Carcinogenesis vol.26 no.2 pp.293–301, 2005
doi:10.1093/carcin/bgh333

Down-regulation of the receptor for advanced glycation end-products (RAGE) supports non-small cell lung carcinoma

Babett Bartling*, Hans-Stefan Hofmann, Bernd Weigle[1], Rolf-Edgar Silber and Andreas Simm

Clinic of Cardiothoracic Surgery, Martin Luther University Halle-Wittenberg, Ernst-Grube-Strasse 40, D-06120 Halle/Saale, Germany and [1]Institute of Immunology, Technical University Dresden, Dresden, Germany

*To whom correspondence should be addressed. Tel: +49 345 557 3314; Fax: +49 345 557 7070;
Email: babett.bartling@medizin.uni-halle.de

The receptor for advanced glycation end-products (RAGE) is a transmembrane receptor of the immunoglobulin superfamily. Several ligands binding to RAGE have been identified, including amphoterin. Experimental studies have given rise to the discussion that RAGE and its interaction with amphoterin contribute to tumour growth and metastasis. However, none of the studies considered a differential transcription profile in cancer that might change the interpretation of the study results when comparing RAGE in tumours with histologically normal tissues. Here we show that RAGE is strongly reduced at the mRNA and even more so at the protein level in non-small cell lung carcinomas compared with normal lung tissues. Down-regulation of RAGE correlates with higher tumour (TNM) stages but does not depend on the histological subtypes, squamous cell lung carcinoma and adenocarcinoma. Subsequent overexpression of full-length human RAGE in lung cancer cells (NCI-H358) showed diminished tumour growth under some conditions. While proliferation of RAGE-expressing cells was less than that of cells expressing the cytoplasmic domain deletion mutant ΔcytoRAGE or mock-transfected NCI-H358 in monolayer cultures, RAGE cells also formed smaller tumours in spheroid cultures and *in vivo* in athymic mice compared with ΔcytoRAGE cells. Moreover, we observed a more epithelial growth of RAGE-expressing, but also of ΔcytoRAGE-expressing, cells on collagen layers, whereas mock NCI-H358 cells kept their tumour morphology. This observation was supported by immunofluorescence analyses demonstrating that RAGE preferentially localizes at intercellular contact sites, independent of expression of the cytoplasmic domain. Thus, down-regulation of RAGE may be considered as a critical step in tissue reorganization and the formation of lung tumours.

Introduction

Cancer arises from an ongoing accumulation of molecular and cellular changes that allow tumour initiation and progression in multicellular organisms (1). The final cancer phenotype encompasses a broad collection of characteristic features, including alterations in the transcriptional profile. Recent investigations have documented changes in expression of a pattern of genes compared with histologically normal tissues (2,3). Some of these alterations can be detected in several types of cancer, whereas others occur in a small subset of tumours only (4). This has led to conflicting conclusions about the number of distinct mechanisms that are required for malignant transformation and the neoplastic phenotype. One of the mechanisms necessary to promote malignancy seems to be the cellular response resulting from activation of the multiligand receptor for advanced glycation end-products (RAGE) (5). RAGE is composed of an extracellular region containing three immunoglobulin domains followed by a transmembrane domain and a short cytoplasmic region (6). Although intracellular binding partners have not yet been identified, this region appears to be essential for RAGE signalling (7,8). Several ligands for the engagement of RAGE have been found, including amyloids, advanced glycation end-products, members of the S100 family of calcium-binding proteins and amphoterin (also known as high mobility group I DNA-binding protein, HMG-1). Binding of these ligands to RAGE contributes not only to perturbation of cell homeostasis under pathophysiological conditions (9), but also to cell migration and differentiation (10,11). Intracellular signalling pathways induced by RAGE include the activation of small GTPases (Cdc42 and Rac) (7), mitogen-activated protein kinase and c-Jun N-terminal kinase (5) and extracellular signal-regulated kinases 1 and 2 (12), as well as activation of transcription factors like nuclear factor κB (NF-κB) (7) or the cAMP response element-binding factor (13).

In tumour biology it has been suggested that RAGE engagement through the binding of amphoterin localized outside the cell promotes tumour growth and metastasis (5). This has been observed in primary C6 glioma cells from rats and in a murine Lewis lung carcinoma model. The major effect of the RAGE–ligand interaction with amphoterin appears to be migration, rather than proliferation, of neoplastic cells, as suggested in studies involving colorectal and pancreatic carcinoma cells (14,15). Although the level of RAGE expression correlates directly with the metastatic potential of these tumour cells, there is some conflict about the biological role of RAGE activation in cancer. Studies using neuroblastoma and melanoma cells clearly show that binding of the C-terminal motif of amphoterin to RAGE inhibits invasive migration in transendothelial migration assays and suppresses metastasis *in vivo* (16). Some of these conflicts may have a basis, firstly, in the fact that RAGE interacts with several extracellular ligands that can be produced by the cell or by neighbouring/circulating cells and therefore influence each other. Moreover, most RAGE ligands also fulfil intracellular functions independent of RAGE. Secondly, all previous studies considered a high level of RAGE and amphoterin in tumours and ignored

Abbreviations: AdCa, adenocarcinoma; ATI, type I lung alveolar; CPD, cumulative population doubling; DCL-1, deleted in liver cancer; DMEM, Dulbecco's modified Eagle's medium; GFP, green fluorescent protein; NF-κB, nuclear factor κB; NSCLC, non-small cell lung carcinomas; PBS, phosphate-buffered saline; PI, propidium iodide; RAGE, receptor for advanced glycation end-products; SCCa, squamous cell lung carcinoma.

an altered expression profile that occurred in a large variety of tumours (2,3,17). However, RAGE is highly expressed in a range of normal tissues and especially in lung tissue (18). Therefore, our study aimed to quantify the expression of RAGE in human lung tissue compared with that in non-small cell lung carcinomas (NSCLC). Comparisons with different histological subtypes of NSCLC, tumour stage and degree of differentiation were intended to give some indication of the biological role of RAGE in lung cancer. Subsequent experimental analyses were created to clarify the effect of human RAGE in a lung carcinoma model *in vitro* and *in vivo*.

Materials and methods

Patients involved in study

Lung specimens from tumour and paired non-tumour tissue of 34 NSCLC patients, who underwent pulmonary resection surgery, were included in this study, which was approved by the local ethics committee. Patients were grouped according to the size of the primary tumour (T), nodal involvement (N) and distant metastasis (M) to TNM stages I–IV according to the guidelines for TNM staging (19). The number of cancer cells in tumour samples was estimated by microscopy after hemalum and eosin staining (Merck) of tissue sections. All specimens analysed had ≥60% cancer versus stromal cells. In addition to primary lung cancer, we analysed lung tumours from distant metastases ($n = 2$ kidney; $n = 3$ colon), pulmonary lymph nodes, benign lung tumours (i.e. hamartoma, $n = 4$) and malignant tumours from other organs, as given in Table I.

Cell culture conditions and reagents

The lung cancer cell lines used (NCI-H358, NCI-A549 and NCI-H322) correspond to NSCLC (ATCC cell bank). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen Life Technology) at 37°C in a 10% CO_2 atmosphere. Collagen was prepared from rat tail in 0.1% acetic acid and layered onto cell culture plates at 2 mg/ml in DMEM. Migration assays were performed using 8 μm pore membrane inserts (Thincerts; Greiner Bio-One) and the number of migrated cells was estimated after AlamarBlue staining (Bioscource). 5-Aza-2′-deoxycytidine (Sigma), an inhibitor of DNA methylation, was applied at 10 μM for 3 days and replenished on a daily basis. Efficient inhibition of DNA methylation was controlled by expression analysis of deleted in liver cancer (DCL-1) mRNA as described recently by Yuan *et al*. (20). We used scriptaid at 2.5 μM (Biomol) to inhibit histone deacetylation (21).

