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Isolation of highly pure alveolar epithelial type I and type II cells from rat lungs

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There are no ideal cell lines available for alveolar epithelial type I and II cells (AEC I and II) at the present time. The current methods for isolating AEC I and II give limited purities. Here, we reported improved and reproducible methods for the isolation of highly pure AEC I and II from rat lungs. AEC I and II were released from lung tissues using different concentrations of elastase digestion. Macrophages and leukocytes were removed by rat IgG 'panning' and anti-rat leukocyte common antigen antibodies. For AEC II isolation, polyclonal rabbit anti-T1α (an AEC I apical membrane protein) antibodies were used to remove AEC I contamination. For AEC I isolation, positive immunomagnetic selection by polyclonal anti-T1α antibodies was used. The purities of AEC I and II were 91$\pm$4 and 97$\pm$1%, respectively. The yield per rat was $\sim 2\times10^6$ for AEC I and $\sim 33\times10^6$ for AEC II. The viabilities of these cell preparations were more than 96%. The protocol for AEC II isolation is also suitable to obtain pure AEC II (93–95%) from hyperoxia-injured and recovering lungs. The purified AEC I and II can be used for gene expression profiling and functional studies. It also offers an important tool to the field of lung biology.
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The alveolar epithelium is composed of type I and II pneumocytes (AEC I and II). AEC I and II are morphologically and functionally different. AEC I are squamous in shape, with a diameter of $\sim$50–100 μm and a volume of $\sim$2000–3000 μm^3.[1] AEC I cover $\sim$95% of the surface area of the lung and are important for gas exchange. Recent studies indicated that AEC I play active roles in water permeability and the regulation of alveolar fluid homeostasis.[2,3] AEC II are cuboidal, with a diameter of $\sim$10 μm and a volume of $\sim$450–900 μm^3.[1] AEC II occupy only $\sim$5% of the surface area. They produce, secrete, and recycle lung surfactant. AEC II can be also converted to AEC I to repair damaged epithelium after lung injury or during fetal lung development.

Given the importance of AEC I and II in lung functions, it is necessary to isolate enriched populations of AEC I and II with sufficient viability and purity for functional studies. The method for AEC II isolation developed by Dobbs *et al*[4] has been used by most investigators. However, the purity of the isolated AEC II is only 80–89%. Although those cell preparations may be appropriate for studying lung surfactant metabolism, they are not pure enough for gene expression profiling. A few reports attempted to obtain higher purities of AEC II. Weller and Karnovsky[5] reported a 90% pure AEC II from rats using Percoll gradient centrifugation, while Abraham *et al*[6] isolated 90–95% pure AEC II by using rat IgG panning and rabbit IgG coated BioMag beads. AEC I have been studied to a lesser extent. Recently, a few laboratories have isolated AEC I with limited purities or yields using AEC I-specific monoclonal antibodies produced in their laboratories. The typical purities were 60 $\sim$86%.[2,3,7]

In order to isolate a specific type of cells, reliable methods to identify the cells are needed. AEC II can be identified by the staining of lamellar bodies using the modified Papanicolaou staining[8] or a fluorescence dye, Nile red.[9] In early studies, AEC I were identified based on their morphology by electron microscope.[7] The availability of AEC I and II specific markers make it possible to identify AEC I and II by immunostaining. These cell markers include T1α,[10,11] aquaporin 5 (AQP-5)[12] and caveolin-1[13] for AEC I, and RTII 70[14], LB180[15] and surfactant proteins[16] for AEC II.

In this study, we first compared different methods for the identification of AEC I and II. We then generated polyclonal antibodies against T1α using a synthetic peptide. Utilizing this antibody, we developed improved and reproducible methods for

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the isolation of highly pure AEC I and II. Our AEC II isolation protocol is also suitable to isolate highly pure AEC II from hyperoxia–injured rat lungs. The isolated cells preserve their functions such as surfactant secretion for AEC II and the ability to form a thin monolayer for AEC I.

Materials and methods

Materials

Sprague–Dawley (SD) rats were purchased from Charles River Breeding Laboratories (Wilmington, MA, USA). Oklahoma State University Animal Use and Care Committee approved all animal surgeries used in this study. DNase I, papain, rat IgG and phorbol 12-myristate 13-acetate (PMA) were from Sigma-Aldrich (St Louis, MO, USA). DEXTRAN 40 was from Amersham Biosciences (Piscataway, NJ, USA). Nylon meshes (160, 37 and 15 μm) were from Tetko (Elmsford, NY, USA). Porcine pancreas elastase in a lyophilized form (Cat #, LS002294) was from Worthington Biochemical (Lakewood, NJ, USA). Bovine serum albumin (BSA), normal goat serum, goat anti-mouse IgG (H + L, heavy and light chains), goat anti-syrian hamster IgG (H + L) and Cy3-conjugated goat anti-mouse IgG (H + L) were from Jackson ImmunoResearch Laboratories (West Grove, PA, USA); Alex 488-conjugated donkey anti-goat IgG were from Molecular Probes (Eugene, OR, USA). Goat anti-SP-C antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal antibodies against p180 lamellar body protein (LB180) were from Berkeley antibody company (Richmond, CA, USA). Polyclonal rabbit anticaveolin-1 and anti-AQP-5 antibodies were from Transduction Laboratories (Lexington, KY, USA) and Alpha Diagnostic (San Antonio, TX, USA), respectively. Monoclonal anti-rat leukocyte common antigen antibodies (anti-LC) were from Accurate (Westbury, NY, USA). Monoclonal hamster anti-mouse T1α antibodies (8.1.1) was from Developmental Studies Hybridoma Bank (University of Iowa). Monoclonal antibodies (E11) against T1α were a kind gift from Dr Antoinette Wetterwald (University of Berne, Switzerland), and Dr Mary C Williams (Boston University). Monoclonal antibodies specific for RTII70 and RTI40 were a kind gift from Dr Leland G Dobbs (University of California, San Francisco, CA USA). Goat anti-rabbit IgG BioMag® beads were from QIAGEN (Valencia, CA, USA). CELLection™ Pan Mouse IgG kit, sheep anti-rat IgG magnetic beads and Dynabeads® M-450 Epoxy beads were from Dynal Biotech (Lake Success, NY, USA). RPMI 1640 Medium containing 25 mM HEPES buffer was from Irvine Scientific Inc. (Santa Ana, CA, USA). Fetal bovine serum (FBS) was from Life Technologies (Paisley, PA, USA). FBS was inactivated at 56°C for 1 h and stored at −20°C.

