstrated that a significant number of genes underwent up-regulation in response to acute hemodilutional anemia. The most striking increases were observed for the inflammatory chemokines, C–X–C ligand 1, 10, and 11. This pattern of gene expression suggested that pro-inflammatory mechanisms had been activated within the cerebral cortex of anemic rats. Subsequent analysis confirmed that TNFα, IL-6, and MCP-1 protein levels were also up-regulated in cerebral cortical tissue. Up-regulation of these proteins has been associated with increased leukocyte and monocyte migration into the brain.[13,14] Local production of these cytokines within the brain (TNFα, IL-6) is strongly supported by the results of our previous study wherein anemia did not increase the levels of circulating cytokines above control values.[8] Thus, local up-regulation of inflammatory mediators and subsequent leukocyte

Table 3 Genes showing an increased expression with anemia and anesthesia at 24 hr

Gene no.	Anemia[a]	Anesthesia[a]	Gene product	Function—description
Immune & inflammatory response				
BF418957	2.0	1.8	Complement component 1, q subcomponent alpha polypeptide,	Complement activation/phosphate transport
AW434057	2.0	2.1	Complement component 1, q subcomponent beta polypeptide,	Complement activation
BI285347	2.2	1.7	Complement component 4, gene 2	
NM_017260	3.7	3.9	Arachidonate 5-lipoxygenase activating protein	Leukotriene metabolism
BI285865	2.0	1.6	Immunoglobulin superfamily member 9 (predicted),	Dentrite morhpogenesis
AJ249701	2.8	2.3	RT1 class Ia, locus A1	Antigen presentation, endogenous antigen
AI639117	2.2	1.9	B-factor, properdin	Complement activation/proteolysis
BF411036	2.3	1.7	Interferon regulatory factor 7	Negative regulation of transcription from RNA polymerase II promoter
Angiogenesis				
BI275292	2.8	1.9	Angiopoietin 2	Signal transduction/vascular endothelial growth factor receptor binding
Vascular homeostasis				
BI298314	3.0	2.0	von Willebrand factor	Blood coagulation/tissue regeneration
AW915763	3.1	2.6	Serine (or cysteine) peptidase inhibitor, clade G, member 1	Complement and coagulation cascades
NM_019905	2.1	1.3	Annexin A2	Fibrinolysis
Cellular biology				
NM_053587	18.5	25.8	S100 calcium binding protein A9 (calgranulin B)	Cell–cell signaling/Calcium ion binding/Inflammatory response
MN_053822	24.9	25.2	S100 calcium binding protein A8 (calgranulin A)	Calcium ion binding/Inflammatory response
AI639014	1.8	2.8	Foveolin precursor	Positive regulation of cell proliferation
NM_031140	2.5	1.5	Vimentin	Cell motility
L12458	2.2	1.9	Lysozyme	Cytolysis
NM_133298	1.7	2.1	Glycoprotein (transmembrane) nmb	Cell adhesion
AI176360	2.1	1.5	Palmdelphin	Regulation of cell shape
Metabolism				
AF394783	2.9	2.6	Sulfotransferase family 1A, phenol-preferring,	Sulfur metabolism member 1
Ion transport				
NM_012532	2.9	2.7	Ceruloplasmin	Ion transport/homeostasis
Protein biology				
BI288424	2.0	1.9	Similar to RIKEN cDNA 2610029K21	Nucleic acid binding
NM_053582	2.0	1.4	Lipocalin 7	Proteolysis/transport
Signal transduction				
NM_019386	3.1	3.0	Transglutaminase 2, C polypeptide	G-protein coupled receptor protein signaling/apoptosis/ positive regulation of cell adhesion
Others				
AI177589	3.6	3.0	Sortilin-related receptor, L (DLR class)	Receptor mediated endocytosis A repeats-containing
BF393825	2.3	2.6	Dendritic cell inhibitory receptor 3	
NM_021909	2.1	2.3	FXYD domaine-containing ion transport regulator 5	Extracellular space
BG37585	1.8	2.1	Leucine-rich alpha-2-glycoprotein 1	Extracellular space

