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Advances in Brief

Differential Messenger RNA and Protein Expression of the Receptor for Advanced Glycosylated End Products in Normal Lung and Non-Small Cell Lung Carcinoma[1]

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Abstract

The receptor for advanced glycosylated end products (RAGE), a member of the immunoglobulin superfamily, was one of the cDNA subtraction clones that was found to be differentially expressed in human lung and the corresponding tumor tissue. In nine additional matched normal/tumor pairs, a strongly reduced or missing expression, not only on a transcriptional level but also on a protein level, was demonstrated in the non-small cell lung carcinoma tissue. Because amphoterin, a physiological ligand of RAGE that is highly expressed in the lung, mediates cell differentiation via RAGE, a down-regulation of the receptor may be considered as a critical step in lung tumor formation. Furthermore, we determined the complete reading frame of RAGE.

Introduction

To identify new genes that become inactivated during lung tumorigenesis, we performed cDNA subtraction hybridization with normal lung and tumor cDNA libraries from a NSCLC[3] patient (1). The 63-bp sequence of one of the cDNA clones that was differentially expressed in normal and tumor tissue was identical to the 3′ end of RAGE cDNA (2). RAGE was originally purified from bovine lung and endothelial cell extracts using a glycosylated BSA binding assay (3). An incomplete human RAGE cDNA, having an open reading frame of 1215 nucleotides, was isolated from a lung cDNA library (4). The gene that is located on chromosome 6p21.3 (5) was sequenced by cosmid walking from MHC class III to class II (6). cDNA and amino acid sequence analysis classified RAGE as a new member of the immunoglobulin superfamily of cell surface molecules. Abundant RAGE protein expression was observed in bovine lung, smooth muscle, and heart. Low amounts of protein were also detected in liver, kidney, and neuronal tissue (7). In contrast, RAGE mRNA expression was mainly restricted to the lung (2, 7). In addition to advanced glycosylated end products, two physiologically relevant ligands for RAGE have been described recently. One of them, amphoterin, was identified by sequential chromatography of bovine lung extract (8). Amyloid-β peptide, the second putative ligand, which has been postulated to be central to the pathology of Alzheimer's disease, was used to purify RAGE from protein extracts of cultured endothelial cells or bovine lung (9).

Despite its high abundance in normal lung, the physiological function of RAGE in this organ is yet unknown. Multiple tissue Northern and Western analyses revealed considerable differences between RAGE mRNA and protein amounts in lung, skeletal muscle, and heart (7). Therefore, the relationship between RAGE mRNA and protein expression in human lung and NSCLC tissues is of particular interest. We performed Northern and Western analyses from nine matched normal/NSCLC tissues. Furthermore, we identified the complete reading frame of the RAGE gene. Our results suggest that the down-regulation of the RAGE gene is a common event during lung tumor formation.

Materials and Methods

Normal/Tumor Lung Tissue and NSCLC Cell Lines. Normal and corresponding tumor lung tissues from nine NSCLC patients were placed in liquid nitrogen immediately after resection.

RNA Extraction and Northern Blotting. Total RNA was extracted from pulverized normal/tumor tissue as described (10). Twenty μg of total RNA of matched normal/tumor samples were fractionated on a 1% agarose gel containing formaldehyde and transferred to Hybond-N nylon membranes (Amersham). Northern blots were hybridized with ^{32}P-labeled 1.4-kb RAGE cDNA (provided by Dr. Kathrin Jansen, Merck, Sharp & Dohme, Westpoint, PA) for 16 h at 65°C in 1% BSA, 1 mM EDTA, 0.5 M NaH_2PO_4 (pH 7.2), and 7% SDS. The filters were washed in 2× SSC-1% SDS at 65°C for 10 min and in 0.1× SSC-0.1% SDS at 65°C for 10 min and exposed to film for 3 days at −70°C.

Protein Extraction and Western Blotting. About 0.1 g of each of the nine paired normal and tumor tissues was pulverized, homogenized in 0.8 ml of 20% trichloroacetic acid using a sonic Dismembrator (Fisher Scientific, Pittsburgh, PA), and kept on ice for 10 min. The samples were centrifuged at 12,000 × *g* for 2 min and washed three times in ice-cold acetone followed by drying. After resuspension in 200 μl of SDS-PAGE sample buffer without reducing agents, the samples were boiled for 5 min and spun down. About 100 μg of extract from each of the supernatants were applied to nonreduced SDS-PAGE (10%), and after electrophoresis, proteins were transferred electrophoretically to Immobilon-P membranes for 1 h at 300 mA. Western blots were reacted with 40 μg/ml rabbit antihuman RAGE IgG, which was generously provided by Dr. Kathrin Jansen. Immunoreactive bands were identified using peroxidase conjugated antirabbit IgGs (Bio-Rad), the ECL Kit (Amersham), and Kodak X-OmatAR film.