Identification of AEC I and II

AEC II were identified by the modified Papanicolaou or Nile red staining and immunocytochemistry using AEC II-specific antibodies. The modified Papanicolaou staining was performed as described by Dobbs.[8] The Nile red staining was carried out according to the method of Liu *et al*.[9] For immunocytochemistry, isolated AEC II were mounted onto glass coverslips using a cytospin apparatus, and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C overnight. The coverslips were blocked using 6% normal goat serum in 0.1 M phosphate buffer (pH 7.4) for 1 h at room temperature. To increase sensitivity, antigen retrieval was carried out by boiling the coverslips in 25 mM Tris-HCl (pH 9.5) containing 5% urea for 10 min in a microwave oven. AEC II were incubated with anti-RTII70 and anti-LB180 antibodies (1:100 dilution), followed by incubation with the secondary antibody, Cy3-conjugated anti-mouse IgG (1:200 dilution). The negative controls were treated the same as above except for the omission of the primary antibodies.

AEC I were identified by immunocytochemistry as described above using AEC I-specific antibodies: monoclonal antibodies against T1α (E11, 1:100 dilution), polyclonal rabbit anticaveolin-1 antibodies (1:200 dilution) or anti-AQP-5 antibodies (1:200 dilution), and their corresponding secondary antibodies, Cy3-conjugated anti-mouse IgG (1:200 dilution) or Alex 488-conjugated anti-rabbit IgG (1:2000 dilution).

Generation of Polyclonal Rabbit Anti-rat T1α Antibody

The antibodies against rat T1α were raised in rabbits using a synthetic peptide corresponding to 83–94 amino acids of rat T1α (H_3N- C T S D H D H K E H E S T -COOH) (Custom Service, Affinity Bio-reagents, Golden, CO, USA). The T1α peptide used was selected based on software predictions for solvent accessibility, hydrophilicity, antigenicity, post-translational modification sites, and sequence uniqueness (BLAST search). This antibody was purified by affinity chromatography and used for AEC I and isolation. The specificity of the antibodies was tested by Western blot.

Dynabead Conjugation

Goat anti-mouse IgG were conjugated to Dynabeads according to the manufacturer's instructions. Briefly, Dynabeads, M-450 Epoxy, were washed and resuspended in 0.1 M phosphate buffer (pH 7.4). In all, 10 μg of antibodies were added to 1×10^7 Dynabeads ($4\text{-}8 \times 10^8$ beads/ml) and mixed well. The mixture was incubated for 30 min at 4°C by end-to-end rotation. To block the remaining active sites, an

equal volume of 0.2% BSA in 0.1 M phosphate buffer (pH 7.4) was added, and the mixture was incubated for 16 h at 4°C. The conjugated beads were stored at 4°C before use.

Isolation of AEC II

The lungs from male SD rats (200–250 g) were perfused at 37°C with solution A (0.9% NaCl, 0.1% glucose, 30 mM HEPES, 6 mM KCl, 0.1 mg/ml streptomycin sulfate, 0.07 mg/ml penicillin G, 0.07 mg/ml EGTA, 3 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH 7.4). The lungs were then lavaged eight to 10 times at 37°C using solution B (solution A plus 3 mM $MgSO_4$ and 1.5 mM $CaCl_2$). The lungs were digested by instilling 7–8 ml elastase (3 U/ml in solution A) at 37°C and incubating for 12–14 min. This process was repeated two times. After chopping with a McIlwain tissue chopper (Brinkmann, Westbury, NY, USA) for two to three times, the cell suspension was mixed with 100 μg/ml DNase I, incubated for 5 min at 37°C with gentle rotation, and filtered through 160 and 37-μm nylon mesh once, and 15-μm nylon mesh twice. The cells were incubated in two rat IgG-coated polystyrene bacteriological 100 mm Petri dishes (1.5 mg rat IgG/dish) sequentially at 37°C, 30 min each. The unattached cells were centrifuged at 250 g for 8 min and resuspended with 10 ml solution C (RPMI 1640 Medium containing 25 mM HEPES, 1% FBS and 100 μg/ml DNase I) at a concentration of $10 \sim 20 \times 10^6$ cells/ml. To remove the remaining macrophages, the cells were incubated with rat IgG (40 μg/ml) at room temperature for 15 min with gentle rotation. After being washed twice with solution C, the cells were incubated with sheep anti-rat IgG magnetic beads (100 μl/rat) for 15 min at 4°C. The beads were removed by a magnetic device. To remove leukocyte and AEC I contaminations, the cells were incubated with anti-LC (40 μg/ml) and rabbit anti-rat T1α (40 μg/ml) at 4°C for 40 min, followed by incubation with goat anti-mouse IgG Dynabeads (100 μl/rat) and goat anti-rabbit IgG BioMag® beads (500 μl/rat). After removing the beads, the resultant cells were used for the evaluation of cell yield, viability, and purity as well as surfactant secretion.