Construction of expression vectors and transfection

Full-length human RAGE cDNA (accession no. XM_004205) and ΔcytoRAGE cDNA (without the cytoplasmic domain, amino acids 367–404) were cloned into the mammalian expression vector pIRES2-EGFP (Clontech). Recombinant plasmids or the vector alone were purified with a plasmid isolation kit (Qiagen, CA) and transfected into NCI-H358 cells using Effecten transfection reagent (Qiagen). For stable expression, cells were selected for G418 resistance (750 μg/ml Genetecin; Calbiochem) and for green fluorescent protein (GFP) fluorescence using a FACSVantage cell sorter (Becton Dickinson). Immunoblot analysis with the anti-RAGE antibody proved the correct overexpression of each cDNA.

Cell growth

NCI-H358 cells were harvested from 80% confluent monolayers by brief trypsinization using 0.05% trypsin and 0.5 mM Na_2EDTA. For proliferation experiments, cells were seeded at a density of 2×10^3 per well in a 12-well tissue culture plate and cultured for 12 days in regular medium, which was exchanged every 4 days. Proliferation was estimated on given days by counting the total number of living cells using a Multisizer™3 Coulter Counter® (Beckman Coulter). The cumulative population doubling (CPD) was calculated as: $CPD = (\ln \text{cell number}_{\text{day x}} - \ln \text{cell number}_{\text{day 1}})/\ln 2$ (22).

For spheroid culturing, a 5× stock solution of methylcellulose (25 mg/ml in DMEM; Sigma) was mixed with NCI-H358 cells to a final number of 500 cells/spheroid and seeded into a single well of a 96-well suspension culture plate. The medium was partially exchanged after 5 days. Twenty spheroids were cultured per experiment. The diameter of each spheroid was estimated using a Zeiss Axiovert microscope equipped with Metamorph 4.6.5 software (Visitron Systems). Spheroid volumes were calculated as the product of $4/3\pi \times \text{diameter}^3$ and subsequently averaged.

Reverse transcription–PCR

Isolation of total RNA and cDNA synthesis have been described previously (23). PCR was established for common amplification of all RAGE isoforms ($sense_1$, 5′-TGA ACA CAG GCC GGA CAG AAG-3′; $antisense_1$, 5′-CTC TTA GCT GGC ACT TGG ATG GG-3′) (GenBank accession no. D28769), for specific detection of the secretory RAGE isoforms ($sense_2$, 5′-GAT CCC CGT CCC ACC TTC TCC TGT AGC-3′; $antisense_2$, 5′-CAG AAA ACC AGG AGG AAG AGG AGG AGC GTG-3′) (24) and for detection of the N-terminally truncated isoform ($sense_3$, 5′-AGC CGG AAC AGC AGT TGG AG-3′; $antisense_1$) (25). PCR amplification for analysis of several ligands of RAGE was performed according to Kuniyasu *et al*. (14) and Teratani *et al*. (26). An equal quantity of synthesized cDNA was determined by amplification of 18S rRNA (sense, 5′-GTT GGT GGA GCG ATT TGT CTG G-3′; antisense, 5′-AGG GCA GGG ACT TAA TCA ACG C-3′). A one fifth volume of the cDNA reaction was used for PCR containing 1.5 mM $MgCl_2$, 5 pmol each primer, 10 μM each dNTP and 1 U r*Taq*DNA polymerase (Promega). After PCR amplification and gel electrophoretic separation the intensity of PCR products was evaluated using AIDA 2.0 software (Raytest). All mRNA expression values were normalized to 18S rRNA.

Purification of recombinant RAGE fragments and generation of anti-RAGE antibodies

To generate a monoclonal mouse anti-RAGE antibody the internal BamHI sites of human RAGE cDNA were used to subclone the fragment coding for amino acids 90–347 of immature RAGE into expression vector pQE-32 (Qiagen). Overexpression of the recombinant RAGE fragment with an N-terminal 6×His tag was performed in *Escherichia coli* M15p(REP4) (Qiagen), as recommended by the manufacturer. Cells were harvested by centrifugation at 3000 *g* and 4°C for 10 min and resuspended in a 1/25 volume of lysis buffer (8 M urea, 100 mM Na_2HPO_4, 10 mM Tris–HCl, pH 8.8, 0.1% Triton X-100, 25 mM imidazole). After brief sonication, cellular debris was pelleted at 13 000 *g* and 4°C for 30 min. Purification was carried out by mixing the cleared lysate with pre-equilibrated Ni-NTA resin (Qiagen) on a rotator for 1 h, followed by washing four times with lysis buffer and eluting with lysis buffer containing 150 mM imidazole. The purification process was analysed by SDS–PAGE, followed by Coomassie blue staining and immunoblotting using mouse anti-penta-His mAb (Qiagen) as primary antibody and horseradish peroxidase-conjugated anti-mouse IgG (DAKO) as secondary antibody. The purified His-tagged RAGE fragment was refolded by stepwise dialysis against buffer (100 mM Na_2HPO_4, 10 mM Tris–HCl, pH 7.4, 0.1% Triton X-100) and protein yield was determined by Bradford protein assay (Bio-Rad) after centrifugation at 22 000 *g* and 4°C for 1 h. The recombinant RAGE fragment was used to immunize BALB/c mice. Hybridomas were generated by fusion of spleen cells with X-63AG8 myeloma cells according to standard procedures and screened for reactivity with recombinant RAGE fragments by ELISA and immunoblotting. Positive wells were established and cloned twice by limiting dilution. An anti-RAGE monoclonal antibody recognizing recombinant RAGE fragments, bovine RAGE from acetone lung powder (Sigma) and High Five insect cells stably transfected with human full-length RAGE cDNA cloned into pIZ/V5-His vector (Invitrogen) in immunoblots was designated A11 (IgG_{2a}).

To generate a polyclonal rabbit anti-RAGE antibody the intracellular domain of human RAGE (amino acids 375–405) including a C-terminal cysteine residue for coupling was synthesized using Fmoc chemistry with several repeated acylation steps. The peptide was purified by reverse phase chromatography (C18 column) and verified by mass spectroscopy. Immunization followed a standard protocol. The keyhole limpet haemocyanin-conjugated peptide was s.c. injected into rabbits. After 3 weeks the animals were boosted with a further aliquot of the conjugate, and 6 weeks after immunization the reactivity of the antiserum was tested by immunoblotting using RAGE peptide and lung protein lysate.

Table I. Characteristics of lung cancer patients included in this study

	n	TNM staging		
		I	II	III–IV
Non-small cell lung carcinoma				
Squamous cell carcinoma	18	8	4	6
Adenocarcinoma	16	9	2	5
Benign lung hamartoma	4			
Lung metastases	9			

Immunoblot analysis

For protein analysis, samples were homogenized in lysis buffer (10 mM Tris–HCl, pH 7.4, 80 mM KCl, 1 mM Na_2EDTA, 2% SDS) containing complete protease inhibitor (Roche Diagnostics). Protein concentration was measured by the BCA protein assay (Sigma). A total of 50 μg protein was separated by SDS–PAGE. In the case of patient tissues, the sample volume was additionally readjusted to an equal protein concentration by the densitometrically determined value of the total protein loading per lane in a Coomassie blue stained gel. The use of housekeeping proteins does not seem to be reliable for normalization of tumours compared with histologically normal samples, because of the different levels of expression frequently detected (see The Cancer Genome Anatomic Project at cgap.nci.nih.gov/SAGE/AnatomicViewer). Proteins from the analytical gel were blotted onto a nitrocellulose membrane. Thereafter, the membrane was blocked with 6% non-fat dry milk in TBS-T buffer (200 mM Tris–HCl, pH 7.5, 300 mM NaCl, 0.15% Tween 20) and incubated with the anti-human RAGE antibody (A11, 1:1000 in TBS-T). The monoclonal antibody to GFP was purchased from Roche Diagnostics. Bound antibodies were visualized by horseradish peroxidase-conjugated secondary antibodies (Dianova) and ECL^{plus} detection (Amersham Bioscience). All analytical gels contained loading samples for internal signal correction. The intensities of visualized signals were densitometrically analysed and are given as optical density units.

