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Type II Alveolar Epithelial Cells in Lung Express Receptor for Advanced Glycation End Products (RAGE) Gene

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Messenger RNA of receptor for advanced glycation end products (RAGE) is abundantly expressed in the lung. However, cell types expressing RAGE mRNA in the lung have not been identified. In order to elucidate the function of RAGE in pulmonary tissue, we have identified a cell type expressing RAGE mRNA by *in situ* hybridization and compared its expression level of RAGE mRNA by RNA blot analysis of isolated cells. *In situ* hybridization revealed that RAGE mRNA was intensely and specifically visualized in alveolar epithelial type II (AT-II) cells, and weakly in alveolar macrophages. The expression of RAGE mRNA in the primary culture of AT-II cells was at a high level, but that in alveolar macrophages isolated from alveolar lavage was under the level of detection by RNA blot analysis. These results showed that RAGE mRNA is specifically expressed in AT-II cells, and suggested that RAGE makes a substantial contribution to the function of AT-II cells in the lung.

Advanced glycation end products (AGEs), final products of nonenzymatic glycation and oxidation of proteins or lipids, are found in several tissues. The amount of AGEs increases with age, and their accumulation is accelerated in diabetes. AGEs alter structure and function of proteins, and exert influence on vasculer proliferation with their receptor through the release of cytokines or growth factors (1). Since the receptor for advanced glycation end products (RAGE) was cloned as a receptor for AGEs (2), much work has been done to clarify the mechanism of RAGE in the relation to AGEs. These results suggest that AGEs-RAGE interaction generates cellular oxidant stress triggering hyperpermeability of endothelial cells, which results in vascular disorder. (3) However, several recent papers argue that AGEs might be an accidental ligand for RAGE. Amphoterin and beta-amyloid peptide were newly identified as ligands for RAGE in nervous systems

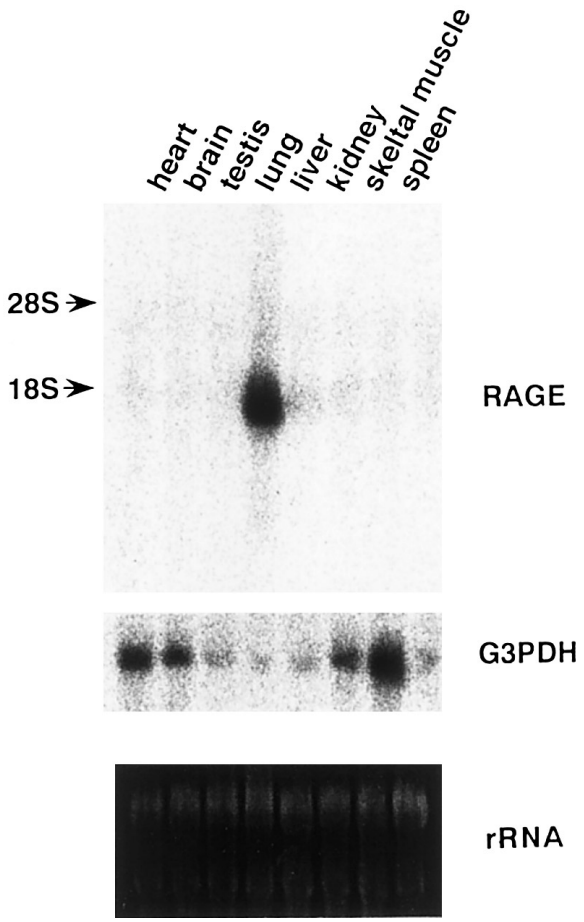

FIG. 1. Tissue distribution of RAGE mRNA expression in the rat. Abundant expression was observed in the lung. 20 μg of total RNA was applied in each lane.