To isolate AEC II from hyperoxia-injured and recovering lungs, rats ($\sim$250 g) were exposed to $>$95% O_2 in a chamber for 48 h as previously described[17] and allowed to recover in normal air for 0, 1, 3, 5, and 7 days. AEC II were isolated from these rats as described above.

Isolation of AEC I From Rat Lungs

Lungs from male SD rats (250–300 g) were perfused via the pulmonary artery with RPMI 1640 medium containing 25 mM HEPES, pH 7.2 (solution D) at 37°C. Lungs were lavaged at 37°C with phosphate-buffered saline (PBS, pH 7.4) containing 5 mM EGTA and 5 mM EDTA. Then, they were instilled with 10 ml solution D containing 10% DEXTRAN 40 and 4.5 U/ml elastase at 37°C for three times, 10 min each, removing the solution outside the lung prior to each addition of elastase. Following elastase digestion, the lung tissue was dissected in solution D containing 20% FBS and 100 μg/ml DNase I and chopped twice. The lung fragments were gently agitated for 2 min at 37°C and filtered once through 160 and 37-μm nylon mesh. The resultant cell suspension was centrifuged for 12 min at 250 g and resuspended in solution D. After panning on a rat IgG-coated bacteriological Petri dish (1.5 mg/dish) for 30 min at 37°C, the cells were further incubated with rat IgG (40 μg/ml) and anti-LC (40 μg/ml) at room temperature for 15 min with end-to-end rotation. The cells were washed twice with solution C, followed by the incubation with sheep anti-rat IgG Dynabeads (100 μl/rat) and goat anti-mouse IgG Dynabeads (100 μl/rat) at 4°C for 20 min. After removing beads, the cells were then incubated with rabbit anti-rat T1α antibodies (40 μg/ml) at 4°C for 40 min and washed three times with solution C. AEC I were separated from contaminated cells by the incubation with goat anti-rabbit IgG BioMag® beads (500 μl beads per rat) at 4°C for 15 min. The AEC I were released from the beads either by incubation with 50 μg/ml papain at 37°C for 1 h, or by culturing cells in MEM containing 10% FBS for 16–20 h. The isolated cells were used for the evaluation of cell yield, viability, and purity.

For comparison purposes, two monoclonal anti-T1α antibodies: mouse anti-rat T1α antibodies (E11) and hamster anti-mouse T1α (8.1.1) were used to replace rabbit anti-rat T1α antibodies. For E11 antibodies, we used goat anti-mouse IgG Dynabeads and Cellection™ Pan anti-mouse IgG kit. After cells were incubated with E11 antibodies, unattached antibodies were washed away by solution C without DNase I. For 8.8.1 antibodies, we used goat anti-hamster IgG Dynabeads. In another experiment, anti-RTII70 antibodies were used to remove AEC II contamination during the leukocyte removing step.

Assessment of Cell Viability and Purity

The cell viability was determined by trypan blue dye exclusion. AEC II purity was determined by the modified Papanicolaou staining or immunocytochemistry with monoclonal anti-LB180 antibodies. AEC I was determined by immunostaining with monoclonal anti-T1α (E11) or rabbit anticaveolin-1 antibodies. At least 500 cells were counted for purity evaluation. In some cases, the cells were double-labeled using anti-SP-C antibodies/Alex 488 anti-goat IgG and anti-T1α (E11) antibodies/Cy3 anti-mouse IgG.

Electron Microscopy Studies

AEC I and II were fixed in 1% paraformaldehyde in 0.1 M. sodium phosphate buffer (pH 7.4) overnight at 4°C. The cell pellets were washed three times in 0.1 M sodium phosphate buffer, 20 min each. Then, the cells were placed into 1% aqueous osmium overnight at 4°C. After being washed with water three times (20 min each), the cells were placed into 2% sodium malicate buffered uranyl acetate at 4°C for 2 h, washed briefly with water, and dehydrated through graded acetone series (30, 50, 70, 90, 95, and 100%) on ice for 10 min each. Then, the cells were put into 1:1 100% acetone: regular polybed solution capped under a fume hood. After a 3-day treatment, the cap was removed and the cells exposed outside under a fume hood overnight. Finally, the cells were embedded in fresh polybed, polymerized in a 60°C oven for 48 h, and examined in a JEOL JEM 100 CXII transmission electron microscope.

Surfactant Secretion Assay

The secretion of lung surfactant from isolated AEC II was performed as previously described.[9] Briefly, AEC II were labeled with [^{3}H]choline overnight. After being washed, the cells were preincubated in 1 ml of MEM for 30 min. The secretagogues (0.1 mM ATP, 0.1 μM PMA, and 10 μM terbutaline) were added and culture continued for 2 h. The lipids in the media and cells were extracted with chloroform–methanol. The secretion was calculated as (dpm in medium/dpm in medium + dpm in cells) $\times$ 100%.

