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Hemoglobin is Expressed in Alveolar Epithelial Type II Cells

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Abstract

Hemoglobin is the main oxygen carrying heme protein in erythrocytes. In an effort to study the differential gene expression of alveolar epithelial type I and type II cells using DNA microarray technique, we found that the mRNAs of hemoglobin α - and β -chains were expressed in type II cells, but not in type I cells. The microarray data were confirmed by RT-PCR. The mRNA expression of both chains decreased when type II cells trans-differentiated into type I-like cells. Immunocyto/histochemistry revealed that hemoglobin protein was specifically localized in type II cells of a lung cell mixture and rat lung tissue. The endogenous synthesis of hemoglobin in alveolar epithelial cells suggest that hemoglobin may have unidentified functions other than oxygen transport in the lung.

Keywords

Hemoglobin; Trans-differentiation; Alveolar Epithelial Cells

Introduction

Alveolar epithelium of the lung is primarily composed of two kinds of cells: type I cells (AEC I) and type II cells (AEC II). The AEC I are large squamous cells while AEC II are cuboidal in shape [1]. Even though the number of AEC II is roughly twice as much as type I cells, AEC II occupy only a small percentage of alveolar surface area (<5%) and are mainly limited to the corners of alveoli. These cells are also functionally different. AEC I are the main epithelial component of the thin air–blood barrier. AEC II have important secretory capacities, such as the secretion of the lung surfactant, and are the progenitor cells for the terminally differentiated AEC I [2;3]. Because of the difference in the functions of these two cell types, there are a large number of genes and proteins differentially expressed in these cells and many of them have been used as markers for the specific cell type [4–6].

Hemoglobin is one of the most abundant heme-binding proteins and consists of two α - and two β -globin polypeptides, which form a heterotetramer with a heme moiety in each monomer. The primary function of hemoglobin has been attributed to oxygen binding and transport in the blood circulation. Other heme binding proteins like cytochromes, extracellular peroxidases, and cytoglobin, neuroglobin, and myoglobin have roles in catalysis, oxygen consumption, cytoprotection and oxygen sensing [7–10]. In this paper, we report, for the first time, the expression of hemoglobin in AEC II of the lung. This finding is novel in the way that no other known cell population, other than erythrocytes and its progenitors, was reported to express this protein under normal physiological conditions. Further functional studies in this direction may uncover unidentified roles of hemoglobin in the alveolar epithelium.

Materials and Methods

Isolation and culture of AEC II

AEC II were isolated from pathogen free male Sprague-Dawley rats (250–275g) by the improved method as previously described by us [11]. In brief, the perfused lungs were lavaged and digested with elastase (3 units/ml, Worthington Biochemical Corporation, Lakewood, NJ). The cell mixture was filtered through 160- and 37- μ m nylon mesh once, and 15- μ m nylon mesh twice, and plated on rat IgG-coated plates to remove macrophages. The unattached cells from IgG plates were further incubated with anti-Leukocyte Common Antigen (anti-LC, 40 μ g/ml) (Accurate, Westbury, NY), rat IgG (70 μ g/ml), and anti-T1 α antibodies for 30 minutes at 4° C. The cells were then incubated for 20 min with anti-rat and anti-mouse IgG-conjugated magnetic beads (DynaL Biotech, Lake success, NY). A magnetic field was applied to remove the cells attached to the magnetic beads. The purity of AEC II was >96 as determined by modified Papanicolaou staining and the viability was >98%. Freshly isolated cells were directly used for DNA microarray analysis and RT-PCR. AEC II were also seeded onto 35 mm tissue culture-treated plastic dishes at a density of 1.3×10^6 cells per dishes in Minimum Essential Medium (MEM) with 10 % fetal bovine serum and cultured for 1 to 7 days. In this culture system, the trans-differentiation of AEC II to AEC I-like cells becomes evident from day 3 onward and was nearly complete by day 5.