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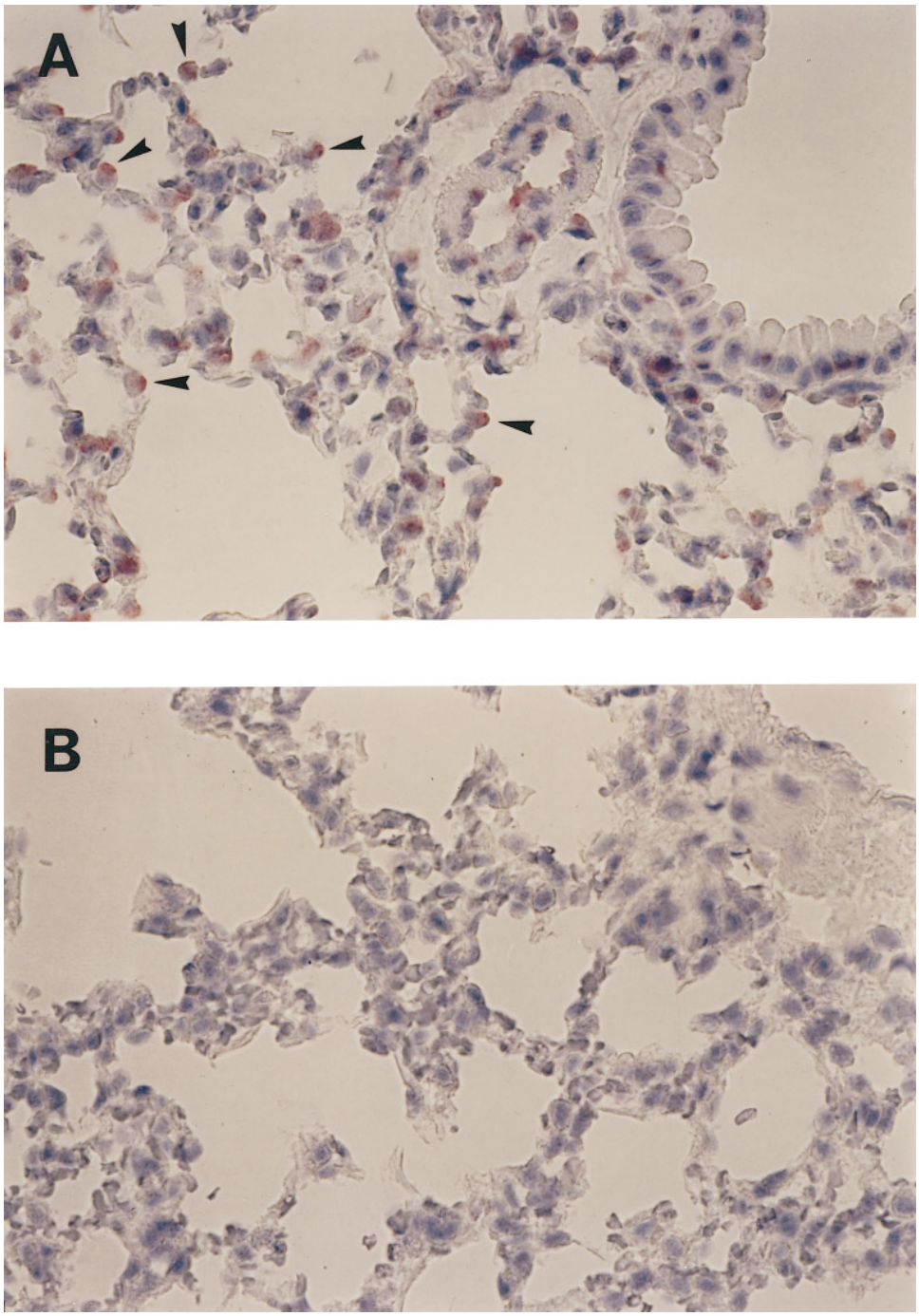

FIG. 2. A: The distribution of RAGE mRNA in mouse lung by *in situ* hybridization. RAGE mRNA was highly expressed in AT-II cells (arrow head) B: No staining was seen with a sense cDNA probe used as the negative control. (Original magnification: ×400).

(4,5). The physiological ligands for RAGE are now under investigation.

RAGE mRNA has been reported to be distributed ubiquitously in various cells (for example, endothelial cells, macrophages, and neurons). RAGE mRNA is also highly expressed in the lung (6),which strongly suggests the existence of a native physiological ligand different from AGE in the lung as well as in the nervous system. However, little is known about the localization of RAGE mRNA in pulmonary tissue. In this study, we demonstrate an abundant expression of RAGE mRNA in AT-II cells, which play many roles in maintaining pulmonary function, including the secretion of surfactants.

