Prognostic Potential and Tumor Growth–Inhibiting Effect of Plasma Advanced Glycation End Products in Non–Small Cell Lung Carcinoma

Babett Bartling,1 Hans-Stefan Hofmann,1,2 Antonia Sohst,1 Yvonne Hatzky,1 Veronika Somoza,3 Rolf-Edgar Silber,1 and Andreas Simm1

1Department of Cardio and Thoracic Surgery, University Hospital Halle (Saale), Martin Luther University Halle-Wittenberg, Halle (Saale), Germany; 2Department of Thoracic Surgery, Hospital Barmherzige Brüder Regensburg, Regensburg, Germany; and 3Research Platform for Molecular Food Science, University of Vienna, Austria

The plasma fluorescence related to the standard fluorescence of advanced glycation end products (AGEs) is a simple measurable blood parameter for distinct diseases but its importance in human cancer, including non–small cell lung carcinoma (NSCLC), is unknown. Plasma samples of 70 NSCLC patients who underwent resection surgery of the tumor were analyzed for the distinct AGE-related fluorescence at 370 nm excitation/440 nm emission. In a retrospective study, we tested the prognostic relevance of this AGE-related plasma fluorescence. The effect of circulating AGEs on the NSCLC growth was studied experimentally in vitro and in vivo. NSCLC patients with high (> median) AGE-related plasma fluorescence were characterized by a later recurrence of the tumor after curative surgery and a higher survival rate compared with patients with low plasma fluorescence (25% versus 47% 5-y survival, P = 0.011). Treating NSCLC cell spheroids with patients' plasma showed an inverse correlation between the growth of spheroids in vitro and the individual AGE-related fluorescence of each plasma sample. To confirm the impact of circulating AGEs on the NSCLC progression, we studied the NSCLC growth in mice whose circulating AGE level was elevated by AGE-rich diet. In vivo tumorigenicity assays demonstrated that mice with higher levels of circulating AGEs developed smaller tumors than mice with normal AGE levels. The AGE-related plasma fluorescence has prognostic relevance for NSCLC patients in whom the tumor growth-inhibiting effect of circulating AGEs might play a critical role.

Address correspondence and reprint requests to Babett Bartling, Klinik für Herz- und Thoraxchirurgie, Universitätsklinikum Halle (Saale), Ernst-Grube-Str. 40, D-06120 Halle (Saale), Germany. Phone: +49 345 345 557 3314; Fax: +49 345 345 557 7070; E-mail: babett.bartling@medizin.uni-halle.de.

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associated with oxidative stress (10,11), tobacco smoking (12) and an impaired detoxification of AGE precursors (13). Furthermore, the physiological amount of circulating AGEs depends on nutritional habits including an increased intake of AGE-rich (thermally processed) food (7,14,15) and sugar molecules with higher responsiveness to AGE formation (fructose instead of glucose) (16). Thus, several intrinsic and extrinsic factors determine the biological level of AGEs for each person. Elevated AGE levels are discussed mostly to play an adverse role in the process of degenerative diseases (15,17), but the threshold between physiological and pathophysiological range of the circulating AGEs has not yet been defined. Moreover, a number of nutritional studies indicate a favorable effect of food-derived AGEs, such as an enhanced antioxidant capacity and increase in chemopreventive enzymes (18–20).

The importance of AGEs in lung carcinomas is studied for extracellular matrix proteins showing an impact of the AGE-modified tissue matrix on the invasive migration of cancer cells (21). This observation is also supported by clinicopathological studies indicating a reduced tumor progression in response to age (22) and diabetes mellitus (23–25), both of which are characterized by an increase in AGE-modified tissue matrix (26). In contrast, the clinical importance of the circulating AGEs in patients with lung carcinomas or other types of cancer is largely unknown. Therefore, we were interested in the importance of the AGE-related fluorescence for the prognosis of NSCLC patients after curative surgery and, if so, in the impact of circulating AGEs on the NSCLC growth.