Results

Identification of AEC I and II

In order to identify AEC I and II, we first compared different methods for the identification of these cells. As shown in Figure 1, all methods showed specific staining. However, we recommend the modified Papanicolaou staining for AEC II identification (Figure 1A1 and A2) because it was highly specific and relatively easy and fast. Nile red, a fluorescence dye, stains lamellar bodies in AEC II (Figure 1A3 and A4), but this staining needs the optimization of dye concentrations and is not always reproducible. Immunostaining using anti-LB180 (Figure 1A5 and A6) and anti-RTII70

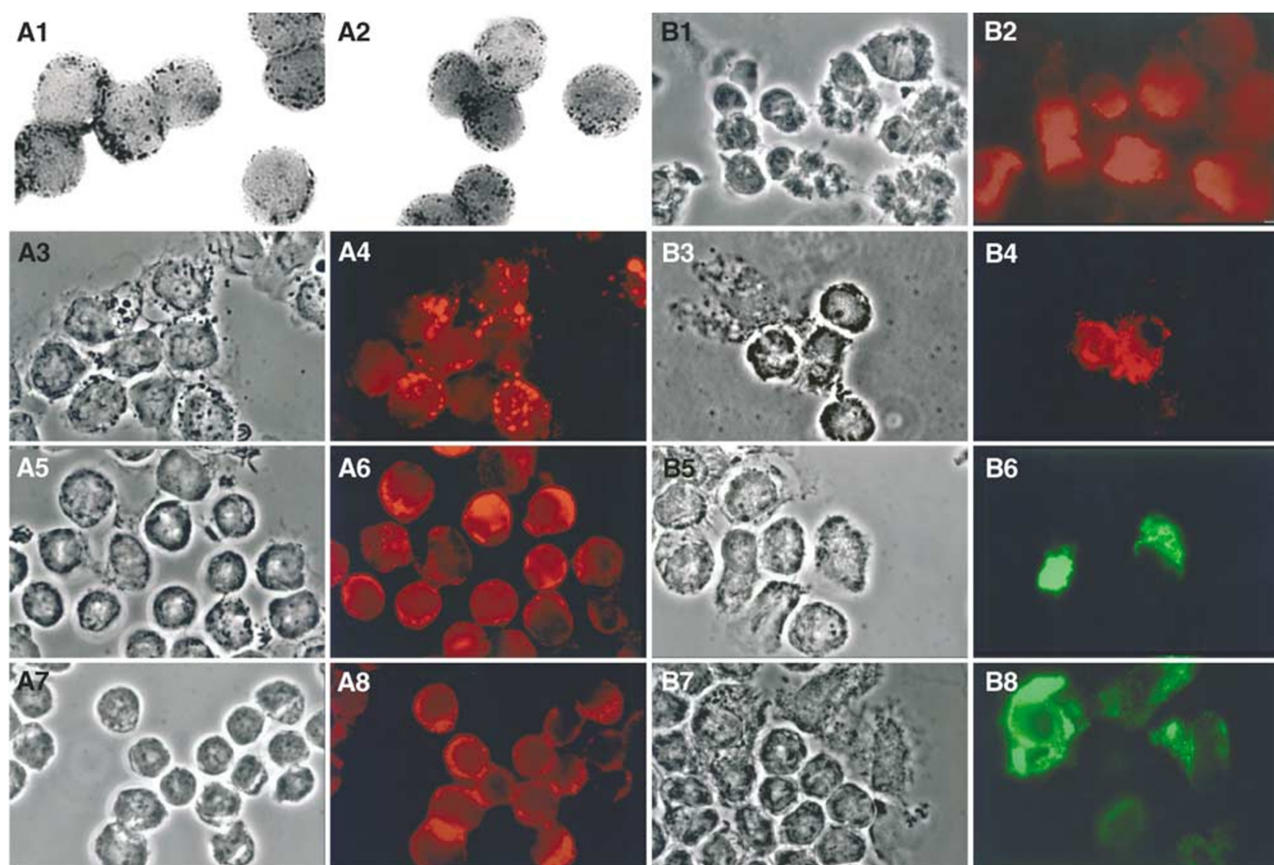

Figure 1 AEC I and II identification. A mixed cell preparations or purified AEC I and II were mounted onto glass coverslips using a cytospin apparatus and used for immunocytochemistry, the modified Papanicolaou and Nile red staining. Panels A1 and A2: the modified Papanicolaou staining to show two different field of the same slide; Panels A3 and A4: the Nile red staining; Panels A5 and A6: LB180 antibodies; Panels A7 and A8: anti-RTII70 antibodies; Panels B1 and B2: E11 antibodies; Panels B3 and B4: anti-RTI40 antibodies; Panels B5 and B6: anticaveolin-1 antibodies; Panels B7 and B8: anti-AQP-5 antibodies. Panels A3, A5, A7, B1, B3, B5, and B7 are phase contrast images; Panels A4, A6, A8, B2, B4, B6, and B8 show fluorescence images. Scale bar, 10 μm.

antibodies (Figure 1A7 and A8) was AEC II specific, but it took a relatively longer time.