Cell cycle analysis

NCI-H358 cells were always collected on day 2 after seeding (i.e. at the start of exponential growth). Nuclear DNA content was analysed after staining with propidium iodide (PI) (Sigma) and subsequent flow cytometry. Briefly, cells were fixed in ice-cold 70% ethanol and incubated with PI solution [50 μg/ml PI, 0.5 mg/ml RNase A, 0.1% sodium citrate, 0.1 mM Na_2EDTA in phosphate-buffered saline (PBS) pH 7.2] for 1 h at 4°C. The DNA content of cells was measured using a FACSCalibur flow cytometer equipped with CellQuest Pro software (Becton Dickinson). The number of proliferating cells in the S and G_2 cell cycle phases was estimated using MultiCycle software (Phoenix Flow Systems).

Immunohistochemistry and immunocytochemistry

Lung samples were fixed in buffered 4% formalin and embedded in paraffin. Sections of 8 μm were cut, dewaxed and rehydrated. Non-specific protein binding was blocked with 5% bovine serum albumin in PBS. For RAGE immunostaining the rabbit antiserum (1:200 in PBS) was applied in a humidified chamber at 37°C for 2 h, followed by detection with the LSAB staining system and DAB plus substrate solution (DAKO). Omission of the primary antibody served as a negative control. In the case of NCI-H358 immunofluorescence, cells grown on class coverslips were fixed in 4% formaldehyde for 10 min at 4°C with or without subsequent membrane permeabilization in 0.3% saponin (control with the membrane impermeable nuclear dye Hoechst 33342). They were then blocked with 5% bovine serum albumin and stained for 1 h with the monoclonal mouse primary antibody (A11, 1:400 in PBS) at room temperature. RAGE detection was performed with Alexa Fluor®594 anti-mouse IgG secondary antibody (Molecular Probes Europe) and fluorescence microscopy. Slides were embedded in Mowiol mounting medium (Calbiochem) containing 1% thimerosal (Sigma).

In vivo tumorigenicity assay

Female athymic mice (NMRI-nu) were obtained from Harlan Winkelmann (Borchen, Germany) and maintained under clean room conditions in sterile isolator cages. Xenograft experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* after approval by the local Animal Care Committee. NCI-H358 tumour cells were cultured and harvested from 80% confluent monolayers as described above. Samples of 2×10^6 cells per tumour were s.c. implanted into the upper flank of the mice. The lengths and widths of the tumours were measured twice a week for the duration of the experiment using a sliding calliper. Tumour volumes were calculated as the product of $4/3\pi \times (\text{length}/2) \times (\text{width}/2)^2$, corresponding to the equation for an ellipsoid. Mice were killed at day 29 and tumours were dissected and weighed.

Statistical analysis

For patient analyses all tumours and the paired control tissue were analysed in one experimental setting with a three-fold determination of RNA and a two-fold detection of protein samples for each patient. Student's paired *t*-test was used for statistical comparison of both evaluation groups. The ANOVA procedure was applied for multiple comparisons followed by the Tukey test in the case of pairwise analyses. Dunnett's test was used for the evaluation to a control group in the case of experimental investigations. Regression analyses were carried out and the coefficients of each proliferation or growth curve were calculated according to the given formula and subsequently subjected to multiple analyses (SigmaStat and SigmaPlot software; Jandel Corp., San Rafael, CA). All data are reported as means ± SEM ($n \geq 3$ in all cases) and P values <0.05 were accepted as indicating a significant difference.

Results

RAGE expression in lung cancer and other tumours

Investigations of RAGE expression revealed that the RAGE mRNA level is markedly decreased in tumour samples of NSCLC patients. Overall mRNA expression was similarly down-regulated in squamous cell lung carcinoma (SCCa) and adenocarcinoma (AdCa) compared with non-tumour control lung tissue derived from the same patient (Figure 1A). These data were confirmed at the protein level (Figure 2). Although there was less expression of RAGE in NSCLC patients with TNM stage III–IV than in TNM I patients (Figure 3), the altered expression is not related to malignant phenotype in general. Lung tumours from benign neoplasms (hamartomas) were also characterized by a strongly reduced RAGE level (Figure 1A) and the low expression of RAGE in NSCLC was shown to depend on tumour differentiation stage. However, detailed analysis did not reveal differentiation-dependent changes in RAGE mRNA values, with 2.47 ± 1.09 in stage II NSCLC ($n = 11$) and 2.20 ± 0.87 in stage III NSCLC ($n = 23$). Because lung tissue is a major source of RAGE expression (18), we also analysed tumour specimens derived from other organs, as well as paired non-tumour controls. As demonstrated in Figure 1B, the level of RAGE is commonly reduced in different types of solid tumours. Metastases from distant tumours revealed a clear RAGE reduction compared with individual lung specimens (Figure 1A). These descriptive investigations clearly show that down-regulation of RAGE seems to be a common event in neoplastic tissues.

In addition to the receptor RAGE, secretory RAGE isoforms exist that lack the transmembrane domain (24). Therefore, it might be possible that reduced levels of full-length RAGE in NSCLC result from differential transcription of the RAGE gene towards one of those secretory isoforms. However, isoform-specific PCR analysis showed that not only the receptor but also the sRAGE1 splice variant is down-regulated in most lung tumours (see for example Figure 1C). While sRAGE1 mRNA is always detectable, expression of the soluble RAGE variants, sRAGE2 and sRAGE3, is less induced in tumours. Direct PCR quantification of the secretory isoforms is, however, not possible and is misleading because of competitive amplification of the PCR products. In contrast to these C-terminally modified isoforms, transcription of the RAGE variant lacking the N-terminal extracellular domain (25) cannot be detected (data not shown).

Blockade of gene transcription by aberrant promoter methylation is a hallmark of cancer progression (1). Therefore, we treated the lung adenocarcinoma cell line NCI-H358 with 5-aza-2′-deoxycytidine, an inhibitor of the methylation process. As a positive control we showed transcription of the novel C-type lectin receptor DCL-1, which is diminished in lung neoplasms as a result of DNA hypermethylation but can be reinduced by 5-aza-2′-deoxycytidine in a range of cell lines (20). While DCL-1 transcription is also activated in NCI-H358 cells, RAGE mRNA expression cannot be reinduced in the presence 5-aza-2′-deoxycytidine (Figure 3). Moreover, inhibition of histone deacetylation is frequently used as an activator of transcription. However, blockade of deacetylation with scriptaid (27), alone or in combination with