MATERIAL AND METHODS

Patients and Animals

Plasma samples were obtained from 78 consecutive NSCLC patients who underwent a pulmonary resection surgery during the hospitalization prior to the surgical procedure. Eight patients were excluded from the analysis because they either died due to a non–tumor-related disease or within 30 d after surgery. Tumor stages were classified according to the TNM system (1) and patients’ data were recorded up to 5 years after surgery. This study was approved by the Ethics Committee of the Medical Faculty (Halle [Saale], Germany).

Blood and tissue samples were also obtained from female athymic mice (NMRI nu/nu; Harlan Winkelmann, Borchen, Germany) who had received a specific diet for modifying the blood AGE level. All mice were kept under clean room conditions in sterile isolator cages. At an age of about 4 wks, 13 mice of the control group received standard diet (70% w/w) protein-free standard chow [C1004; Altromin, Lage, Germany], 15% casein, 15% starch), whereas 13 mice of the AGE group received an AGE-rich diet (70% protein-free standard chow, 15% casein, 15% bread crust as AGE-rich compound). All mice were fed each day with an average of about 6 g of the diet per capita. The preparation and partial characterization of the AGE-rich bread crust from rye/wheat-mixed bread was described earlier (27). After a feeding period of 21 d, mice were killed by cervical dislocation. Blood was removed after thoracotomy and a drop of the blood was used for preparing blood films on glass slides. The remaining blood was transferred to microvettos. Lung and liver were removed, frozen in liquid nitrogen and stored at −80°C until use. The local Animal Care and Use Committee, Halle (Saale) approved this study.

In Vivo Tumorigenicity Assay

Female athymic mice (NMRI nu/nu; Harlan Winkelmann) were used for the in vivo tumorigenicity assay. According to the feeding experiments described above, mice received a specific diet for modifying the blood AGE level. At an age of 4 wks, 16 mice of the control group received a standard diet and 16 mice of the AGE group received an AGE-rich diet. After a prefeeding period of 14 d, NSCLC cells were injected subcutaneously into each upper flank of athymic mice. Thus, 32 tumors in each diet group were studied. The National Cancer Institute (NCI) cell line H358 (ATCC, Manassas, VA, USA) was used as NSCLC cell line. H358 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (Invitrogen, Karlsruhe, Germany) at 37°C in a humidified 10% CO₂ atmosphere. For tumor cell injection, H358 cells were harvested from 80% confluent monolayers by brief trypsinization, washed and resuspended in ice cold PBS. Then, 2 × 10⁶ H358 cells per site were implanted subcutaneously, and the respective diet was continued during the period of tumor growth. Length and width of the tumors were measured twice a week for the duration of the experiment using a sliding caliper. Individual tumor volume was calculated as the product of 4/3π × (length/2) × (width/2)² that corresponds to the equation of an ellipsoid. Mice were killed at day 25 of the tumor growth, and the tumors were dissected carefully and weighed. One aliquot of the tumor tissue was frozen in liquid nitrogen and stored at −80°C until used. Another aliquot was fixed in 4% phosphate-buffered formaldehyde and embedded in paraffin. Throughout the experiment, all mice were kept 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 of the local Animal Care Committee, Halle (Saale).

Blood Analyses

Blood films on glass slides were analyzed for the cytological composition of the blood samples following Giemsa staining and light microscopy. The AGE-specific fluorescence was determined for serum samples of the mice and plasma samples of the NSCLC patients. Mice serum was diluted 1:5 and human plasma was diluted 1:20 in PBS (optimal dilution was tested before); 100 μL of each sample was transferred per well of
AGE-RELATED PLASMA FLUORESCENCE IN NSCLC