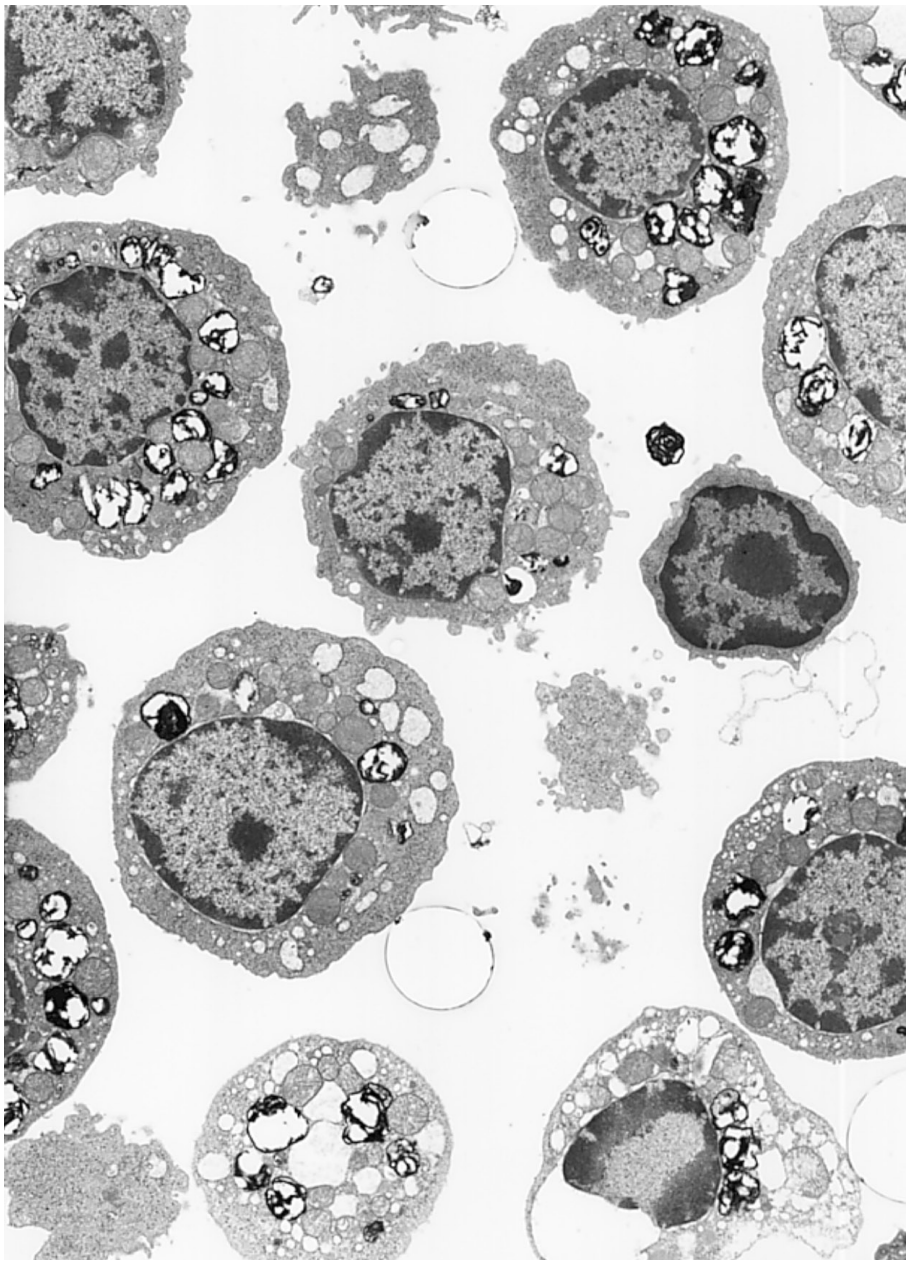

FIG. 3. Electron microscopic evaluation of cultured AT-II cells. Over 90% of the cells were identified as AT-II cells by the presence of lamellar bodies.