Isolation of AEC I

AEC I was isolated according to our recently published protocol [11]. The perfused lungs were digested three times with 4.5 U/ml elastase at 37°C for 10 minutes. After being filtered through 160- and 37- μ m nylon mesh, the resulting cell mixture was panned on a rat IgG-coated bacteriological Petri dish for 30 min at 37°C. The cells were then incubated with rat IgG (40 μ g/ml) and anti-LC (40 μ g/ml), followed by the incubation with sheep anti-rat IgG Dynabeads (100 μ l/rat) and goat anti-mouse IgG Dynabeads (100 μ l/rat) at 4°C for 20 min. The beads were removed and the cells were further incubated with rabbit anti-rat T1 α antibodies (40 μ g/ml) at 4°C for 40 min. AEC I were collected by the incubation with goat anti-rabbit IgG BioMag beads. The purity of AEC I was >90% and the viability was >95%.

Isolation of lung cell mixture

The procedure was the same as for the isolation of AEC I. The cell mixture was collected after filtering and cytospinned onto microscopic glass slides. The cells were fixed with 4% paraformaldehyde and used for immunocytochemistry.

Microarray analysis

The total RNAs from isolated AEC II and AEC I were extracted using TRI reagents (Molecular Research Center, Cincinnati, OH) and used for cDNA synthesis and 2-step microarray hybridization with 3DNA 50 Expression kit (Genisphere Inc., Hatfield, PA). Total RNA was reverse-transcribed with Cy3- or Alexa 647-specific primers. The cDNA, thus obtained, was purified with the Microcom YM-30 columns (Millipore, Billerica, MA) and mixed with 2x formamide hybridization buffer (50% formamide, 6x SSC, 0.2% SDS) for hybridization at 42° C for 48 hours. The slides were washed and incubated with Cy3- and Alexa 647-specific capture reagents at 42°C for 2 hours. The slide was scanned by a laser confocal scanner, ScanArray Express (PerkinElmer Life and Analytical Sciences, Boston, MA). Hybridization images were analyzed using GenePix pro 4 software (Axon Instruments, Inc. Union City, CA). Data analysis were performed using our in-house software Realspot [12] and SAM package. The details of microarray experiments and the whole data set will be published elsewhere (Chen et al.).

Reverse Transcription- Polymerase Chain Reaction

PCR primers were as follows: the β chain of hemoglobin, Forward, 5'-TGTGACAAGCTGCATGTGGAT-3', Reverse, 5'-TGACCATTGCACAAAGACAAGA-3', α -chain of hemoglobin, Forward, 5'-CCACTCTGAGCGACCTGCAT-3', Reverse, 5'-GGTGCTCACAGAGGCAAGGA-3', 18S RNA, forward, 5'-TCCCAGTAAGTGCGGGTCATA-3', Reverse, 5'-CGAGGGCCTCACTAAACCATC-3'. One microgram of total RNA was reverse-transcribed into cDNA using Super script II (Invitrogen, Carlsbad, CA). The PCR was performed using the following conditions: 96° for 5 minutes, followed by denaturation at 94° for 30 seconds, annealing at 60° for 30 seconds, and extension at 72° for 30 seconds. The PCR product was then visualized by agarose gel electrophoresis.

Immunocytochemistry and Immunohistochemistry

Paraffin embedded tissue sections of perfused rat lung were dewaxed using xylene, rehydrated using descending grades of ethanol, and washed in phosphate buffer saline (pH 7.4). Antigen retrieval was done by boiling the slides for 15 minutes in 20 mM citrate buffer (pH 6.0). The subsequent steps were the same for both immunocytochemistry and immunohistochemistry. Briefly, the fixed tissues or cytopspinned cells were permeabilized with 0.4% Triton X-100 for 20 minutes and blocked for one hour in 10% fetal bovine serum. The slides were then incubated with mouse anti-LB-180 (1:200 dilution) (Covance, Richmond, CA) and rabbit anti-rat hemoglobin (1:300 dilution) (Biogenesis, Poole, England) antibodies for overnight at 4°C. The slides were then washed and incubated with Alexa 568-conjugated anti-rabbit and Alexa 488-conjugated anti-mouse secondary antibodies (Jackson Immuno Res, PA). Slides were then washed and mounted on an antifade medium (5% n-propyl gallate and 80% glycerol in PBS) and viewed through a Nikon Eclipse E600 fluorescence microscope.