RT-PCR Analysis. RT-PCR analysis was used to demonstrate the more upstream transcription start site of the RAGE mRNA. cDNA synthesis was performed at 37°C for 60 min in a volume of 20 μl, containing 5 μg of total RNA from normal human lung, 1× M-MuLV-RT buffer (Life Technologies, Inc.), 1 mM each dNTP, 10 units of RNase Inhibitor (Boehringer Mannheim), 0.5 μM RAGE mRNA-specific 3′ reverse primer (5′-TTC CCA TCC AAG TGC CAG CT-3′), and 200 units of M-MuLV-Reverse Transcriptase (Life Technologies). RT-PCR was performed in a volume of 20 μl using 2 μl of the first strand cDNA product, 1× PCR buffer (Life Technologies), 10 pmol of both forward primer (5′-GCA TGA ATT CCT AGC ATT CC-3′) and reverse primer (5′-CTC AAG GCC CTC CAG TAC TAC TCT-3′), 0.5 mM each dNTP, 1.5 mM $MgCl_2$, and 1 unit of Taq polymerase (Life Technologies). PCR conditions were: 1 cycle of 94°C for 5 min, 60°C for 3 min, and 72°C for 3 min, followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 3 min.

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[3] The abbreviations used are: NSCLC, non-small cell lung carcinoma; RAGE, receptor for advanced glycosylated end products; RT-PCR, reverse transcriptase-PCR.

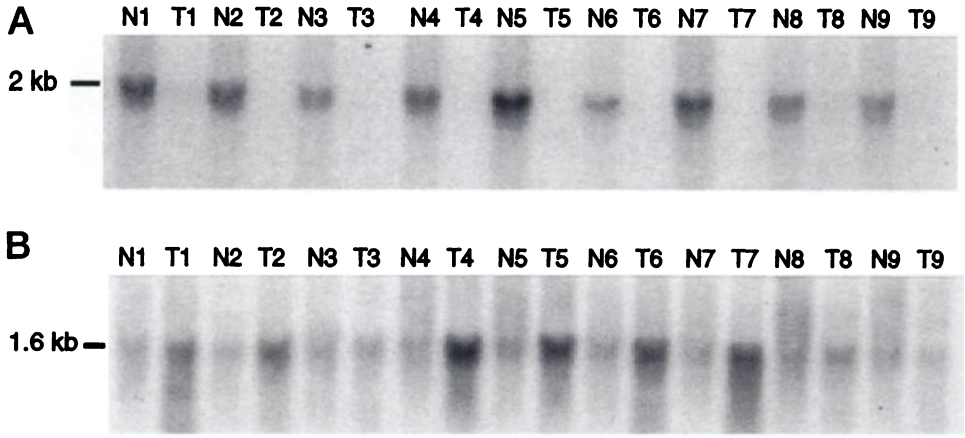

Fig. 1. *A*, RAGE gene expression in normal/tumor RNA pairs from nine NSCLC patients. *B*, Northern blot was hybridized with a glyceraldehyde-3-phosphate dehydrogenase probe as a control. *Lanes N*, total RNA from normal lung tissue of patients; *Lanes T*, total RNA from tumor tissue of patients; *numbers*, patient numbers.

Results and Discussion

To provide evidence that the inactivation of the RAGE gene is a common event in NSCLC, we examined RAGE gene expression at both the transcriptional and translational levels in nine paired normal/tumor tissues. Five patients suffered from squamous cell carcinoma, three patients suffered from adenocarcinoma, and one suffered from large cell carcinoma. Northern analysis of total RNA from the nine NSCLC patients using a 1.4-kb human RAGE cDNA as a probe demonstrated a transcript of approximately 2 kb in normal lung. In the corresponding tumors, the RAGE mRNA was clearly absent (Fig. 1).

Because the start codon of the published RAGE cDNA sequence was missing (4), we applied RT-PCR to obtain the complete reading frame of the RAGE gene. On the basis of the human genomic sequence of RAGE (6), we identified an ATG start codon that is 93 nucleotides further upstream of the predicted start site (4–6). As shown in Fig. 2*A*, we could amplify a 1.35-kb PCR product, beginning 41 bp upstream and ending 1309 bp downstream of the ATG codon. Sequence analysis confirmed the RT-PCR result. As a consequence, the RAGE protein consists of 31 additional amino acids at its NH_2-terminal end (Fig. 2*B*).

In addition to total RNA, protein was also extracted from the same nine normal and tumor tissues. Western blot analysis using polyclonal rabbit antihuman RAGE antibodies and antirabbit IgGs demonstrated high levels of RAGE protein in normal human lung tissue (Fig. 3). In most cases, a strong single band of M_r ~55,000 was observed, which is consistent with previous results (9). Moreover, we could show that the absence of the RAGE transcript correlated with the absence (tumor nos. 1–7) or the decrease (tumor nos. 8 and 9) of RAGE protein in human lung tumor tissue. The weak background of protein in tumors 8 and 9 may be explained by contaminating normal cells. As shown in Table 1, the differential expression of RAGE did not depend on tumor type, stage, and grade. Therefore, the down-regulation of RAGE points toward a common event in NSCLC. With respect to the receptor's importance in lung tumor development, the cell types in which RAGE is expressed are of particular interest. Previous data demonstrated the presence of RAGE antigen in a variety of tissues,