MATERIALS AND METHODS

Cell and culture. Type II alveolar epithelial cells were isolated from male, six week old, Wistar-imamichi rats (Imamichi Institute for animal reproduction, Japan) (7). Briefly, the animals were anesthetized, and pulmonary perfusion with phosphate buffered saline (PBS) was performed. After lavage via the trachea to remove alveolar macrophages, 10 ml of dispase II at 2000U/ml was instilled via the trachea and incubated for 30 min. The lung was removed from the large airways, minced and stirred. The tissue was filtered successively through cotton gauze, 150-μm, and 15-μm filters. The cells from the filtrate were suspended in Dulbecco's modified Eagle's medium (DMEM, Sigma) and were applied for 1 h to plates coated with rat IgG (Sigma) to eliminate Fc receptor+ cells. The non-adherent cells, which were predominantly type II alveolar epithelial cells, were then plated in DMEM supplemented with 10% fetal bovine serum (FBS) in tissue-culture grade plastic dishes and incubated at 37°C under 10% CO_2. Alveolar macrophages were isolated from the above solution of alveolar lavage by centrifugation.

Reverse transcription (RT) and polymerase chain reaction (PCR). Total RNA was extracted by using RNeasy (QIAGEN, Germany). RT-PCR was performed using specific primers and the RT product from human smooth muscle cells. Specific primers for PCR were designed according to the human RAGE sequence deposited in GenBank (accession no. D28769). The sequences of the primers are as follows: 5′-ATGGAAACTGAACACAGGCC-3′ (sense) and 5′-CAC-

ACATGTCCCCACCTTAT-3′ (antisense). The PCR product was subcloned into a TA cloning vector (Invitrogen) for a probe of RNA blot analysis and *in situ* hybridization.

RNA blot analysis. Total RNA was separated by electrophoresis using a 1.5% agarose gel containing 2.2 M formaldehyde and transferred to a nylon membrane. The membrane was prehybridized in 50% formamide, 5× SSPE (1× SSPE is 0.15 M NaCl, 15 mM NaH_2PO_4, pH 7.0, 1 mM EDTA), 5× Denhardt's solution, 100 μg/ml denatured salmon sperm DNA, and 1% SDS at 42°C for 2 h and hybridized with the ^{32}P-labeled cDNA probe at 42°C for 18 h in the same buffer for prehybridization without salmon sperm DNA. The membrane was washed with 2× SSC (1× SSC is 0.15 M NaCl, 15 mM sodium citrate) at 55°C for 5 min, twice with 2× SSC, 1% SDS at 55°C for 30 min, and exposed to a photostimulatable imaging plate. The radioactivities of the hybridized fragments were quantified by a Fuji Imaging Analyzer (BAS 2000, Fuji Photo Film Co., Japan).

In situ hybridization. Probes were labeled with digoxigenin (DIG)-dUTP (Boehringer Mannheim, Germany) by asymmetric PCR using sense or antisense primer. The lung of BALB/c mice (Charles Liver Japan) was fixed with 4% paraformaldehyde in PBS, and embedded in paraffin. Sections (4 μm) were mounted onto polyL-lysine (MW=150,000 SIGMA) treated slides. Before hybridization, slides were deparaffinized, hydrated, rinsed with 0.1 M PB (phosphate buffer), pH 7.4, treated with proteinase K (5 μg/ml) in 0.1 M PB for 10 min at 37°C, refixed with 4% paraformaldehyde in PBS, rinsed with 0.1 M PB, pH 7.4, treated with 0.2 N HCl for 10 min, rinsed with 0.1 M PB, pH 7.4, de-hydrated, and dried. Hybridization was carried out at 50°C for 18 h in a solution composed of 50% formamide, 10 mM Tris-HCl (pH 7.4), 1× Denhardt's solution, 10% dextran sulfate, 0.6 M NaCl, 0.25% SDS, 1 mM EDTA and DIG-labeled probe. After hybridization, slides were sequentially washed at 37°C in 5× SSC for 2 min, 2× SSC/30% formamide for 10 min, and 2× SSC for 1 min. After blocking with 3% BSA/PBS, slides were incubated with PBS containing a 1:500 dilution of anti DIG-POD Fab fragments (Boehringer Mannheim) at room-temperature for 1 h. The color was then developed using 3-amino-9-ethylcarbazole (AEC), and nuclear staining was carried out using Mayer's hematoxylin as counter-stain.