AEC I can be identified by immunocytochemistry with AEC I-specific antibodies including monoclonal antibodies against T1α (E11, Figure 1B1 and B2 or RTI40, Figure 1B3 and B4), rabbit polyclonal antibodies against caveolin-1 (Figure 1B5 and B6) or AQP-5 (Figure 1B7 and B8). The E11 and RTI40 antibodies recognize the same protein, T1α. The T1α is an apical membrane protein of AEC I and has a short hybrophobic transmembrane domain, a short cytoplasmic tail and a large extracellular domain.[18] Caveolin-1 is an integral membrane protein located in the cytoplasmic side and AQP-5 is a water channel and has six transmembrane domains and relatively a small portion of extracellular domain. Both are highly expressed in AEC I.[18]

Yield, Viability and Purity of Isolated AEC II

Since most of AEC I–specific antibodies are not commercially available or are expensive to purchase for the cell isolation purpose, we generated polyclonal antibodies against T1α using a synthetic peptide. This antibody named rabbit anti-rat T1α specifically recognizes a ~40 kDa protein on the plasma membrane of lungs (Figure 2). Using this antibody, we developed a reproducible method for AEC II isolation. The purity of AEC II during various stages of isolation was as follows: after elastase digestion, 50%; after rat IgG 'panning', 85%; after the negative selection with anti-rat IgG beads and anti-LC, the purity was 90 and 93%, respectively. The purity and viability of the final isolated AEC II preparations was 96.50±1.39% and 98.74±0.52 from normal rats, respectively (Table 1). The contamination by AEC I was less than 1% as determined by double labeling with polyclonal goat anti-SP-C and monoclonal mouse anti-T1α (E11) antibodies (Figure 3). Macrophages and lymphocytes accounted for less than 0.5% as evaluated by Giemsa staining (data not shown). The rest of the contaminated cells may be Clara cells because their sizes were similar to AEC II, but no lamellar bodies can be seen after the modified Papanicolaou staining.

When the rabbit anti-rat T1α antibodies were replaced by two monoclonal anti-T1α antibodies, E11 and hamster anti-mouse T1α, similar yields and purities of AEC II were obtained. Furthermore, our protocol was also suitable to isolate AEC II from the injured lungs. The purities of AEC II isolated from hyperoxia-injured and recovering lungs was 93–95%, the yields 18–40 × 10^6, and the viability 92–94% per rat (Table 2).

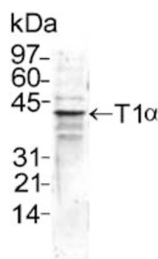

Figure 2 Specificity of rabbit anti-rat T1α antibodies. In all 40 μg of plasma membrane protein from lung tissue were resolved on a 10% SDS-PAGE gel, transferred onto nitrocellulose paper, and probed with rabbit anti-rat T1α antibodies. The arrow points to the T1α band.

Yield, Viability and Purity of the Isolated AEC I

In order to isolate AEC I, the elastase concentration was increased from 3 U/ml for the isolation of AEC II to 4.5 U/ml. to release AEC I. Since AEC II (~50%) exist in the cell mixture, positive immunomagnetic selection was chosen to isolate AEC I using rabbit anti-rat T1α antibodies. The purity of the AEC I preparations was 91.17±3.83%, the yield ~2.0 × 10^6, and the viability 96.46±1.65% per rat (Table 3). The contamination by AEC II was 1–2% as determined by double labeling with polyclonal goat anti-SP-C and monoclonal mouse anti-T1α (E11) antibodies (Figure 3). Macrophages and lymphocytes accounted for less than 0.5% as evaluated by Giemsa staining (data not shown). Most contaminations in AEC I preparations was not identifiable. A similar purity of AEC I was obtained using E11 antibodies. Inclusion of a procedure for a negative selection to remove AEC II by anti-RTII70 antibodies only slightly improved the purity (Table 3). Hamster anti-mouse T1α antibodies only produced ~40–50% purity of AEC I preparations, probably because of their low affinity for rat AEC I.

Table 1 Yield, purity and viability of AEC II isolated from rat lungs using different anti-T1α antibodies

Antibody	n	*Yield (× 10^6)*	*Purity (%)*	*Viability (%)*
Rabbit anti-rat T1α	5	33.88±5.64	96.50±1.39	98.74±0.52
E11	5	32.14±9.00	96.80±0.94	98.80±0.45
Hamster anti-mouse T1α	5	33.00±4.24	96.50±2.12	94.50±3.54

The purity of these AEC II preparations was determined by the modified Papanicolaou staining. The numbers represent mean ±s.d.