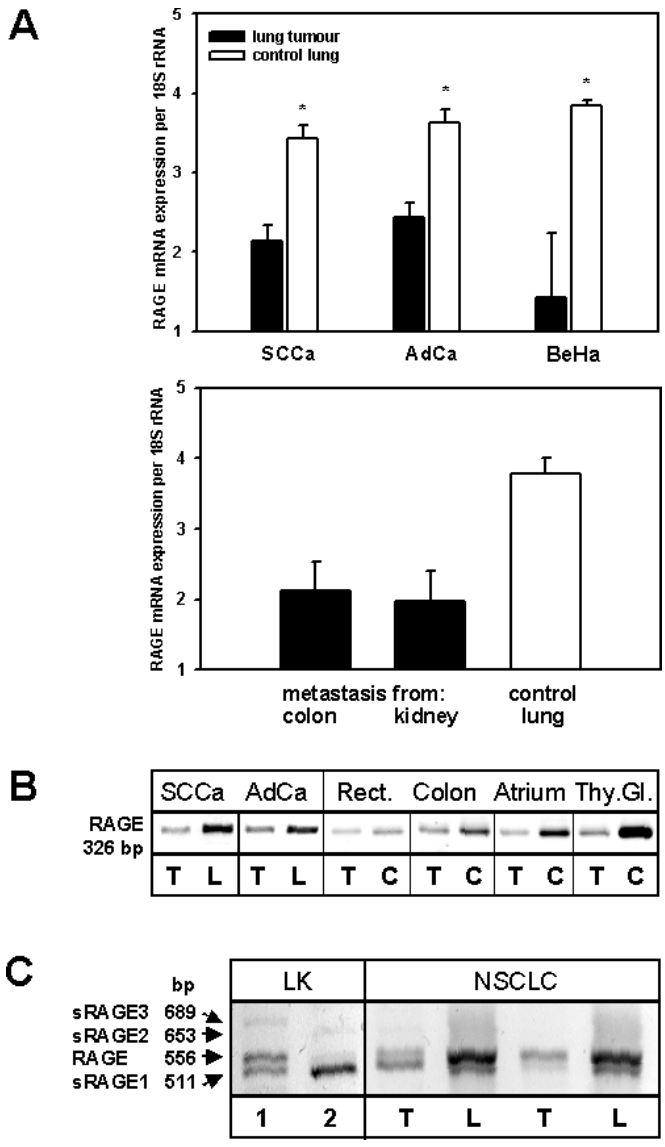

Fig. 1. (**A**) RAGE mRNA expression adjusted to 18S rRNA in lung tumours and non-tumour control lungs are shown in the upper graph. $^{*}P < 0.05$ versus control lung from the same patient. SCCa, squamous cell lung carcinoma; AdCa, adenocarcinoma; BeHa, benign lung hamartoma. In the lower graph, lung metastases from distant tumours are evaluated per mean mRNA of all control lungs. (**B**) Representative gel electrophoresis of PCR products for RAGE demonstrates low mRNA level in SCCa and AdCa tumour tissue (T) compared with the paired control lung (L). Tumour specimens from colon, rectum (Rect.), right atrial myxoma (Atrium) and thyroid gland (Thy.Gl.) are also shown in comparison with individual control tissue (C). (**C**) Gel electrophoresis of an isoform-specific PCR for secretory RAGE splice variants (sRAGE) indicates strongest amplification for sRAGE1 mRNA in NSCLC (T) and normal lung (L). Material from pulmonary lymph nodes (LK1 and 2) was used as a positive control.

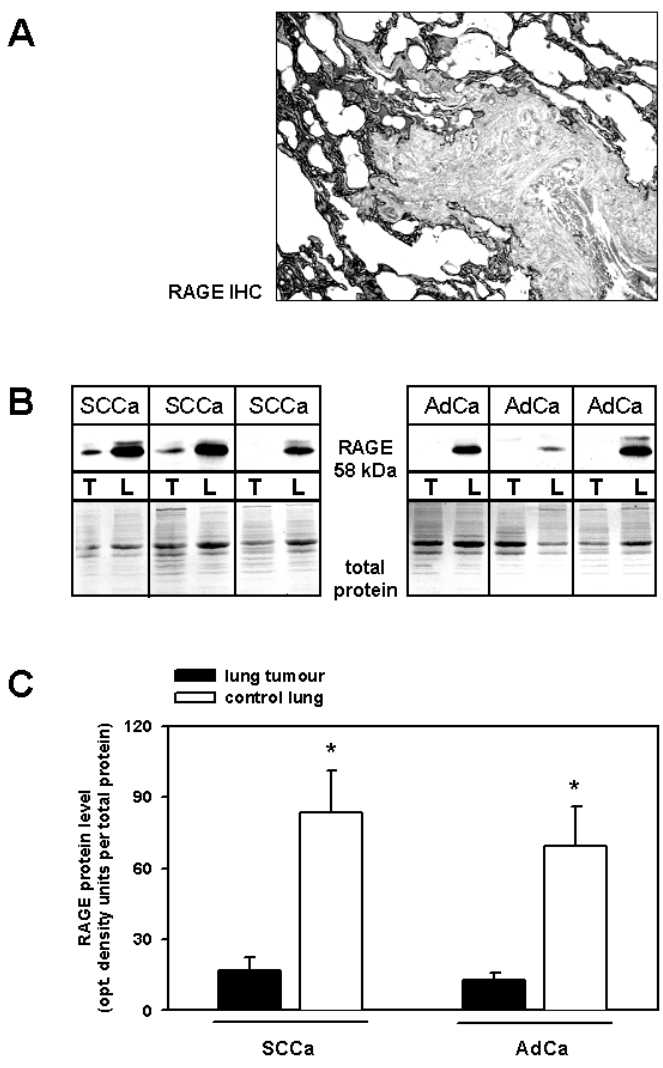

Fig. 2. (**A**) Immunohistochemistry of a representative lung adenocarcinoma sample indicates positive staining for RAGE in the surrounding non-tumour area, whereas tumour cells remained unstained. (**B**) Additionally, immunoblot analyses show a strong RAGE signal in the control lung (L), but less or no detection in the tumour specimens (T) from NSCLC patients with squamous cell lung carcinoma (SCCa) and adenocarcinoma (AdCa). Loading of the total protein amount is shown below. (**C**) Immunoblot quantification of NSCLC samples confirms reduced RAGE expression in AdCa and SCCa at a significant level. $^{*}P < 0.05$ versus control lung. Opt. density units, optical density units.

5-aza-2′-deoxycytidine, did not result in transcriptional reinduction of RAGE (data not shown). These experimental data suggest that down-regulation of RAGE in NSCLC does not result from typical changes during tumour progression.

While assessing which of the known RAGE ligands are expressed in lung tissue and whether there is a broad failure of the RAGE pathway in NSCLC, we showed expression of amphoterin, S100A1, S100A12, S100B and S100P. The mRNAs for amphotherin, S100A1 and S100P could be detected in human lung tumours and normal lung tissues using PCR analysis, but S100B and S100A12 mRNAs were not expressed (data not shown).

Proliferation of RAGE-expressing lung cancer cells in vitro and in vivo

On the basis of the low level of RAGE in NSCLC and other neoplasms it might be that down-regulation of RAGE supports tumour growth. Therefore, we performed *in vitro* experiments to study the impact of RAGE on the proliferation of lung cancer cells. Initially we tested the RAGE level in different human lung cancer cell lines (NCI-H322, NCI-A549 and NCI-H358). All cell lines were characterized by a low level of RAGE mRNA and even a lack of protein expression, compared with human lung tissue (data not shown). Finally, we chose NCI-H358 for overexpression of full-length human RAGE and of ΔcytoRAGE, which lacks the cytosolic domain

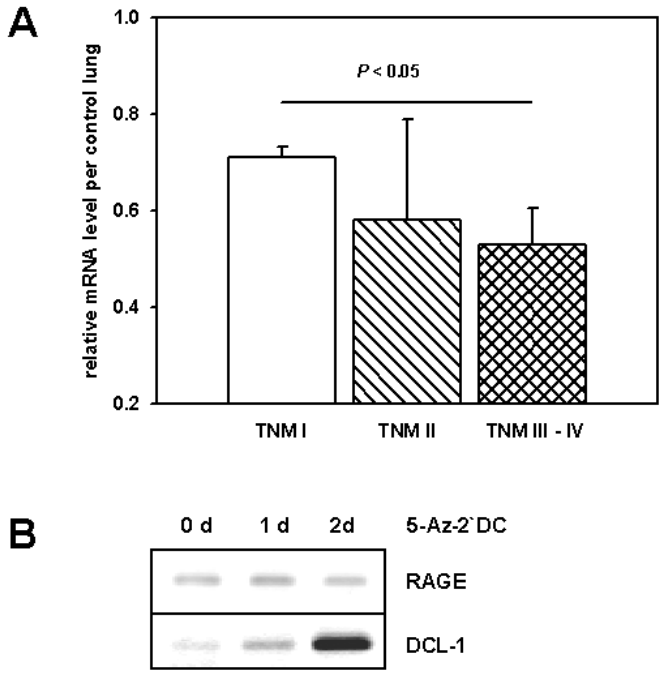

Fig. 3. (**A**) TNM stage-dependent expression of RAGE was evaluated from mRNA analyses of all NSCLC patients in relation to the RAGE level in the paired lung control. This ratio revealed a significant down-regulation of RAGE in NSCLC with increasing TNM stage. (**B**) Reduced RAGE mRNA expression cannot be induced by treatment of human lung carcinoma cells (NCI-H358) with 5-aza-2′-deoxycytidine, an inhibitor of DNA methylation, whereas DCL-1 mRNA (positive control) is re-induced in the presence of 5-aza-2′-deoxycytidine.