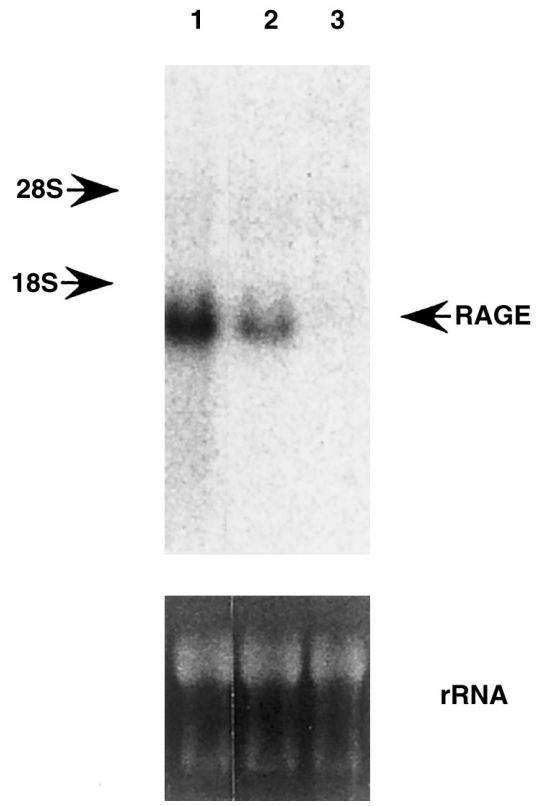

FIG. 4. RNA blot analysis of RAGE mRNA expression in primary cultured AT-II cells and alveolar macrophages. 10 μg of total RNA was applied in each lane. Arrows indicate 28S and 18S RNA. Lane 1, adult rat lung tissue; lane 2, primary cultured AT-II cells from adult rat lung; lane 3, alveolar macrophage from adult rat.

RESULTS

Abundant Expression of RAGE mRNA in Lung Alveolar Epithelial Type II Cells

In order to examine the expression profile of RAGE mRNA in various mouse and rat tissues and cultured cells, we obtained a partial fragment of human RAGE cDNA by RT-PCR. The 285-bp fragment was amplified by PCR, with a primer set corresponding to human RAGE cDNA. In RNA blot analysis, using this fragment as a probe, a single transcript of approximately 1,500 nucleotides was observed in total RNA from lung tissue of the rat and mouse. These results coincided with a previous finding on human tissue (6), and suggested that this probe cross-hybridized with both rat and mouse RAGE mRNAs (data not shown). RAGE mRNA was highly expressed in rat lung compared with other tissues (Fig. 1). The expression pattern is in close agreement with previous data from human tissues (6). The precise distribution of RAGE mRNA in the lung was investigated by *in situ* hybridization, and RAGE mRNA was found to be expressed highly in AT-II cells and weakly in alveolar macrophages (Fig. 2).

RAGE mRNA Expression in Primary Culture of AT-II Cells

In order to confirm the result of *in situ* hybridization, we isolated AT-II cells and examined their expression level of RAGE mRNA. Purity of the isolated AT-II cells was evaluated by electron microscopy, and over 90% of cells were confirmed to be AT-II cells by the presence of the typical lammelar body (Fig. 3). In RNA blot analysis, the expression of RAGE mRNA in the primary culture of AT-II cells was at a high level, while that in alveolar macrophages isolated from alveolar lavage was undetectable (Fig. 4). We also examined the expression levels of RAGE mRNA in other cells by RNA blot analysis, for example, human umbilical vein endothelial cells and rat peritoneal macrophages. Although these cells were reported to express RAGE mRNA (6), the expression level was too low to be detected by RNA blot analysis (data not shown). These data showed that RAGE mRNA is specifically expressed in AT-II cells.