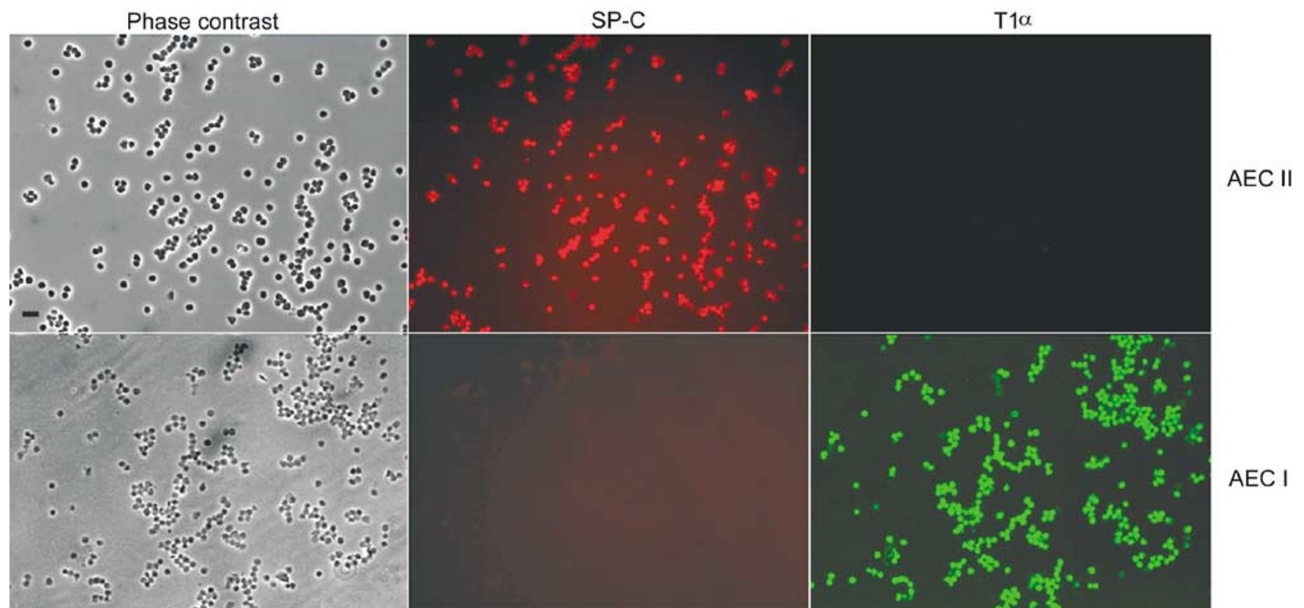

Figure 3 Purities of type I and II cells. Cytocentrifuged AEC II and I preparations were double-labeled with anti-SP-C antibodies/Alex 488 anti-goat IgG and anti-T1α (E11) antibodies/Cy3 anti-mouse IgG. Upper panels, AEC II; Lower panels, AEC I. Scale bar 20 μm.

Table 2 Yield, purity, and viability of freshly isolated AEC II from hyperoxia-injured and recovering lungs

Recovery days	N	*Yield ($\times 10^6$)*	*Purity (%)*	*Viability (%)*
0	4	28.65±6.46	95.00±0.96	93.50±0.58
1	4	40.00±4.08	94.00±2.06	92.00±2.31
3	4	18.13±6.29	94.75±0.50	93.50±3.51
5	4	29.50±7.00	93.00±1.15	93.75±1.26
7	4	28.20±7.32	94.25±1.50	93.25±1.71

Rats were exposed to >95% O_2 for 48 h and allowed to recover in normal air for 0, 1, 3, 5, and 7 days. Rabbit anti-rat T1α antibodies or E11 were used to remove AEC I. The purity of these AEC II preparations was determined by the modified Papanicolaou staining. The numbers represent mean ±s.d.

Table 3 Yield, purity and viability of AEC I isolated from rat lungs using different antibodies

Antibody	N	*Yield ($\times 10^6$)*	*Purity (%)*	*Viability (%)*
Rabbit anti-rat T1α	7	2.02±0.91	91.17±3.83	96.46±1.65
E11+anti-RTII70	4	1.64±0.74	90.75±0.96	95.62±1.19
E11 only	3	1.87±0.64	88.60±1.62	97.00±1.00
Hamster anti-mouse T1α	3	0.17±0.04	45.00±7.07	94.50±2.12

The purity of these AEC I preparations was determined by immunocytochemistry with AEC I-specific antibodies, E11. The numbers represent mean ±s.d.

Morphology and Functional Studies of Isolated AEC I and II

Transmission electron microscopy analysis revealed that the isolated AEC II had typical features of AEC II in *in vivo* lungs including lamellar bodies and microvilli (Figure 4a). The image also captured a lamellar body that was undergoing exocytosis. The isolated AEC I showed small nuclei and thin cytoplasmic extensions (Figure 4b), consistent with previous observations.[7,19] To determine whether the isolated AECs preserve their cell functions, we assessed surfactant secretion from AEC II and the ability of AEC I to form a thin monolayer. As shown in Figure 5, surfactant secretion from AEC II was enhanced ~five fold in the presence of secretagogues, indicating a functional AEC II. When they were cultured on the plastic dishes for 5 days, AEC I formed a monolayer (Figure 6a), suggesting that the isolated AEC I can be cultured *in vitro*. This is not because of overgrowth of possible contaminations such as macrophages or fibroblasts. Because these cultured cells can be stained with the AEC I-specific cell marker, caveolin 1 (Figure 6b). A similar result