...was added to a final concentration of 28 randomly selected NSCLC patients completed after 24 h. Thereafter, plasma formation of H358 spheroid was well suspension culture plate (Greiner). per spheroid into a single well of a 96-calf serum (Invitrogen). Then, H358 cells 5 mg/mL methylcellulose (Sigma, Straubenhardt, Germany). Briefly, 1 μL of the serum sample was spotted in triplicate onto nitrocellulose membranes using the Minifold Spot Blot Unit (Schleicher & Schüll, Dassel, Germany). After blocking the membrane with 6% nonfat dry milk in TBS buffer containing 0.15% polyethyleneorbitan monolaurate, CML levels were detected by using a rabbit polyclonal anti-CML antibody (ED Schleicher, University of Tuebingen, Germany) followed by a horseradish peroxidase-conjugated anti–rabbit IgG antibody (Dianova, Hamburg, Germany) and enhanced chemiluminescence detection (Amer sham Bioscience, Buckinghamshire, UK). The signals were evaluated by use of the LAS 3000 computer-based imaging system (FUJI Film; Tokyo, Japan) and AIDA 3.5 software (Raytest, Straubenhardt, Germany).

Cell Culture

For three-dimensional cell culture, spheroids were formed from H358 lung carcinoma cells in DMEM containing 5 mg/mL methylcellulose (Sigma, Deisenhofen, Germany) and 2.5% fetal calf serum (Invitrogen). Then, H358 cells were seeded to a final number of 300 cells per spheroid into a single well of a 96-well suspension culture plate (Greiner). The formation of H358 spheroid was completed after 24 h. Thereafter, plasma of 28 randomly selected NSCLC patients was added to a final concentration of 7.5%. We studied 10 spheroids per individual plasma for 2 d. The diameter of each spheroid was estimated by using the Axiostar M200 microscope (Carl Zeiss, Jena, Germany) equipped with Spot Camera and Metamorph 4.6.5. software (Visitron Systems, Puchheim, Germany) and subsequently averaged. The spheroid volume was calculated as a product of 4/3π × diameter³ and the mean increase in the spheroid volume was determined for the individual plasma samples in three time-independent experiments. Accordingly, we studied the effect of serum taken from mice with or without AGE-rich diet as well as AGE-modified FCS (AGE-FCS). AGE-FCS was generated by incubating FCS (Invitrogen) with 20 mmol/L glyoxal (Sigma, Deisenhofen, Germany) and control FCS without glyoxal at 37°C for 2 d. Thereafter, control FCS and AGE-FCS were extensively dialyzed against water using ZelluTrans dialysis tubing (Roth, Karlsruhe, Germany). The AGE-related fluorescence was determined as described above.

We also studied the effect of AGE-FCS on the cell proliferation and migration in two-dimensional assays. As the FCS supply of cells in two-dimensional cultures is better than in spheroids, we used less FCS in these assays (5% control FCS or AGE-FCS; tested before). H358 cell proliferation (5,000 cells per well of a 96-well culture plate) was spectrophotometrically determined after 3 d using the alamarBlue reagent (Biosource Europe, Nivelles, Belgium). Moreover, the migration/proliferation of H358 cells was studied in an in vitro wound scratch model. Six scratches per experiment were placed into a monolayer of confluent cells by a pipette tip to generate defined cell-free areas. The distances between the two edges of the migrating/proliferating cells were estimated microscopically. Ten distances per scratch were always measured and finally averaged.

Expression Analyses

For RNA expression analysis, total RNA was isolated with Trizol reagent (Invitrogen), cleaned up (RNeasy Mini Kit; Qiagen, Hilden, Germany) and submitted to high-density oligonucleotide microarray (Affymetrix, Santa Clara, CA, USA) as described previously for human lung tissues (29) and H358 cells (30). The intensity of the hybridization signals was scanned with the GeneArray Scanner 7G and analyzed by using the GeneChip Operating System. For protein expression analysis, standard immunoblot was performed as described (30). Monoclonal mouse anti–Ost-48 antibody was purchased from Santa Cruz Biotechnology (sc-74408; Santa Cruz, CA, USA).