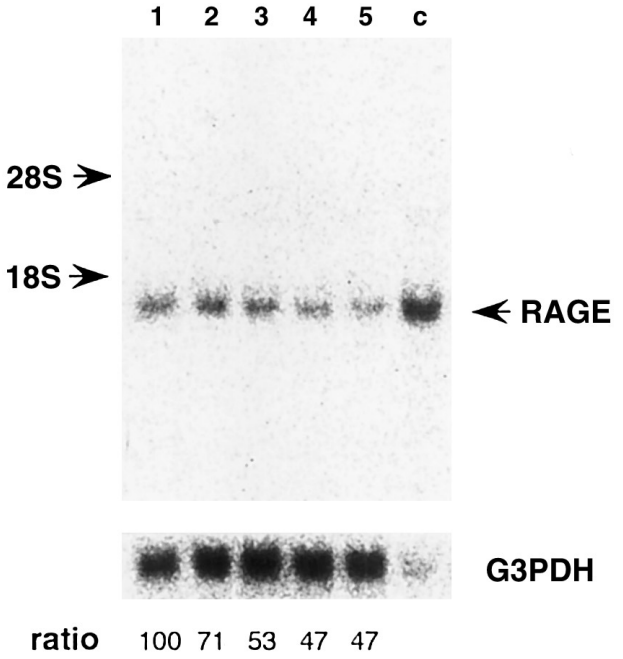

FIG. 5. RAGE mRNA expression in AT-II cells after one week of culture at various densities. 10 μg of total RNA were applied. Cells were cultured under various cell densities (lane 1: 8×10^4 cells/cm^2, lane 2: 4×10^4 cells/cm^2, lane 3: 2×10^4 cells/cm^2, lane 4: 1×10^4 cells/cm^2, lane 5: 0.5×10^4 cells/cm^2). and harvested on the seventh day. Lane 6: adult rat lung tissue. RAGE mRNA level was normalized to the G3PDH mRNA level, and expressed as a percentage of the level of lane 1.

Effect of Cell Density on the Level of RAGE mRNA Expression in Vitro

AT-II cells are reported to differentiate into alveolar type I epithelial (AT-I) cells morphologically and functionally when they are seeded at low density *in vitro* (8,9,10). To test the possibility that the amount of RAGE mRNA may change during the differentiation process of AT-II cells, the expression levels of RAGE mRNA by AT-II cells were examined after one week of culture at various densities. The expression of RAGE mRNA decreased significantly when the cells were cultured at lower density (Fig. 5). We also observed that AT-II cells in the culture at lower density lost their characteristic morphological features and were judged to have differentiated into AT-I cells (data not shown). Thus, these results strongly suggest that RAGE is involved in the specific cellular function of AT-II cells, but not of AT-I cells.

DISCUSSION

The results of this study demonstrated that RAGE mRNA is abundantly expressed in AT-II cells in the lung compared with other tissues and cells. We found that the expression level of RAGE mRNA in AT-II cells was much higher than that in cells which were previously reported to express RAGE mRNA. It is of interest that RAGE mRNA is expressed at low levels in various tissues and cells, but that it is highly expressed specifically in AT-II cells. We suspect that the expression of RAGE mRNA may be a major characteristic of AT-II cells.

At present, the role of RAGE in AT-II cells is not clear, and a physiological ligand for RAGE in the alveolar space has not yet been identified. Amphoterin, which is a ligand for RAGE in the nervous system, is also expressed in the lung. Therefore, it may be a candidate as a ligand for RAGE in the lung. AT-II cells are known as the principal cells that produce the surfactants which reduce the surface tension in alveoli and that serve as stem cells for alveolar epithelium (11). RAGE may be responsible for those functions of AT-II cells. It is critical to identify a physiological ligand for RAGE in the lung, which is expected to be different from AGE, in order to elucidate the function of RAGE.

The examination of the expression pattern of a molecule in detail provides powerful information on the function of the molecule. Our finding that RAGE mRNA is abundant specifically in AT-II cells will lead to the clarification of the role of RAGE through the study of AT-II cells.

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