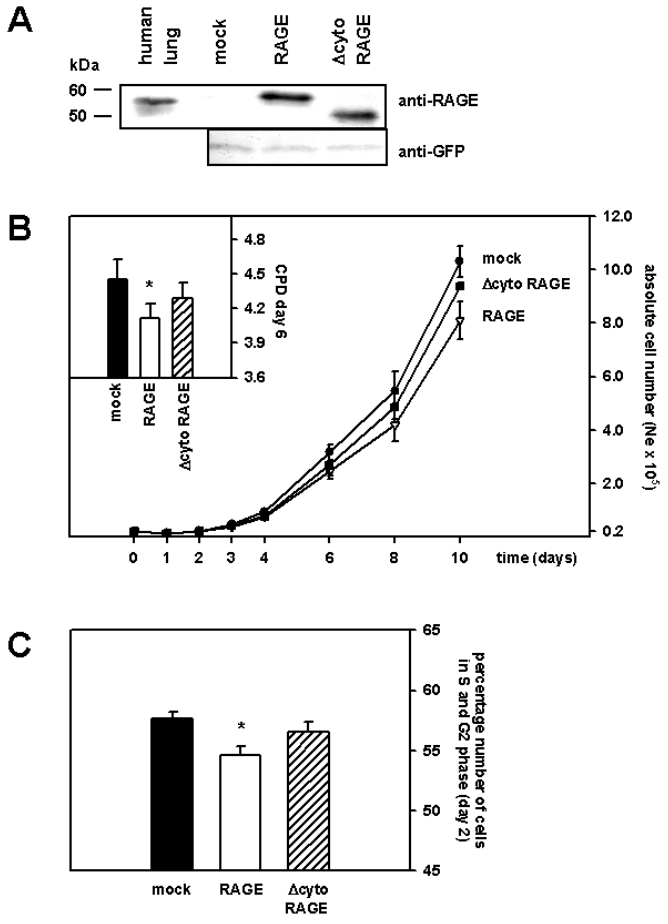

Fig. 4. (**A**) Full-length human RAGE and ΔcytoRAGE lacking the intracellular domain were inserted into the pIRES2-EGFP expression vector. Subsequent overexpression in NCI-H358 cells is demonstrated by immunoblot analysis compared with mock-transfected cells and human lung lysates. Antibody detection of GFP indicates equal protein loading. (**B**) Stable expression of RAGE results in reduced proliferation of NCI-H358 cells with a significant reduction in CPD after day 6 (left graph). (**C**) Cell cycle analyses revealed fewer RAGE-expressing cells in the S and G_2 phases at the beginning of exponential proliferation (day 2 after seeding). $^*P < 0.05$ versus mock control.

(Figure 4A). The stable expression of RAGE and ΔcytoRAGE cDNA in NCI-H358 had no influence on the level of amphoterin and S100A1 *in vitro*, as determined by PCR analysis (data not shown). As demonstrated in Figure 4B, overexpression of RAGE resulted in diminished proliferation of NCI-H358 cells *in vitro*. DNA analyses confirmed this observation, detecting a lower number of cells in the S and G_2 cell cycle phases for RAGE-transfected NCI-H358 compared with mock control cells (Figure 4C).

Cell behaviour and proliferation might be strongly coupled with cell–cell contacts and, consequently, we established a 3-dimensional spheroid cell culture to investigate proliferation of tumour cells in a more cancer-related *in vitro* model. Here we also observed diminished growth of RAGE-transfected cells compared with mutant ΔcytoRAGE NCI-H358 cells (Figure 5A). Surprisingly, spheroid growth of mock-transfected control cells was not significantly altered, as seen in monolayer cultures (Figure 5A). The s.c. implantation of NCI-H358 lung cancer cells confirmed this observation, showing that tumours derived from RAGE-transfected cells developed more slowly than those from ΔcytoRAGE-expressing cells, but they were not different from mock controls (Figure 5B). These data imply that RAGE re-expression in cancer cells does not exclusively impair tumour growth. Rather, it suggests that for RAGE signalling more factors are necessary, which are actively neutralized in mutant ΔcytoRAGE cells but not in normal NCI-H358 cells.

Cell morphology and subcellular localization of RAGE

Epithelial cells in the airways of the lung are attached to the basal lamina, a fibre network consisting of several extracellular compounds, including collagen. Therefore, we cultured NCI-H358 cells on collagen layers and observed a spreading, epithelial-like growth of RAGE- and ΔcytoRAGE-expressing cells that was not seen for NCI-H358 controls (Figure 6). This observation can be confirmed for spheroid culturing of RAGE- and ΔcytoRAGE-expressing cells, which, when placed on non-coated plastic dishes, start spreading after 7–10 days. This may be explained by accumulation of extracellular matrix compounds that are produced by the cell itself. However, overexpression of RAGE did not induce increased cell migration across a pore membrane as assessed by *in vitro* migration assays, whereas ΔcytoRAGE transfection did (data not shown).

Subsequently, immunocytochemistry was performed to determine the localization of RAGE in NCI-H358 cells. Fluorescence microscopy after staining with an antibody directed against the extracellular domain showed a strong membrane localization in single cells along with a clear redistribution of RAGE towards intercellular contact sites at higher cell densities (Figure 7, series I). The surface distribution of RAGE frequently appears scattered without a distinct boundary

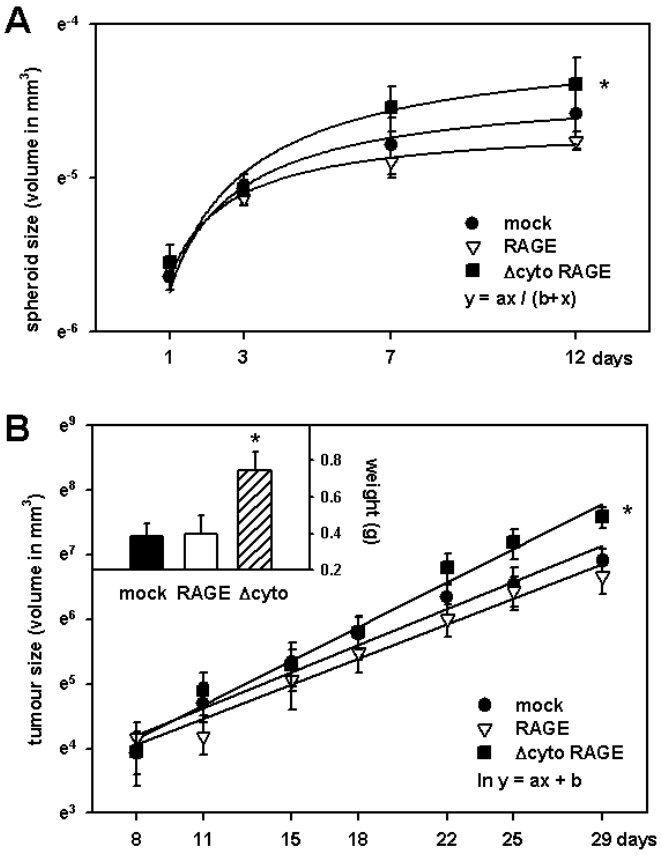

Fig. 5. (**A**) NCI-H358 spheroids consisting of 500 cells were cultured in single wells ($n = 20$ for each experiment). Spheroid growth was evaluated by estimating the mean volume on given days, which shows a reduced size of RAGE- but not of ΔcytoRAGE-expressing spheroids. $^{*}P < 0.05$ versus RAGE for the mean coefficient *a* calculated from regression for each experiment ($n = 8$). (**B**) Increased tumour growth of ΔcytoRAGE-transfected NCI-H358 cells after s.c. injection into athymic mice. $^{*}P < 0.05$ indicates differences for the mean coefficient *a* calculated from regression of ΔcytoRAGE and RAGE cells ($n \geq 7$). Final tumour weight additionally indicates larger tumours derived from ΔcytoRAGE-expressing NCI-H358 cells. $^{*}P < 0.05$ versus RAGE tumours and mock controls.

around the cell. This scattered RAGE staining can be detected with and without cell permeabilization, suggesting a clear membrane localization. Although the cytosolic domain is lacking in ΔcytoRAGE-expressing cells, the truncated receptor is also preferentially localized at cell–cell contacts (Figure 7, series II). NCI-H358 control cells were almost negative for RAGE staining, as already determined by immunoblotting (Figure 4A). Altogether, this observation suggests that RAGE signalling is not required for the formation of intercellular contacts.