Mouse Tissue Analyses

The vascularization of tumor tissues from the xenografts was determined for tissue sections by staining of endothelial cells for the von Willebrand Factor (vWF) using a rabbit polyclonal anti-vWF (F3520; Sigma, St. Louis, MO, USA) and labeled-streptavidin-biotin detection (DAKO, Hamburg, Germany). Slides were embedded and the vessel density per area was evaluated for two sections each tumor by using the Axiostar microscope (Carl Zeiss) equipped with Spot Camera and Metamorph 4.6.5. software (Visitron Systems). In addition, we isolated and quantified the total protein of mouse lung and liver by SDS lysis. This protein was subjected to the slot blot procedure and subsequent antibody staining for carboxymethyl lysine (CML) as entirely described for the mouse serum. Data were calculated as arbitrary units (aU) per μg of the protein.

STATISTICS

Patient data are given either as the median (M) with interquartile range or as the total or percentage number (n). Experimental data are reported as mean ± SD or SEM. All statistical calculations and data presentations were performed by use of the SigmaStat 3.5 and SigmaPlot 10 software (Systat Software Inc., San Jose, CA, USA). The appropriated statistical test is given in the legend of tables and figures respectively. P values ≤0.05 indicate a significant difference.
RESULTS

Plasma Fluorescence and Patients’ Outcome

The retrospective clinical study included plasma samples from NSCLC patients treated by pulmonary resection surgery. None of the patients had distant metastasis, resection stage R2 or died owing to a non–tumor-related disease. The plasma fluorescence was measured at the standard AGE-related fluorescence (370 nm excitation/440 nm emission), and the median plasma fluorescence had been determined with 1.08 μg/mL AGE-BSA (range: 0.73 to 1.61 μg/mL AGE-BSA). According to this median value, the NSCLC patients were divided into patients with either low or high plasma fluorescence. Subsequent analyses of the clinical-pathological data did not find significant differences for age, gender, smoking status, secondary diseases (diabetes mellitus, renal failure) and NSCLC-related parameters such as T and N stages (Table 1). In contrast, we revealed that NSCLC patients with high plasma fluorescence had a significantly better outcome (relapse-free interval and survival) after curative surgery than patients with low plasma fluorescence (Figure 1A). Even within the group of NSCLC patients without nodal involvement (N0), the 5-year survival had been determined as only 30% for patients having low plasma fluorescence compared with a 5-year-survival rate of about 70% for patients with high plasma fluorescence ($P = 0.015$).

On the basis of our observation (see Figure 1A and Table 1), NSCLC patients were also subdivided into quartiles (QI to QIV) according to the level of their plasma fluorescence. Subsequent Kaplan-Meier analyses showed that particularly patients with very low AGE fluorescence (QI) had the lowest survival rate (Figure 1B). Because QI patients did not show a lower body mass index (median BMI of 25.6 compared with median BMI of 26.0 for all patients), a low dietary intake of AGEs due to malnutrition is unlikely for this patient group. Although the survival of NSCLC patients was improving along with an increasing plasma fluorescence (QI to QIII), the survival of patients with a very high plasma fluorescence (QIV) was diminished again (see Figure 1B).

Plasma Fluorescence, Advanced Glycation End Products and Tumor Growth In Vitro

Our clinical data suggest that plasma compounds mediating the AGE-related fluorescence may have an influence on the NSCLC progression. Therefore, we used the multicellular spheroid model to analyze the effect of patients’ plasma on the tumor growth in vitro depending on the individual’s plasma fluorescence. Tumor spheroids were formed from the NSCLC cell line H358, which is suitable for this in vitro growth assay because H358 cells form stable spheroids without