$p < 0.0125$ ANOVA, Benjamini correction, threshold greater than to 2.0

[a] Fold increase compared with control

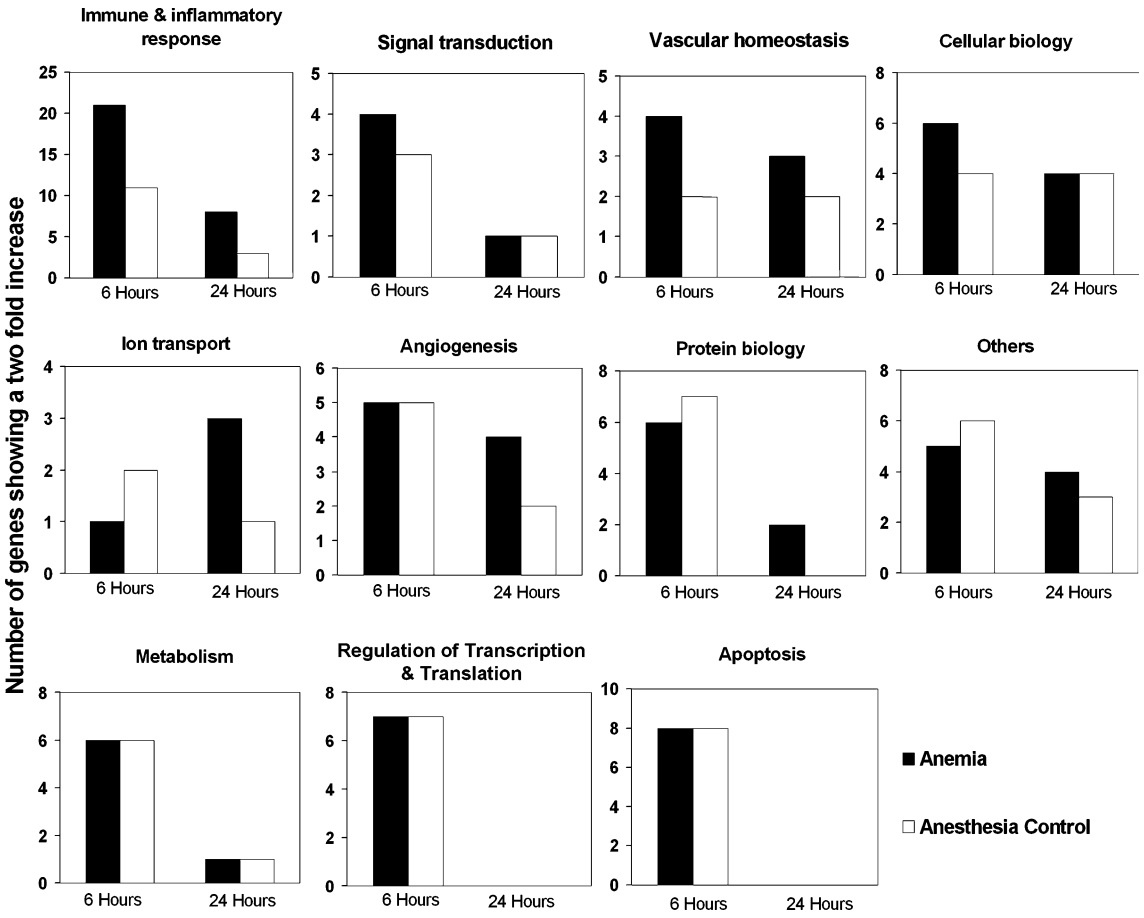

Fig. 1 Genes showing a twofold change in the cerebral cortex representing the different biological processes defined using GeneSifter™ software at 6 and 24 hr after acute hemodilutional anemia (Anemia) and anesthesia + surgical stress (Anesthesia Control) at 6 and 24 hr in the cerebral cortex for the different biological processes defined using GeneSifter software

migration into the brain may contribute to anemia-induced cerebral injury.

In addition, strong up-regulation of a number of different genes was observed at 6 hr (prostaglandin E synthase, uridine phosphorylase 1 and apoptotic mediators, P-selectin, CCAAT/enhancer binding protein (C/EBP) beta and delta, S100 calcium binding protein A9, and metallothionein 1a). By 24 hr, most of these genes were no longer up-regulated. The two exceptions were S100 calcium binding protein A9 and angiopoietin 2, which remained elevated at 6 and 24 hr. After 24 hr, additional genes demonstrated up-regulation that was not expressed at 6 hr (S100 calcium binding protein A8, von Willebrand factor, and Annexin A2).