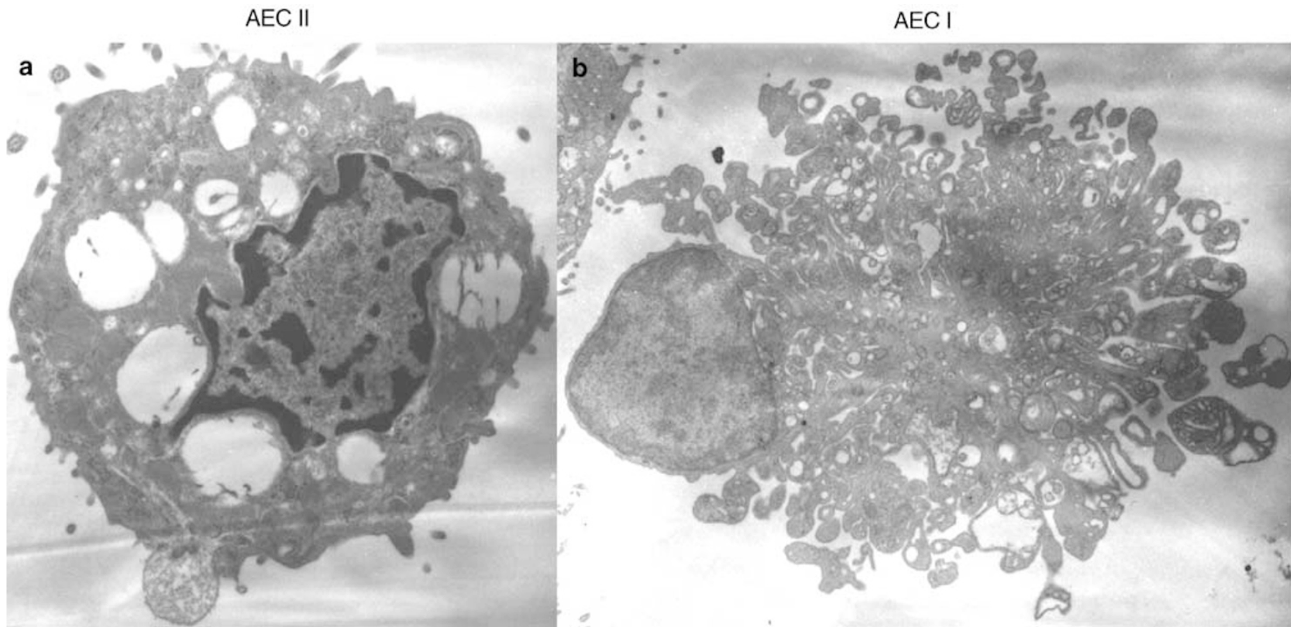

Figure 4 Electron microscopy images of AEC I and II. Original magnifications: (**a**) AEC II × 7200; (**b**) AEC I × 4800.

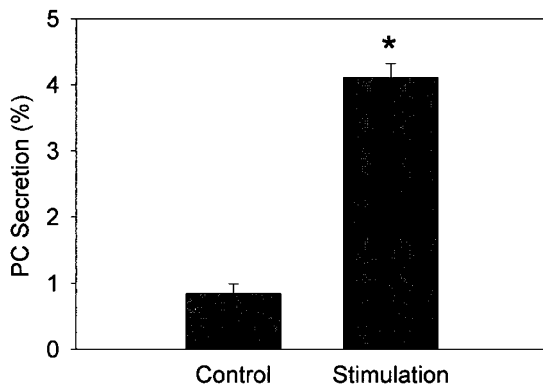

Figure 5 Surfactant Secretion from isolated AEC II. [^{3}H]choline-labeled and overnight-cultured AEC II were stimulated without (control) or with 0.1 mM ATP, 0.1 μM PMA, and 10 μM terbutalin (stimulation) for 2 h. The phosphatidylcholine secretion was expressed as (dpm in medium/dpm in medium and dpm in cells) × 100%. *$P<0.001$ *vs* control.

was obtained with anti-T1α antibodies (data not shown). The viability of the 5-day cultured AEC I was >98% as determined by trypan blue exclusion.

Discussion

In this study, improved and reproducible methods for the isolation of highly pure AEC I and II were developed using rabbit anti-rat T1α antibodies. The purities for AEC I and II were significantly improved compared to the current methods. This is the first report to use polyclonal anti-T1α antibodies for lung pneumocyte isolation. In addition, different antibodies against T1α protein (rabbit anti-rat T1α, E11, and hamster anti-mouse T1α) were also compared for AEC I and AEC II isolation. Although three of the T1α antibodies were suitable to remove AEC I by negative selection during the isolation of AEC II, the polyclonal rabbit anti-T1α antibodies were found to be most efficient in the isolation of AEC I by positive selection. Although rat T1α and mouse T1α share a 78% amino-acid homology in the coding region,[20] the use of hamster anti-mouse T1α antibodies resulted in only ~45% purity of AEC I (Table 3). This may be due to the low affinity of hamster anti-mouse T1α antibodies to rat T1α.

The main contaminations during AEC II isolation are red blood cells, leukocytes, and AEC I. Several steps were used to ensure the removal of these cells: (i) lung perfusion was critical to remove blood cells; (ii) different concentrations of elastase digestion were used to selectively release AEC I and II from the tissue; (iii) alveolar macrophages were removed by endobronchial lavage; (iv) most leukocytes were removed by two rat IgG pannings and magnetic bead selection with rat IgG and anti-LC antibodies; (v) finally, AEC I were removed by anti-T1α antibodies. In an early study, Weller and Karnosky[5] used anti-LC 'panning' to remove leukocytes However, we found that it is less efficient compared to the magnetic bead selection used in our protocol. The contamination by macrophages and lymphocytes in our AEC II preparations was less than 0.5%, but Weller and Karnosky[5] reported 2% macrophages and lymphocytes in their AEC II preparations. AEC I contamination was less than 1%. The rest of the contaminated cells may be Clara cells. Low AEC I contaminations in our AEC II preparations may be due to low concentration of elastase for selective digestion of AEC II and anti-T1α antibodies removal of AEC I.