### Table 1. Patients’ characteristics depending on the plasma AGE-related fluorescence (FL).a

| NSCLC patients parameter | Data | Plasma FL | P valuec |
|--------------------------|------|-----------|----------|
|                          |      | Lowb (n = 36) | Highb (n = 34) |        |
| Plasma FL, μg/mL AGE-BSA| M    | 0.81 (0.73–0.95) | 1.29 (1.21–1.61) | <0.001 |
| Age, y                   | M    | 69 (60–72) | 68 (60–73) | 0.797   |
| Gender                   |      |           |           |         |
| Male                     | n    | 31        | 22        | 0.071   |
| Female                   | n    | 5         | 12        |         |
| Body mass index          | M    | 26 (24–28) | 27 (24–29) | 0.813   |
| NSCLC histology          |      |           |           |         |
| ADCd                     | n    | 11        | 19        | 0.059   |
| SCCe                     | n    | 21        | 12        |         |
| Others                   | n    | 4         | 4         |         |
| Tumor (T) stage          |      |           |           |         |
| T1                       | n    | 5         | 12        | 0.113   |
| T2                       | n    | 20        | 14        |         |
| T3-4                     | n    | 11        | 8         |         |
| Nodal (N) stage          |      |           |           |         |
| N0                       | n    | 15        | 21        | 0.290   |
| N1                       | n    | 9         | 6         |         |
| N2                       | n    | 11        | 7         |         |
| Resection (R) stage      |      |           |           |         |
| R0                       | n    | 31        | 33        | 0.199   |
| R1                       | n    | 5         | 1         |         |
| Smoker                   |      |           |           |         |
| Current                  | n    | 18        | 12        | 0.199   |
| Former                   | n    | 12        | 16        |         |
| None                     | n    | 6         | 6         |         |
| Diabetes mellitus        |      |           |           |         |
| Yes                      | n    | 10        | 9         | 1.000   |
| No                       | n    | 26        | 29        |         |
| Blood glucose, mmol/L    | M    | 5.5 (4.9–7.0) | 5.3 (4.9–6.9) | 0.812   |
| Renal failure            |      |           |           |         |
| Yes                      | n    | 3         | 4         | 1.000   |
| No                       | n    | 33        | 30        |         |
| Blood creatinine, μmol/L | M    | 79 (69–85) | 77 (70–85) | 0.897   |
| Blood urea, mmol/L       | M    | 5.3 (4.6–6.5) | 5.7 (4.6–6.3) | 0.668   |

aData are either given as median (M) with interquartile range or as number of NSCLC patients (n).

bCutoff point at the median plasma FL of all NSCLC patients (1.08 μg/mL AGE-BSA).

CP values were calculated by use of the Student t test (median differences) or the χ² test (homogeneity of variances) as appropriated.

dADC, adenocarcinoma.

eSCC, squamous cell carcinoma.
simultaneous migration of single cells from the united cell structure (21). H358 spheroids were treated individually with plasma of randomly selected NSCLC patients from our clinical study group (Table 1). Subsequent analyses of the spheroid growth revealed an inverse correlation between tumor spheroid growth in vitro and the level of the AGE-related fluorescence of the individual plasma sample (Figure 2A).

This inverse correlation could be caused by AGE modifications of plasma compounds, such as growth factors, thereby impairing their functionality. Therefore, we studied the effect of AGE-FCS (AGE-related fluorescence corresponds to 4.35 μg/mL AGE-BSA versus 1.01 μg/mL AGE-BSA in control FCS) on the proliferation and migration of H358 cells in a proliferation and wound scratch assay, respectively. These assays showed a reduced cell proliferation and migration in response to AGE-FCS (Figure 2B). The antiproliferative effect of AGE-FCS is still more obvious by decreasing the FCS concentration (2% instead of 5%; data not shown). An impact of AGE-FCS on the viability of H358 cells has not been observed. The growth of H358 spheroids also was influenced strongly by AGE-FCS (Figure 2C).

As plasma AGEs also could mediate direct biological functions by interacting with defined receptors, we additionally studied the expression of AGE receptors in human lung carcinoma. This study showed a high mRNA expression of the AGE-Rs 1, 2 and 3 in H358 cells and NSCLC tissues. While both AGE-R1 and AGE-R2 are increased in NSCLC tissues compared with control (healthy) lung, AGE-R3 is not differentially expressed in NSCLC (Table 2). The high expression of AGE-R1 in NSCLC also has been observed by immunoblot analysis (data not shown). In contrast, RAGE was downregulated significantly in H358 cells and NSCLC tissues compared with control lung at mRNA (Table 2) and protein level (31).