Discussion

Recent application of transcriptional profiling to cancers has documented large numbers of differentially expressed genes in tissues derived from cancer patients, compared with those from healthy individuals or histologically normal tissues from the same individual (2,3). This observation has been made for many types of tumours, including lung carcinomas (28). In the present study we have demonstrated that the multiligand receptor RAGE is one of those genes differentially expressed in NSCLC. In paired samples derived from tumour tissue and histologically normal tissue from the same patient RAGE expression is strongly reduced at the mRNA and more strongly reduced or even lacking at the protein level in lung tumours. Moreover, down-regulation of RAGE mRNA is especially strong in patients with a high cancer stage (TNM stage III–IV carcinoma). Investigations of tumour samples and metastases derived from other origins also revealed a reduced level of RAGE in neoplastic tissues, suggesting that down-regulation of RAGE is not restricted to lung neoplasms. Recently, Schenk *et al*. identified polymorphisms in the promoter region of the *RAGE* gene that are associated with NSCLC (29). However, these differences in genotype can only be detected in a subset (21%) of NSCLC patients, which does not correlate with the common transcriptional down-regulation of RAGE observed. Because RAGE transcription is mainly under the control of NF-κB, which is induced in cancer (30), down-regulation of the corresponding transcription factor cannot be responsible for the lower level of RAGE mRNA expression in tumours. A further hallmark of cancer is a blockade of gene transcriptions by aberrant DNA methylation (1), which is why we investigated whether indirect inhibition of DNA hypermethylation with 5-aza-2′-deoxycytidine can re-induce RAGE mRNA expression. However, inhibition of DNA methylation, neither alone nor in combination with the histone deacetylase inhibitor scriptaid, was sufficient to re-induce RAGE transcription in a human lung cancer cell line.

As already mentioned, the decrease in RAGE protein seems to be more pronounced than that in mRNA, suggesting further post-transcriptional regulation. In this context, several truncated isoforms of RAGE have also been identified (24,25). Differential splicing of the precursor RAGE mRNA results in a transmembrane spanning N-truncated and several C-truncated (secretory) isoforms of RAGE which could contribute to a divergent cellular response. However, a change to alternative splicing producing one of those isoforms could not be detected in lung tumours compared with normal lung. While lower RAGE expression due to reduced levels of the gene-activating transcription factors NF-κB and Sp-1 (31) is unlikely in cancer cells, active repression of RAGE expression or other post-transcriptional mechanisms are also possible. With regard to the latter, Caballero *et al*. have recently identified a second polyadenylation sequence in the 3′-untranslated region of the *RAGE* gene generating a mRNA isoform with an adenylate plus thymidylate rich element that contributes to rapid degradation of RAGE mRNA (32).

The major ligand of RAGE in the lung might be amphoterin, which is also highly expressed in lung tissue. Amphoterin is a high mobility group I non-histone DNA-binding protein that can be secreted into the extracellular space during certain stages of development (33) or in necrotic cells, where it triggers inflammation (34). Moreover, amphoterin is secreted in response to pro-inflammatory stimulation of cells with cytokines (35) or endotoxin (36). Although it is unclear to what extent amphoterin is located in the extracellular space of tumour cells, the interaction of amphoterin with RAGE has been suggested to contribute to tumour growth and, especially, to invasive migration and metastasis (5,37). However, no studies using experimental approaches consider RAGE to be differentially expressed in tumours compared with the corresponding normal tissues, as was suggested by Schraml *et al*. some years ago (38). On the basis of this and our observations, whether RAGE takes over this special function in cancer

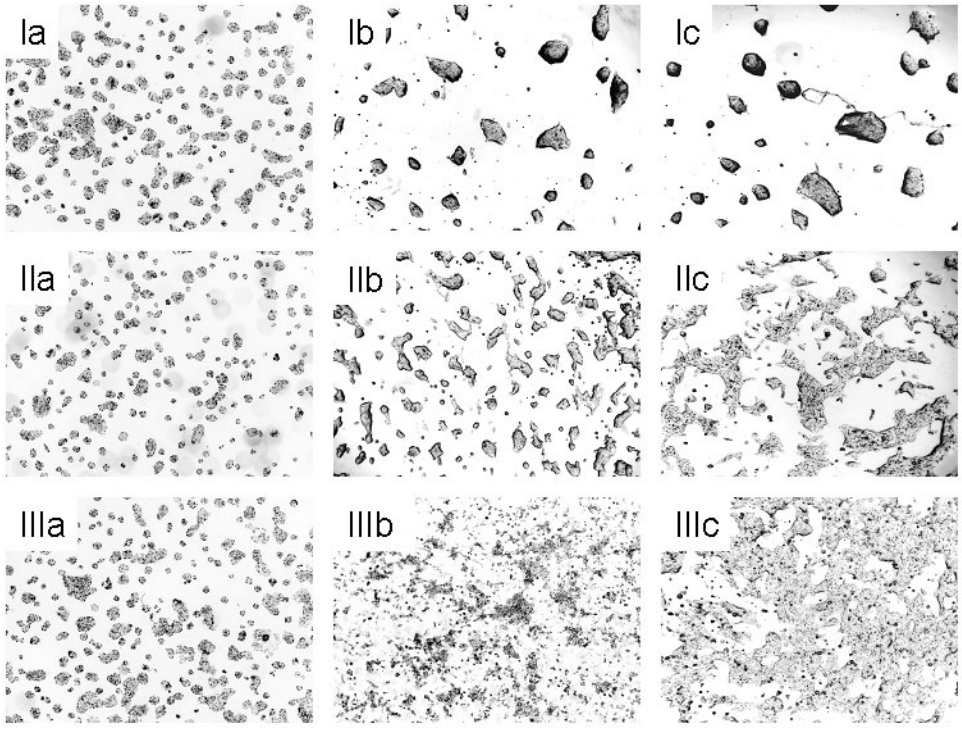

Fig. 6. NCI-H358 cells were cultured on tissue culture dishes without (a, 2 days) and with a collagen layer (b, 2 days; c, 5 days). Mock-transfected NCI-H358 cells (series I) had a rounded shape, whereas cells overexpressing RAGE (series II) and ΔcytoRAGE (series III) showed spreading growth on collagen.

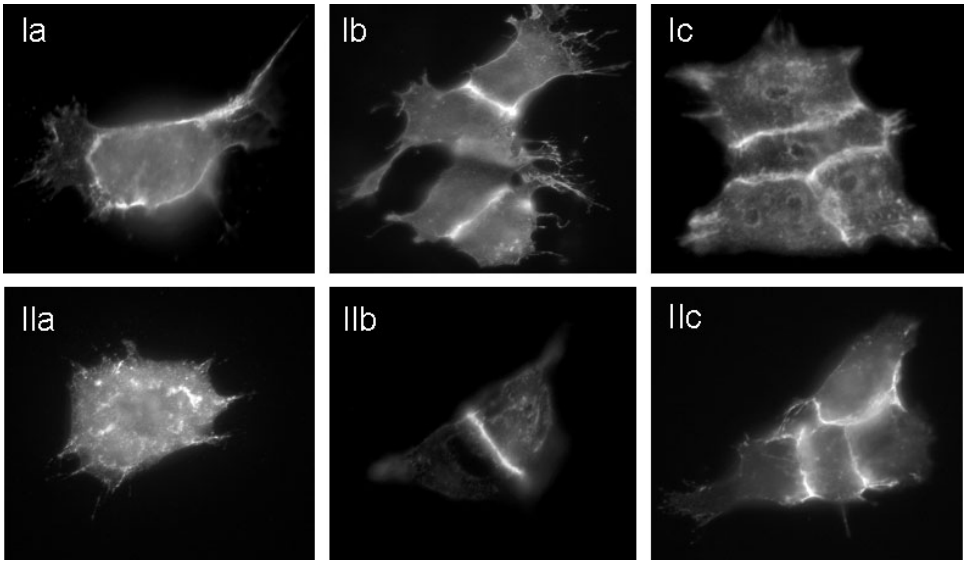

Fig. 7. (**A**) Immunocytochemistry of RAGE-overexpressing NCI-H358 cells indicating membrane localization of RAGE in single cells (Ia) with a clear redistribution of RAGE towards intercellular contact sites (Ib, Ic). Although the cytosolic domain is lacking in ΔcytoRAGE-expressing cells, the truncated receptor is also preferentially localized at cell–cell contact sites (IIb, IIc).

biology as suggested is speculation. Although it has been observed that RAGE levels correlate with the invasive behaviour of gastric cancer cells, we also detected a lower level of RAGE expression in primary tumours derived from the colon and rectum. Moreover, the low level of RAGE expression in metastatic tissue from gastric cancers and other metastases, as well as the reduction in RAGE in benign tumours, contradicts a biological function of RAGE activation in metastasis. In this context, our clinical data, as well as the lack of an increase in migration of RAGE-expressing lung cancer cells (NCI-H358) *in vitro*, support the observation of Huttunen *et al*. that RAGE activation does not contribute to tumour cell migration (16).