Significant changes in gene expression were also caused by surgical and anesthetic procedures (Tables 2 and 3). The magnitude of the changes observed was generally smaller when compared with changes observed after acute hemodilution. It is of interest that none of the genes reported in Tables 2 and 3 exhibited a significant decrease in expression (greater than two-fold). By focusing on the genes that were up-regulated in both groups, we were able to identify those genes whose expression was solely increased because of anemia, thereby excluding the effects of surgical and anesthetic procedures. These data demonstrate a complex biological pattern of cerebral cortical gene expression, which may contribute to important physiological and pathophysiological mechanisms involved in adaptive and maladaptive responses to acute anemia.

Genes involved in inflammation

The dramatic up-regulation of mRNA expression of several chemokines and cytokines may promote leukocyte migration into the brain. These cytokines are often produced under pathological conditions, such as mechanical trauma, multiple sclerosis, Alzheimer's disease, and ischemia.[15–19] In addition, the dramatic increase in prostaglandin E synthase may also mediate a cerebral inflammatory response.[20–22]

Two other pro-inflammatory factors (CCAAT/enhancer binding protein [C/EBP β and δ]) were also up-regulated at 6 hr. The C/EBP family plays an important role in the

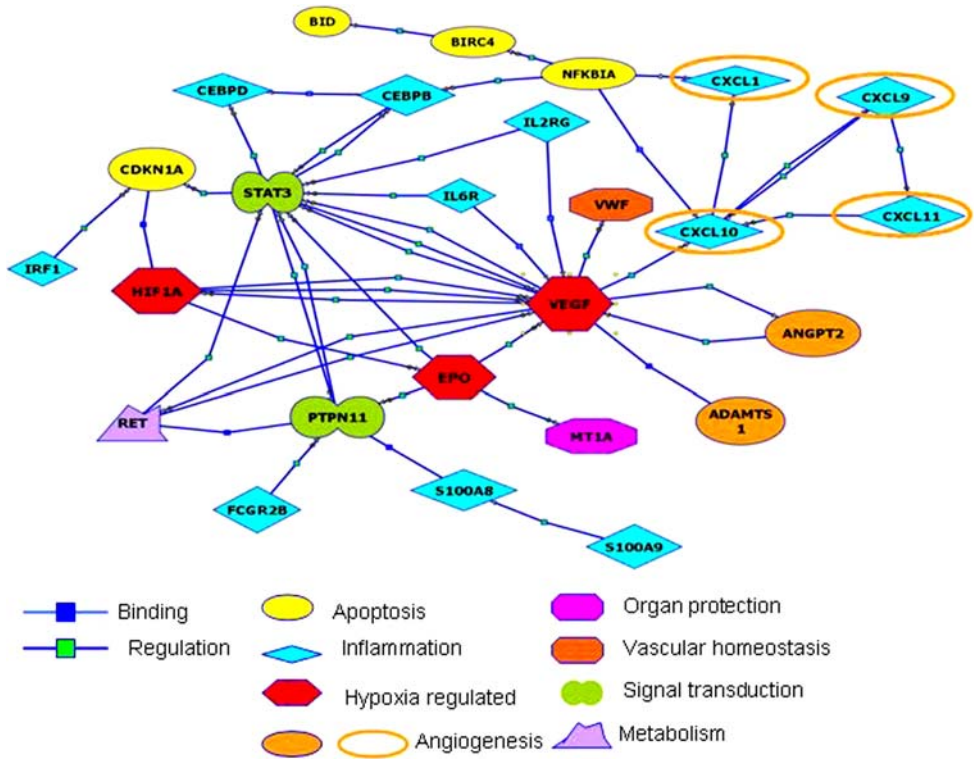

Fig. 2 Pathway analysis of the genes showing at least a twofold change at 6 and 24 hr after acute anemia. One network containing 23 genes was defined using Pathway Architect and is displayed graphically as nodes (genes/protein products) and edges (the connections—binding, positive and negative regulation—between the nodes). Three genes involved in response to hypoxia were also included in the analysis: hypoxia inducible factor-1α (HIF-1A), erythropoietin (EPO), and vascular endothelial growth factor (VEGF). ADAMTS1 = thrombospondin 1; ANGPT2 = angiopoietin 2; BID = BH3 interacting domain death agonist; BIRD4 = baculoviral IAP repeat-containing 4; CDKN1A = cyclin-dependent kinase inhibitor 1A; CEBPB = CCAAT/enhancer binding protein (C/EBP) beta; CEBPD = CCAAT/enhancer binding protein (C/EBP) delta; CXCL1 = chemokine ligand 1; CXCL9 = chemokine ligand 9; CXCL10 = chemokine ligand 10; CXCL11 = chemokine ligand 11; FCGR2B = Fc receptor IgG low affinity III; IL2R = interleukin 2 receptor; IL6R = interleukin 6 receptor; IRF1 = interferon regulatory factor 1; MT1A = metallothionein 1a; NFKBIA = nuclear factor of kappa light chain gene enhancer in B-cells inhibitor alpha; PTPN11 = protein tyrosine phosphatase non-receptor type 11; RET = retinal pigment epithelium 65; S100A8 = S100 calcium binding protein A8; S100A9 = S100 calcium binding protein A9; STAT3 = signal transduction and activator of transcription 3; VWF = von Willebrand factor