**Advanced Glycation End Products and Tumor Growth In Vivo**

Our in vitro data suggest an association between the plasma level of fluorescent AGES and the tumor growth (see Figures 2A–C). Therefore, we established a xenograft model (athymic NMRI nu/nu mice) with a physiologically increased level of circulating AGEs. In the first experiment, athymic mice were fed with AGE-rich food (32,33) without injection of the NSCLC cell line H358 (group 1, Figure 3A). After 21 d of the specific diet, we removed blood and tissue from lung and liver for further analyses. In the case of mice, serum instead of plasma was prepared because of the immediate blood coagulation after thoracotomy. This initial feeding experiment showed that AGE-rich diet mediates an increased level of fluorescent AGES in the mouse serum compared with serum from mice receiving control diet (Table 3). We also determined a slightly increased serum level of the nonfluorescing AGE variant N-ε-carboxymethyllysine (CML) in response to the AGE-rich diet, whereas the tissue level of CML was not influenced by the diet (Table 3). The AGE-rich diet had no impact on the weight and the number of white blood cells (granulocytes, lymphocytes, monocytes) (Table 3).

Our previous studies already demonstrated an antiproliferative effect of AGE-rich food in H358 cells (34). As the gastrointestinal digestion alters the food-AGEs regarding quality and quantity, we now treated NSCLC spheroids from H358 cells with the individual serum of mice receiving either AGE-rich or control diets. In accordance with the in vitro experiments using patients’ plasma (see Figure 2A), we determined an inverse correlation between the growth of H358 spheroids in vivo and the level of AGE-related fluorescence of the individual serum sample (Figure 3B,
This inverse correlation was related mainly to the serum from mice receiving AGE-rich diet (see Figure 3B, above). In contrast, correlating the growth of H358 spheroids in vivo and the CML level of the individual serum did not reach statistical significance (Figure 3B, below). In the second experiment, athymic mice were fed with an AGE-rich diet to study the impact of circulating AGEs on the tumor growth of H358 cells in vivo (group 2, Figure 3A). The in vivo tumorigenicity assay demonstrated that the H358 tumor growth is less pronounced in the group of mice receiving the AGE-rich diet compared with the control group (Figure 4A, above). In other words, mice with an AGE-poor diet (control) developed more frequently large tumors than mice with an AGE-rich diet (Figure 4A, below). In accordance with the different tumor volume, the mean weight of H358 tumors was reduced significantly in response to the AGE-rich diet, whereas the tumor vascularization was not influenced (Figure 4B).
time. A similar tendency was observed for the relapse-free period after surgery. The highest survival rate after 5 years (60%) has been determined for NSCLC patients having a moderately increased AGE-related fluorescence of plasma samples, whereas this positive correlation was partially abolished in patients with a very high AGE-related fluorescence (35%). Although their 5-year survival was still significantly higher than the survival of patients with very low AGE-related plasma fluorescence (10%), the concentration-dependent observation supports the pathophysiological role of highly increased AGE levels (17,36,37). It is well conceivable that an excess of AGEs induces oxidative stress by exhaustion of antioxidative defense systems (10,11), thereby supporting the tumor progression again. This relation has been suggested recently for patients with advanced, compared with early, breast cancer (38). In this context, pathophysiologically high levels in fluorescent AGEs also have been described for blood samples derived from smokers (12) and patients with chronic renal failure (3,6,35) or untreated diabetes mellitus (4,5). However, none of these factors affected the AGE-related plasma fluorescence in our patients’ group. Some contrasting data regarding the plasma level of AGEs may be explained by the changing smoking behaviors of patients during the time of hospitalization, the few number of patients with renal failure, the well-controlled medical regime in the hospital (diabetes patients) and the concomitant NSCLC disease.