To further elucidate the down-regulation of RAGE in tumours we analysed the proliferation of NCI-H358 cells stably overexpressing RAGE and the cytoplasmic domain deletion variant ΔcytoRAGE. Through study of monolayer

cultures we revealed diminished proliferation of RAGE-expressing cells *in vitro*. Our findings are consistent with data from a neuroblastoma cell model showing that full-length, but not the cytoplasmic domain deleted, RAGE mediates the arrest of proliferation after receptor activation (13). Therefore, these results initially suggest a tumour-suppressive action of RAGE in lung cancer. *In vitro* spheroid cultures and *in vivo* tumorigenicity assays in athymic mice confirmed that lung cancer cells overexpressing RAGE also form smaller tumours compared with those overexpressing ΔcytoRAGE. However, they did not differ from normal NCI-H358 cells as observed in monolayer cell cultures. Despite the differences between 2- and 3-dimensional cell proliferation, we did not observe increased tumour formation in the presence of RAGE, an increase which has been seen for C6 glioma cells after injection into athymic mice (5) or for melanoma cells *in vitro* (39). On the contrary, we observed better tumour growth through blockage of RAGE signalling via the cytoplasmic domain deletion mutant ΔcytoRAGE. These conflicting observations in lung cancer cells clearly suggest a more complex network in response to RAGE–ligand interactions that is still poorly understood. In our study we have shown that several RAGE ligands, like amphotherin, S100A1 and S100P, are expressed in lung cancer cells whereas others are not detectable (S100B and S100A12). Taking into account the fact that RAGE can be activated by these ligands, there might be a synergistic effect between the pro-inflammatory action, on the one hand, and the pro-survival effect, on the other, as described for the RAGE-binding proteins S100 and amphoterin (34,40,41). It is also the case that the extracellular level and the constitution of RAGE-binding ligands might be dependent on the location of tumour cells and the microenvironment. Moreover, it has to be remembered that all known ligands of RAGE fulfil intra- and extracellular functions independent of RAGE (34,40). The induced expression of RAGE in cancer cells might change this balance to an unknown degree as a result of a preferred receptor interaction in overexpressing cells.

Despite some conflict regarding the importance of RAGE in tumour growth and invasive behaviour, it has to be borne in mind that the level of RAGE is greatly reduced in malignant tumours derived from lung and other organs and in also benign tumours. Based on immunocytochemistry (which demonstrates preferential localization of RAGE at cell–cell contacts), down-regulation of RAGE in tumours may be considered a critical step in tissue reorganization during tumour formation. Moreover, this intercellular localization clearly depends on the extracellular RAGE domain and can also be observed for mutant ΔcytoRAGE.

In adults normal epithelial cells are polarized and organized broadly into squamous sheets or secretory structures via intercellular attachments. Recently, the receptor RAGE has been identified as a marker protein for type I lung alveolar (ATI) cells which are polarized (42). RAGE is localized at the basolateral membrane of ATI cells and co-localizes with the ERBB2/HER2 receptor-interacting protein, another basolateral protein important for epithelial organization (43). Moreover, RAGE expression is enhanced in parallel with differentiation of ATI cells (43,44). Given that RAGE is associated with differentiation, cell polarity and organization of the epithelium, its decrease in lung tumours might contribute to the loss of polarization and differentiation. This assumption is strongly supported by our observation that RAGE contributes to cell growth as an epithelial layer on collagen, a major compound of the epithelial basement membrane. In contrast, RAGE-deficient lung cancer cells preferentially proliferate as a multicellular complex. Along with the detection of RAGE, and ΔcytoRAGE, at intercellular contact sites, this epithelial cell arrangement seems not to be dependent on expression of the intracellular RAGE domain. This suggests that there are more extracellular factors and/or surface membrane compounds that contribute (in combination with RAGE) to epithelial cell organization.

In summary, our findings indicate that down-regulation of RAGE in human NSCLC might contribute to a loss of epithelial tissue structure and concomitant oncogenic transformation without restriction of cell proliferation in epithelial carcinomas.

Acknowledgements

The authors are very grateful to A.Graul, S.Koitzsch and R.Donath for their technical assistance. We thank Dr A.Hammer for histological investigations of the tumour tissue and Dr R.Schinzel for help with the polyclonal RAGE antibody. This project was supported by a Wilhelm Roux grant of the BMBF (FKZ7/04) and, in part, by SFB 598 TPA5. The work was carried out in the Zentrum für Angewandte Medizinische Grundlagenforschung, Halle/Saale.

References

1. Hanahan,D. and Weinberg,R. (2000) The hallmarks of cancer. *Cell*, **100**, 57–70.
2. Golub,T., Slonim,D., Tamayo,P. *et al*. (1999) Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. *Science*, **286**, 531–537.
3. Yeang,C., Ramaswamy,S., Tamayo,P. *et al*. (2001) Molecular classification of tumor types. *Bioinformatics*, **17**, S316–S322.
4. Bhattacharjee,A., Richards,W., Staunton,J. *et al*. (2001) Classification of human lung carcinomas by mRNA expression profiling reveals distinct adenocarcinoma subclasses. *Proc. Natl Acad. Sci. USA*, **98**, 13790–13795.
5. Taguchi,A., Blood,D.C., del Toro,G. *et al*. (2000) Blockade of RAGE-amphoterin signalling suppresses tumour growth and metastases. *Nature*, **405**, 354–360.
6. Neeper,M., Schmidt,A.M., Brett,J., Yan,S., Wang,F., Pan,Y., Elliston,K., Stern,D. and Shaw,A. (1992) Cloning and expression of a cell surface receptor for advanced glycosylation end products of proteins. *J. Biol. Chem.*, **267**, 14998–15004.
7. Huttunen,H.J., Fages,C. and Rauvala,H. (1999) Receptor for advanced glycation end products (RAGE)-mediated neurite outgrowth and activation of NF-kappaB require the cytoplasmic domain of the receptor but different downstream signaling pathways. *J. Biol. Chem.*, **274**, 19919–19924.
8. Basta,G., Lazzerini,G., Massaro,M. *et al*. (2002) Advanced glycation end products activate endothelium through signal-transduction receptor RAGE: a mechanism for amplification of inflammatory responses. *Circulation*, **105**, 816–822.
9. Schmidt,A.M., Yan,S.D., Yan,S.F. and Stern,D.M. (2001) The multiligand receptor RAGE as a progression factor amplifying immune and inflammatory responses. *J. Clin. Invest.*, **108**, 949–955.
10. Huttunen,H., Kuja-Panula,J., Sorci,G., Agneletti,A., Donato,R. and Rauvala,H. (2000) Coregulation of neurite outgrowth and cell survival by amphoterin and S100 proteins through receptor for advanced glycation end products (RAGE) activation. *J. Biol. Chem.*, **275**, 40096–40105.
11. Hori,O., Brett,J., Slattery,T. *et al*. (1995) The receptor for advanced glycation end products (RAGE) is a cellular binding site for amphoterin. Mediation of neurite outgrowth and co-expression of rage and amphoterin in the developing nervous system. *J. Biol. Chem.*, **270**, 25752–25761.
12. Simm,A., Münch,G., Seif,F., Schenk,O., Heidland,A., Richter,H., Vamvakas,S. and Schinzel,R. (1997) Advanced glycation endproducts stimulates the MAP-kinase pathway in tubulus cell line LLC-PK1. *FEBS Lett.*, **410**, 481–484.
13. Huttunen,H.J., Kuja-Panula,J. and Rauvala,H. (2002) Receptor for advanced glycation end products (RAGE) signaling induces CREB-dependent chromogranin expression during neuronal differentiation. *J. Biol. Chem.*, **277**, 38635–38646.