Results

Hemoglobin was highly expressed in AEC II and not in AEC I

Using our in-house 10K rat gene DNA microarray, we performed gene profiling of freshly isolated AEC I and AEC II. Two of the genes with the highest fold change between AEC II and AEC I were α -chain (NM_013096) and β -chain (NM_033234) of hemoglobin. The ratios between AEC II and AEC I were 13.8 ± 1.2 and 7.4 ± 1.2 , respectively (means $\pm$ SE, n=30) (Fig. 1A). Two other hemoglobin probes (X56327 and M32509) in the 10K set also showed a similar change (9.3 ± 1.2 and 9.1 ± 1.2). The M32509 probe corresponds to the β -chain mRNA 3' end and has a sequence similarity of 96 % to the 3' end of NM_033234. The X56327 probe represents epsilon 2 globin gene. Three related heme proteins, cytoglobin [13;14], neuroglobin [9;13], and myoglobin [10] were not expressed in both cell types in our microarray data set. RT-PCR analysis showed that the mRNAs of hemoglobin α - and β -chains were highly expressed in AEC II, but was not detectable in AEC I (Fig. 1B), validating the microarray data.

The mRNA expression of hemoglobin decreased as AEC II trans-differentiate to AEC I-like cells

Although the purity of AEC II was >96%, the possibility still exists regarding contaminations in our cell preparations. We further examined the mRNA expression of hemoglobin during the trans-differentiation of AEC II to AEC I-like cells. When being cultured on tissue culture treated plastic dishes, AEC II gradually converted to AEC I-like cells. The latter had a similar cell shape as AEC I and express AEC I markers, but lacked AEC II markers. By day 2, the trans-differentiation began and was completed by day 5 to day 7, depending on culture conditions. The cells were collected on Day 0, 1, 3, and 5 and total RNA extracted. RT-PCR

analysis revealed that both chains of hemoglobin were expressed on day 0 and gradually decreased as the trans-differentiation initiated (Fig. 2).

Hemoglobin is specially expressed in AEC II of a lung mixture cell population

To further examine the expression of hemoglobin at the protein level in AEC II, we isolated a population of mixed cells from the rat lung as described in the Materials and Methods, and cytospinned onto microscopic glass slides. This mixture of cells consisted of AEC I, AEC II, Clara cells, ciliated airway epithelial cells, fibroblasts, macrophages, and lymphocytes. LB-180, an AEC II marker, was used to identify AEC II in the mixture. The double labeling with anti-hemoglobin and anti-LB-180 antibodies revealed the co-localization of hemoglobin with LB-180 in AEC II, but not in other cells (Fig. 3a–d). The omission of primary antibodies did not generate any signals (Fig. 3e–h).

Hemoglobin is localized in AEC II of the lung

The gene and protein expression could be potentially affected by cell isolation procedures. To avoid this problem, we directly immunostained rat lung tissue. The rat lung was perfused to remove red blood cells before fixation and sectioning. Once again, LB-180 was used as an AEC II marker. The results revealed that hemoglobin was localized in the corners of the alveoli, which were occupied by AEC II. The hemoglobin staining pattern was the same as LB-180 (Fig. 4a–h). The control without primary antibodies did not have signals (Fig. 4i–l). The result indicated that hemoglobin protein was localized only in AEC II, but not in AEC I and other lung cell types in the normal rat lung. The endothelial cells did not show any staining for hemoglobin while the airway epithelium showed faint positive staining (data not shown).