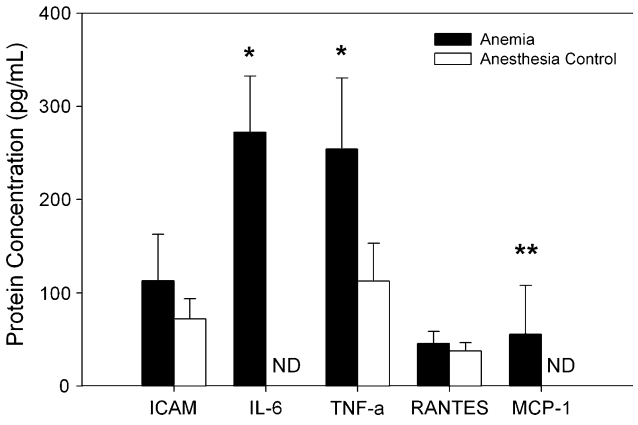

Fig. 3 Cerebral cortical protein levels in anemic rats demonstrate elevated levels of pro-inflammatory cytokine interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-a), and the chemokine monocyte chemoattractive protein-1 (MCP-1). No increased in intracellular adhesion molecule-1 (ICAM-1) or RANTES (Regulated on Activation, Normal T Expressed and Secreted). * $P < 0.001$, ** $P < 0.05$

control of cellular proliferation and differentiation.[23,24] C/EBP β and δ are pivotal transcription factors involved in the regulation of genes encoding many acute phase proteins and cytokines induced after cerebral ischemia reperfusion, e.g., IL-6, IL-1β, and TNFα.[23,25–27] Protein measurements demonstrating an increase in IL-6 and TNFα in the cerebral cortex of anemic rats supports this microarray finding. Astrocytes expressing C/EBP β and δ may produce these cytokines, which in turn can induce the expression of C/EBP β and δ in the surrounding cells. This feed-forward mechanism could serve to augment the inflammatory response following acute anemia.[27]

Finally, P-selectin at 6 hr and von Willebrand factor (vWF) were up-regulated at 24 hr in the anemic cerebral cortex. Low erythrocyte aggregation might trigger endothelium-dependent thrombogenic and inflammatory responses during acute isovolemic hemodilution.[28] Moreover, serum levels of vWF may be a good indicator of

cerebral endothelial injury and poor outcome following cerebral injury.[29,30] Finally, our microarray data showed a significant up-regulation of S100A9, a marker of organ injury at 6 and 24 hr.[31,32] However, this pro-inflammatory molecule did not increase to the same degree as the Anesthesia Control at 24 hr. Clearly, further detailed investigations are required to better understand the roles of numerous genes with novel and complex expression patterns in the cerebral cortex following acute hemodilution.

Genes involved in angiogenesis

Previous experimental studies have provided evidence of VEGF up-regulation,[12,33] angiogenesis, and increased capillary density in anemic animals and humans.[33–35] Our previous studies have demonstrated evidence of increased vascular endothelial growth factor (VEGF) RNA and protein in brain homogenates from anemic rats, suggesting that angiogenesis may be acutely activated. The current microarray data demonstrated that angiopoietin 2 (angiogenic factor) increased progressively after 6 and 24 hr of anemia. Up-regulation of angiopoietin 2, thrombospondin 1, and other angiogenic genes provides additional evidence that angiogenesis may have been activated in the cerebral cortex of anemic rats. Angiopoietin 2 interacts with VEGF to promote new blood vessel formation. In our study, cerebral VEGF levels were significantly elevated, but at a magnitude below our last detection filter (1.8-fold increase). The concomitant increase in VEGF and angiopoietin 2 gene expression in the anemic cerebral cortex may play a role in mediating the increase in capillary density observed during anemia and hypoxia exposure.[35,36] Conversely, angiopoietin 2 up-regulation may play a maladaptive role when expressed alone by promoting apoptosis or increasing permeability of the blood brain barrier.[37] In addition, there is growing evidence that brain chemokines play crucial roles in the neuro-glio-vascular interaction,[38] ischemic injury and repair,[39,40] and angiogenesis.[41] In the current study, one angiogenic (CXCL1) and three angiostatic CXCs (CKCL9-11) were dramatically up-regulated by anemia at 6 hr but not at 24 hr. Further investigations will be required to determine the effect of this response on angiogenesis during anemia.