14. Kuniyasu,H., Chihara,Y. and Kondo,H. (2003) Differential effects between amphoterin and advanced glycation end products on colon cancer cells. *Int. J. Cancer*, **104**, 722–727.
15. Takada,M., Koizumi,T., Toyama,H., Suzuki,Y. and Kuroda,Y. (2001) Differential expression of RAGE in human pancreatic carcinoma cells. *Hepatogastroenterology*, **48**, 1577–1578.
16. Huttunen,H.J., Fages,C., Kuja-Panula,J., Ridley,A.J. and Rauvala,H. (2002) Receptor for advanced glycation end products-binding COOH-terminal motif of amphoterin inhibits invasive migration and metastasis. *Cancer Res.*, **62**, 4805–4811.
17. Schraml,P., Shipman,R., Colombi,M. and Ludwig,C.U. (1994) Identification of genes differentially expressed in normal lung and non-small cell lung carcinoma tissue. *Cancer Res.*, **54**, 5236–5240.
18. Brett,J., Schmidt,A.M., Yan,S. *et al.* (1993) Survey of the distribution of a newly characterized receptor for advanced glycation end products in tissues. *Am. J. Pathol.*, **143**, 1699–1712.
19. Mountain,C.F. (1997) Revisions in the international system for staging lung cancer. *Chest*, **111**, 1710–1717.
20. Yuan,B., Jefferson,A., Baldwin,K., Thorgeirsson,S., Popescu,N. and Reynolds,S. (2004) DLC-1 operates as a tumor suppressor gene in human non-small cell lung carcinomas. *Oncogene*, **23**, 1405–1411.
21. Su,G., Sohn,T., Ryu,B. and Kern,S. (2000) A novel histone deacetylase inhibitor identified by high-throughput transcriptional screening of a compound library. *Cancer Res.*, **60**, 3137–3142.
22. Dell'Orco,R.T., Mertens,J.G. and Kruse,P.F.,Jr (1973) Doubling potential, calendar time and senescence of human diploid cells in culture. *Exp. Cell Res.*, **77**, 356–360.
23. Hofmann,H., Simm,A., Hammer,A., Silber,R. and Bartling,B. (2002) Expression of inhibitors of apoptosis (IAP) proteins in non-small cell human lung cancer. *J. Cancer Res. Clin. Oncol.*, **128**, 554–560.
24. Schlueter,C., Hauke,S., Flohr,A., Rogalla,P. and Bullerdiek,J. (2003) Tissue-specific expression patterns of the RAGE receptor and its soluble forms—a result of regulated alternative splicing? *Biochim. Biophys. Acta*, **1630**, 1–6.
25. Yonekura,H., Yamamoto,Y., Sakurai,S. *et al.* (2003) Novel splice variants of the receptor for advanced glycation end-products expressed in human vascular endothelial cells and pericytes and their putative roles in diabetes-induced vascular injury. *Biochem. J.*, **370**, 1097–1109.
26. Teratani,T., Watanabe,T., Kuwahara,F., Kumagai,H., Kobayashi,S., Aoki,U., Ishikawa,A., Arai,K. and Nozawa,R. (2002) Induced transcriptional expression of calcium-binding protein S100A1 and S100A10 genes in human renal cell carcinoma. *Cancer Lett.*, **175**, 71–77.
27. Keen,J., Yan,L., Mack,K., Pettit,C., Smith,D., Sharma,D. and Davidson,N. (2003) A novel histone deacetylase inhibitor, scriptaid, enhances expression of functional estrogen receptor alpha (ER) in ER negative human breast cancer cells in combination with 5-aza 2′-deoxycytidine. *Breast Cancer Res. Treat.*, **81**, 177–186.
28. Garber,M., Troyanskaya,O., Schluens,K. *et al.* (2001) Diversity of gene expression in adenocarcinoma of the lung. *Proc. Natl Acad. Sci. USA*, **98**, 13784–13789.
29. Schenk,S., Schraml,P., Bendik,I. and Ludwig,C.U. (2001) A novel polymorphism in the promoter of the RAGE gene is associated with non-small cell lung cancer. *Lung Cancer*, **32**, 7–12.
30. Darnell,J.,Jr (2002) Transcription factors as targets for cancer therapy. *Nature Rev. Cancer*, **2**, 740–749.
31. Li,J. and Schmidt,A. (1997) Characterization and functional analysis of the promoter of RAGE, the receptor for advanced glycation end products. *J. Biol. Chem.*, **272**, 16498–16506.
32. Caballero,J.J., Giron,M.D., Vargas,A.M., Sevillano,N., Suarez,M.D. and Salto,R. (2004) AU-rich elements in the mRNA 3′-untranslated region of the rat receptor for advanced glycation end products and their relevance to mRNA stability. *Biochem. Biophys. Res. Commun.*, **319**, 247–255.
33. Muller,S., Scaffidi,P., Degryse,B., Bonaldi,T., Ronfani,L., Agresti,A., Beltrame,M. and Bianchi,M.E. (2001) New EMBO members' review: the double life of HMGB1 chromatin protein: architectural factor and extracellular signal. *EMBO J.*, **20**, 4337–4340.
34. Scaffidi,P., Misteli,T. and Bianchi,M. (2002) Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature*, **418**, 191–195.
35. Czura,C., Wang,H. and Tracey,K. (2001) Dual roles of HMGB1: DNA binding and cytokines. *J. Endotoxin Res.*, **7**, 16625–16635.
36. Wang,H., Bloom,O., Zhang,M. *et al.* (1999) HMG-1 as a late mediator of endotoxin lethality in mice. *Science*, **285**, 248–251.
37. Kuniyasu,H., Oue,N., Wakikawa,A., Shigeishi,H., Matsutani,N., Kuraoka,K., Ito,R., Yokozaki,H. and Yasui,W. (2002) Expression of receptors for advanced glycation end-products (RAGE) is closely associated with the invasive and metastatic activity of gastric cancer. *J. Pathol.*, **196**, 163–170.
38. Schraml,P., Bendik,I. and Ludwig,C.U. (1997) Differential messenger RNA and protein expression of the receptor for advanced glycosylated end products in norma lung and non-small cell lung carcinoma. *Cancer Res.*, **57**, 3669–3671.
39. Abe,R., Shimizu,T., Sugawara,H. *et al.* (2004) Regulation of human melanoma growth and metastasis by AGE-AGE receptor interactions. *J. Invest. Dermatol.*, **122**, 461–467.
40. Donato,R. (2003) Intracellular and extracellular roles of S100 proteins. *Microsc. Res. Tech.*, **60**, 540–551.
41. Arumugam,T., Simeone,D., Schmidt,A. and Logsdon,C. (2004) S100P stimulates cell proliferation and survival via receptor for activated glycation end products (RAGE). *J. Biol. Chem.*, **279**, 5059–5065.
42. Shirasawa,M., Fujiwara,N., Hirabayashi,S., Ohno,H., Iida,J., Makita,K. and Hata,Y. (2004) Receptor for advanced glycation end-products is a marker of type I lung alveolar cells. *Genes Cells*, **9**, 165–174.
43. Bryant,P. and Huwe,A. (2000) LAP proteins: what's up with epithelia? *Nature Cell Biol.*, **2**, E141–E143.
44. Fehrenbach,H., Kasper,M., Tsherinig,T., Shearman,M., Schuh,D. and Mueller,M. (1998) Receptor for advanced glycation endproducts (RAGE) exhibits highly differential cellular and subcellular localisation in rat and human lung. *Cell. Mol. Biol.*, **44**, 1147–1157.

Received August 20, 2004; revised October 27, 2004; accepted November 1, 2004