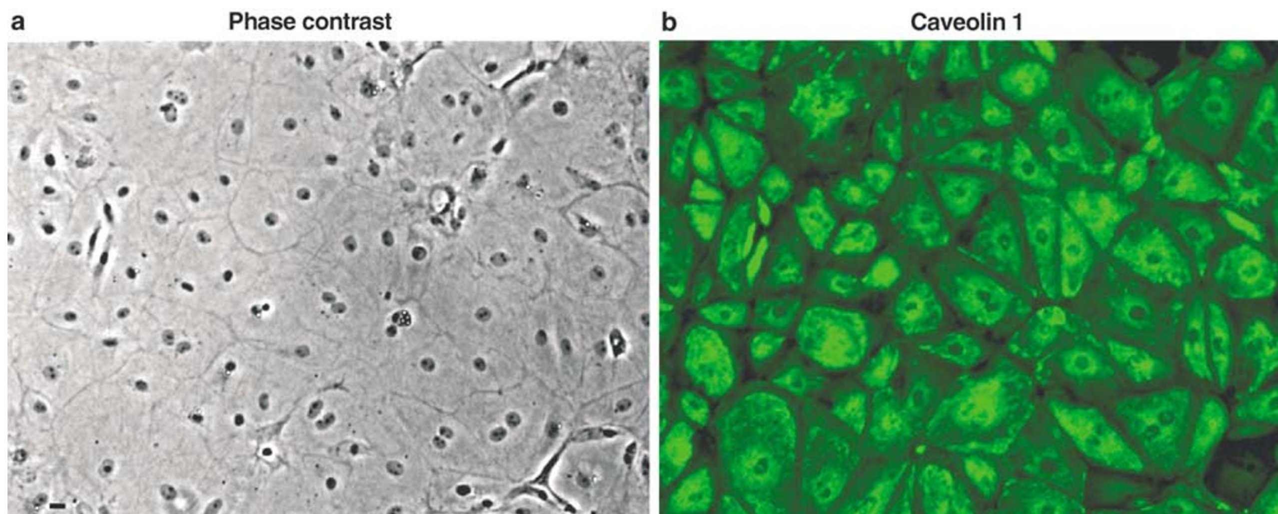

Figure 6 (**a**) Culture of isolated AEC I. The cells were cultured for 5 days, fixed with paraformaldehyde, and (**b**) labeled with polyclonal anticaveolin 1/FITC-conjugtaed anti-rabbit antibodies. Scale bar: 20 μm.

For the isolation of AEC I from rat lungs, Weller and Karnosky[7] used Percoll density gradient centrifugation and obtained a purity of $\sim$88% as evaluated by electron microscopy Dobbs *et al*[3] and Borok *et al*[19] have recently reported methods to isolate AEC I with limited purities or yields. Both laboratories used a discontinuous gradient centrifugation to enrich AEC I, followed by positive selection by using monoclonal antibodies against RTI40 or VIIIB2. Our AEC I isolation protocol does not require a gradient centrifugation. This not only saves time, but also avoids possible adverse effects of Percoll on AEC I. Similar to our AEC II isolation protocol, we removed most macrophages and other leukocytes by rat IgG panning, rat IgG, and anti-LC combined with magnetic bead selection. The time for rat IgG panning step is important because some AEC I attach to the bacteriological plate after a prolonged incubation. We used a 30-min incubation in this step for AEC I cell isolation. Since a significant amount of AEC II exists in the cell mixture after elastase digestion, negative selection is impractical to remove AEC II. Therefore, positive selection was used to isolate AEC I. Maybe this is one of the reasons for a low yield of AEC I ($\sim 2 \times 10^6$/rat) compared to 33×10^6/rat for AEC II. Our AEC I preparations contained $\sim$1–2% AEC II, $<$0.5% macrophages and lymphocytes, and $\sim$6–7% other unidentified cells.

In the present study, we also isolated AEC II from injured lungs. Hyperoxia-mediated lung injury model was used to optimize the conditions to isolate AEC II from injured lungs. Hyperoxia exposure causes inflammation in the lung and has characteristic features of infiltration of phagocytic cells, AEC II hyperplasia, AEC I and endothelial cell injury, and edema, which are commonly observed in several disease conditions. Using our protocol, we reached the purity levels up to $\sim$93–95% of AEC II. Previously, a few groups attempted to isolate the AEC II from hyperoxia-exposed lungs using the method of Dobbs *et al.*[4] Carter *et al*[21] obtained a $\sim$85% pure of AEC II. Buchley *et al*[22] reported up to 95% purity for overnight cultured cells, but the purity of freshly isolated AEC II was not given. Most of the AEC II are in a hyperplasic condition after hyperoxia exposure and overnight culture may cause significant changes at gene expression level compared to the freshly isolated AEC II. Hence our method may be more versatile for the isolation of AEC II from injured lungs.

The present method for isolating AEC II is based on the method of Dobbs *et al*[4] with the addition of negative immunomagnetic selection to remove leukocytes and AEC I contaminations. The isolated AEC II have typical characteristics of AEC II such as lamellar bodies, apical microvilli, etc. The cells also secrete lung surfactant in response to secretagogues. For the AEC I isolation, a positive selection step was used. This raises the concern that the use of anti-T1α antibodies for the positive selection may affect the functions of AEC I. The function of T1α is still unclear, but may be related to transepithelial ion transport.[18] We do not believe that the antibody binding to T1α causes a significant functional or morphologic change of AEC I, but the possibility exists and needs further investigation. Using monoclonal anti-T1α antibodies, Dobbs's group has isolated AEC I with a purity of 60–86%. These cells are highly water-permeable[19] and also are able to transport sodium.[2] Our AEC I preparation not only shows AEC I features of *in vivo* lungs, but also is suitable for culture. The cultured AEC I form a monolayer, further supporting that these AEC I cells isolated using T1α antibodies are functional.

In summary, we developed improved and reproducible methods for AEC I and AEC II isolation using a polyclonal antibody against T1α generated in

our laboratory. The purity of AEC I and AEC II preparations was 91.17±3.83% and 96.50±1.39%, respectively. Our AEC II isolation protocol can also be used to isolate highly pure AEC II from hyperoxia-injured lungs. These cell preparations may be highly valuable for studying gene expression profiling, lung injury and repair, and lung epithelial cell biology.

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