Discussion

Aside from its most important and well-known function, oxygen transport, hemoglobin also has a variety of other functions. These functions include being a molecular heat transducer by virtue of its oxygenation-deoxygenation cycle, the alteration of red blood cell metabolism, hemoglobin oxidation, enzymatic activities, and drug interactions [7]. Since oxygen can be a damaging agent to the cells exposed to it, the oxygen binding hemoglobins may have a role in the protection of cells from the damage [7]. Hemoglobin is also thought to be involved in the protection of cells against nitrosative stress [15;16]. Recent studies have shown that hemoglobin can bind and release NO as a redox reaction, thus functioning in close resemblance to cytochromes [17]. Neuroglobin and cytoglobin, two other members of the vertebrate globin family, also have protective functions including oxygen sensing and scavenging [9;13;14]. Neuroglobin is predominantly expressed in the nervous system while cytoglobin is present in almost all tissues. Even though their amino acid and gene sequences are distinct from each other, their functional structure has striking similarities. The related globins in fungi and bacteria are involved in electron transport and protection from oxidative stress [7].

A long standing notion that hemoglobin gene can only be expressed in the cells of erythroid lineage has been challenged by a recent study, in which the treatment with lipopolysaccharide and interferon- γ led to the activation of the β globin gene in murine macrophages [18]. In the current study, we provide evidence that hemoglobin was specially expressed in AEC II of the lung. Since AEC II are in close proximity to environmental air, blood circulation, and facing the conditions of altered gaseous environment around it, our finding may have an important functional significance. We speculated the following possible functions of hemoglobin in the lung: (i) facilitate oxygen transport across the air-blood barrier; (ii) behave as an oxygen sensor; and (iii) function as oxygen or nitric oxide scavenger and thus protect alveolar epithelium from oxidative/nitrosative stress. Further studies are needed to uncover un-identified functions of hemoglobin in the lung.

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Fig. 1.

The mRNA expression of hemoglobin in alveolar epithelial cells. (A) DNA microarray. The ratios of AEC II and AEC I were from 10K rat gene DNA microarray hybridizations and are presented as means $\pm$ SE (n=30, 5 biological and 6 technical replications). Two probes from the β chains of hemoglobin (NM_033234, and M32509), one from epsilon 2 (X56327), and one from α chain (NM_013096) were shown. (B) RT-PCR. The total RNA from 3 independent AEC II and AEC I preparations were reversed transcribed to cDNA. The α - and β -chain of hemoglobin PCR-amplified for 30 cycles. 18S rRNA was used as a control.

Fig. 2.

The mRNA expression of hemoglobin during the trans-differentiation of AEC II into AEC I-like cells. Freshly isolated AEC II were seeded on plastic dishes in MEM medium containing 10 % FBS and cultured for 0 (D0), 1 day (D1), 3 days (D3), 5 days (D5), and 7 days (D7). Total mRNAs were extracted from cultured cells and RT-PCR was performed to detect α - and β -chains of hemoglobin. 18S rRNA was used as a control. Panel A, representative gels; and panel B, quantitation. The results were expressed as a percentage of control (D0). Data shown are means $\pm$ SE. * $P < 0.05$ ($n = 3$ independent cell preparations). Black bar: α -chain; dotted bar: β -chain.

Fig. 3.

Double-labeling of a lung cell mixture for LB-180 (an AEC II marker) and hemoglobin. A lung cell mixture was cytopinned onto microscopic glass slides. The slides were incubated with rabbit anti-hemoglobin and mouse anti-LB-180 antibodies, followed by incubation with Alexa 568-conjugated anti-mouse and Alexa 488-conjugated anti-rabbit IgG (Panels a–d). The negative control without primary antibodies was shown in Panels e–h. Scale bar: 5 μ m.

Fig. 4.

Double-labeling of rat lung tissue sections for LB-180 (AEC II marker) and hemoglobin. Rat lung tissue sections were incubated with rabbit anti-rat hemoglobin and mouse anti-LB -180 antibodies, followed by incubation with Alexa 568-conjugated anti-mouse or Alexa 488-conjugated anti-rabbit IgG (Panels a–h). The negative control was without primary antibodies (Panels i–l). Scale bars: 20 μm for x40 and 8 μm for 100x.