Our clinical observation suggested a protective effect of plasma AGEs on the tumor progression. This can be supported indirectly by previous studies describing an inhibiting effect of reactive carbonyls, which are potential substances for AGE formation, on tumor growth in vitro (39–41). However, in breast cancer, elevated plasma levels of protein carbonyls were associated with an increased cancer progression (38). Although carbonyls also might affect tumor growth via cellular actions different from AGE

| Characteristics          | Diet                  | P value |
|-------------------------|-----------------------|---------|
|                        | Control (n = 13) | AGE (n = 13) |
| Weight                  |                       |          |
| Start, g               | 12 ± 1.5              | 13 ± 2.5 | 0.175 |
| End, g                 | 20 ± 1.6              | 21 ± 1.9 | 0.140 |
| Serum FL, μg/mL AGE-BSA| 348 ± 65              | 402 ± 87 | 0.042 |
| Serum CML, μg/mL AGE-BSA| 46 ± 1.9             | 49 ± 2.3 | 0.051 |
| Blood                   |                       |          |
| Granulocytes, %         | 26 ± 13               | 27 ± 13  | 0.899 |
| Lymphocytes, %          | 72 ± 13               | 72 ± 15  | 0.905 |
| Monocytes, %            | 1.8 ± 1.9             | 1.8 ± 2.0| 1.000 |
| Tissue                  |                       |          |
| Lung CML, aU/μg         | 12 ± 2.7              | 12 ± 2.5 | 0.903 |
| Liver CML, aU/μg        | 11 ± 4.1              | 9.7 ± 2.0| 0.387 |

Table 3. Characteristics of group 1 athymic NMRI nu/nu mice with AGE-rich diet. a

aAll data are given as mean ± SD. P values were calculated by use of the Student t test.
actions (42,43), further findings again support the antitumorigenic effect of AGEs. In this regard, experimental and pathological studies especially indicate an effect of extracellular tissue matrix modified with AGEs on tumor progression (21,23).

Quantity and quality of the large number of AGE variants are suggested as crucial factors that finally determine the biological role of AGEs (8,17,19), but what does cause the different levels of AGEs, including fluorescent AGEs, in human plasma? Besides dietary habits (14,17,44), a large number of endogenous factors influence the level of fluorescent AGEs in human blood circulation. This includes the degree of glucose and lipid metabolism with the subsequent formation of reactive carbonyls (26), the activity of enzymatic detoxification systems (45), oxidative stress (11), receptor-mediated AGE uptake by cells (46), targeted degradation of AGE-modified proteins by the proteasomal system (47) and renal excretion (48). In normal conditions, generation and clearance of AGEs are in balance sustaining an AGE homeostasis (17). Low plasma levels of fluorescent AGEs, as observed for our NSCLC patients’ group with worse outcome after curative surgery, may result from a low metabolic formation of AGEs, hypoalbuminemia, high detoxification of reactive carbonyls, low dietary intake of AGEs and/or high degradation and excretion of AGEs.

In contrast, moderately higher levels in plasma AGEs may result from a higher metabolic formation of AGEs and/or dietary intake of AGEs. The moderate increase in the AGE-related plasma fluorescence indicated a better disease-free survival of NSCLC patients, suggesting a protective effect of circulating AGEs and/or secondary factors accompanied by an increased level of circulating AGEs on the reoccurrence and progression of NSCLC. Therefore, plasma samples of NSCLC patients were subjected to in vitro studies. In correspondence with the prognostic relevance of fluorescent AGEs, treating NSCLC spheroids with patients’ plasma showed an inverse correlation between the growth of spheroids and the individual AGE-related fluorescence of each sample. This inverse correlation of fluorescent AGEs with the growth of NSCLC spheroids also has been observed for blood samples from mice, whose circulating AGE level was moderately elevated by an established dietary model using AGE-rich bread crust (32,33). AGE-rich bread crust did not induce oxidative stress in NSCLC cells (34), and the diet with bread crust caused an increase in circulating AGE chromophores of 15% that corresponds approximately with the increase in plasma AGE chromophores determined for NSCLC patients with moderately higher levels (1.21 μg/mL AGE-BSA versus the cutoff value at 1.08 μg/mL AGE-BSA). Although the AGE-rich diet also caused a small increase in the nonfluorescent AGE variant CML, the level of plasma CML did not correlate with the spheroid growth. This might be based on the insignificant impact of the single compound CML on the cell proliferation compared with fluorescent AGEs as a set of AGE variants.