Genes involved in organ protection

Our microarray results showed the up-regulation of three organ protective genes, i.e., cyclin-dependent kinase inhibitor 1A (CDKN1A) and metallothionein 1a at 6 hr and annexin II at 24 hr. Annexin II plays a role in promoting glial cell survival and protects neurons and glial cells against hypoxia injury.[42] Metallothioneins (MTs) are small cystein-rich proteins involved in cytoprotection during pathology. Metallothionein isoforms I and II are important for host defense responses, immunoregulation, cell survival, and brain repair.[43–45] Consistent with this notion, experiments using genetically altered mice have suggested that MT-I + II are essential for wound healing and neuronal survival after brain injury secondary to trauma, hypoxia, and ischemia.[46–49] The mechanisms underlying MT's protective effect may relate to a reduction in the inflammatory response associated with central nervous system (CNS) injury.[50,51] It is plausible that the profound up-regulation of MT in the cerebral cortex of anemic rats may play an anti-inflammatory neuroprotective role. We also observed a significant up-regulation of CDKN1A and lipocalin 2 (also named neutrophil gelatinase-associated lipocalin [NGAL]) in the brain of anemic rats. These molecules may be protective by facilitating DNA repair and by decreasing inflammatory response in the brain.[52,53]

The risk of false discovery

A number of lines of evidence suggest that many of our microarray findings reflect true biological responses. First, the microarray data that suggest a strong up-regulation of pro-inflammatory response are supported by our protein measurements (TNFα, IL-6, MCP-1). Second, the current microarray data supports previous RNA and protein data suggesting up-regulation of proteins involved in angiogenesis (VEGF, Ang. 2). Third, many of the genes that are up-regulated at 6 hr were not identified at 24 hr, while a few genes remained elevated at both 6 and 24 hr. Fourth, two control groups were utilized for the comparison to baseline. Fifth, a high degree of stringency was applied to the cut-off for changes in gene expression (greater than two-fold). However, despite these arguments, the possibility that some of our responses represent a degree of false discovery cannot be excluded.

There are several limitations to this study. We have analyzed three groups of rats and assessed changes in gene expression by microarray analysis. Although many novel pathways were identified, this method was not sensitive enough to pick up all identified changes in gene expression that have been previously demonstrated, i.e., inducible and neuronal nitric oxide synthase.[8,12] In addition, this method does not assess important post-transcriptional modification RNA or protein stabilization, which have been observed in the cerebral cortex of anemic rats, i.e., Hypoxia Inducible Factor-1α.[12] However, the microarray analysis did demonstrate evidence of up-regulation of novel genes that were involved with these biological mechanisms. Our protein analysis provided support for activation of inflammatory mechanisms. The lack of down-regulated genes was likely a combination of several factors: (1) The design set the cutoff at a fold change of two, which may have eliminated discovery of down-regulated genes; (2) The experimental time points may not have captured the down-regulated

genes, i.e., earlier than 6 hr or later than 24 hr; (3) The sample size was too small to detect down-regulated genes. However, further experimental studies will be required to confirm the significance of these genes using mRNA (RT-PCR) and protein analysis (Western blot).

Conclusions

This study demonstrates the potential usefulness of DNA microarray technology as an approach for generating hypotheses regarding molecular pathways associated with anemia. We focused our study on genes involved in inflammation, angiogenesis, and organ protection. We have shown changes in the expression levels of numerous genes that have not been previously associated with acute hemodilution. Most of the genes have been previously identified in models of cerebral ischemia and hypoxia and cardiac ischemia reperfusion. Further studies are necessary to characterize these genes and to identify their physiological impact after anemia.

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Conflicts of interest None declared.

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