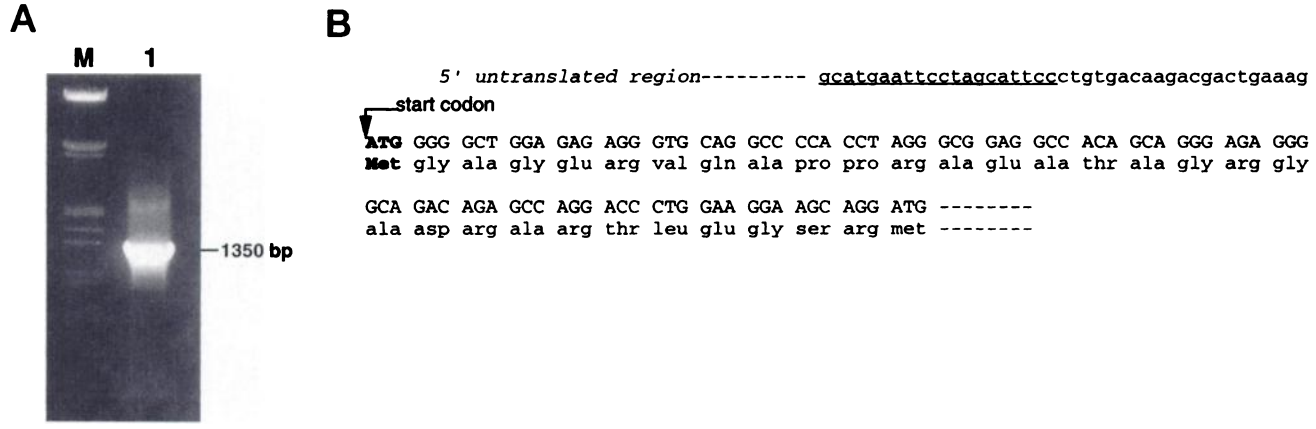

Fig. 2. *A*, amplification of the RAGE cDNA by RT-PCR using total RNA from normal human lung tissue. *Lane M*, λ DNA marker III (Boehringer Mannheim); *Lane 1*, expected 1.35-kb PCR fragment. *B*, 5′ end sequence of the reading frame of the RAGE gene. *Underlined sequence*, forward primer used for RT-PCR; *boldface sequence*, start codon.

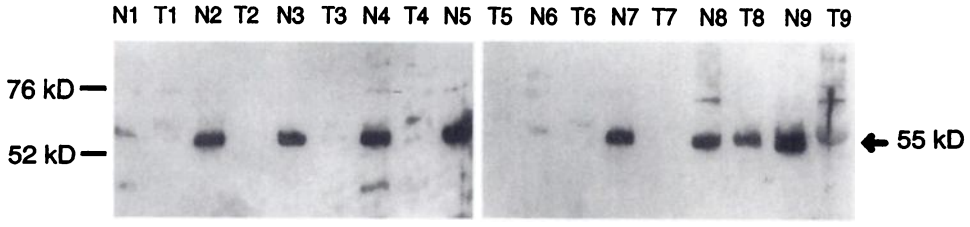

Fig. 3. RAGE protein expression in normal/tumor protein pairs from nine NSCLC patients. *Lanes N*, protein extract from normal lung tissue of patients; *Lanes T*, protein extract from tumor tissue of patients; *numbers*, patient numbers. Coomassie blue staining of the filters used as control showed equal amounts of protein in each lane (data not shown).

Table 1 *Histology and TNM[a] parameters of the nine NSCLC cases*

Patient no.	Tumor type	TNM parameters
1	SCC[b]	$T_2 N_1$
2	LCC[c]	$T_4 N_0$
3	ADC[d]	$T_2 N_0$
4	ADC	$T_1 N_0$
5	SCC	$T_2 N_2$
6	SCC	$T_2 N_0$
7	SCC	$T_2 N_1$
8	ADC	$T_1 N_0$
9	SCC	$T_3 N_0$

[a] TNM, tumor-node-metastasis.
[b] SCC, squamous cell carcinoma.
[c] LCC, large cell carcinoma.
[d] ADC, adenocarcinoma.

including epithelial components of the kidney (7). Because carcinomas are thought to originate from epithelial cells, immunohistochemical analysis will show whether RAGE is expressed in the normal epithelium of the lung.

Although abundantly expressed in the lung, the physiological role of RAGE in this organ is still unknown. Immunoglobulin-like structures that are similar to neural cellular adhesion molecules and the identification of amphoterin and amyloid-β peptide as primary ligands (8, 9) provided a function of RAGE that is totally distinct from its previously suggested role as a central cellular receptor for advanced glycosylated end products. Of the three ligands that bind RAGE, amphoterin showed the highest affinity. Amphoterin, which is also present in the lung (8), was originally discovered in the developing rat central nervous system (11). Amphoterin-RAGE binding mediates both neuronal development and neurite formation of rat cortical neurons. Because RAGE and amphoterin are expressed in the lung, their participation in cellular differentiation is conceivable. Stable transfection studies using suitable cell lines and RAGE gene promoter mutation analysis will address the tumor suppressive properties of RAGE.

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