Nonetheless, our in vitro data indicate the impact of plasma rich in fluorescent AGEs on the NSCLC growth. This has been supported by our in vivo studies showing that the chronic intake of AGE-rich food was associated with a reduced subcutaneous growth of NSCLC cells in about 30% of athymic mice (final tumor weight; number of large tumors). The reduced tumor growth of NSCLC cells in

Figure 4. Effect of AGE-rich diet on the growth of lung carcinoma cells in vivo. (A) Tumor growth of H358 cells subcutaneously implanted into athymic mice receiving either control or AGE-rich diet. The individual regression coefficient is calculated as determinant of the tumor growth per time. In addition, H358 tumors were subdivided in small and large tumors (cut off at the median volume), and the total number and percentage of tumors with either small or large size was calculated. (B) Weight and vascularization of H358 tumors in mice with or without AGE-rich diet. Data are given as mean ± SEM. P values were calculated by the Student t test.
mice without affecting tumor vascularization as well as the inverse correlation between spheroid growth of NSCLC cells and the AGE-related fluorescence of plasma samples, suggest the antiproliferative effect of AGEs. Moreover, circulating AGEs also may have an antimitotic effect as suggested by the data of our wound scratch assay. Although the detailed chemical structures, digestion and absorption of food-derived AGEs are still incompletely understood, it seems to be clear that particularly low-molecular weight AGEs pass the colon (49,50).

Moreover, other compounds than the food-derived AGE chromophores may contribute to the biological effect, because an increased intake of AGEs could also modify the AGE generation in vivo and other factors. In this context, cell surface receptors are identified, which are believed to mediate the biological function of extracellular AGEs (51), among these are the receptors AGE-R1, better known as OST-48, and RAGE, which are the best studied AGE-binding molecules. While the expression of RAGE is strongly downregulated in NSCLC cells and tissues (31), we identified a high expression of AGE-R1, whose activation by AGE-modified serum albumin suppresses the cell signaling cascade induced by the epidermal growth factor (EGF) (52). As EGF-mediated cell signaling contributes to the progression of lung carcinomas (53), the impairment of the EGF receptor signaling by AGE-R1 activation might, at least in part, explain the tumor growth-inhibiting effect of circulating AGEs. However, AGEs also may act independently of AGE receptors. In this regard, we recently showed a reduced activity of the soluble matrix metalloproteinase 2 as well as the growth factor PDGF following AGE modification (54,55). The reduced growth activity of AGE-modified circulating factors also has been demonstrated in this study by a reduced proliferation/migration of NSCLC cells in response to AGE-FCS. Therefore, several types of biological mechanisms might contribute to the antitumorigenic effect of circulating AGE chromophores.

Moreover, it is conceivable that no singular AGE compound acts alone but in concert with other, probably still unknown, AGE chromophores. The quantification of a set of fluorescent AGEs as a prognostic factor is therefore an advantage compared with the quantification of single compounds in human plasma samples. Nonetheless, the question arises, “which of the AGE structures contribute to the tumor growth-inhibiting effect of AGEs?” Pronyl-lysine is one of the few AGEs presently identified as a chemopreventive factor in bread crust (27), which also affects the formation and progression of chemically induced preneoplastic lesions in rat colon (56). This colon cancer model is not completely consistent with the tumorigenicity model used in our study, but it indicates the potential role of this AGE compound in cancer development. In addition, 3-hydroxy-4-[(E)-(2-furyl)methylidene]methyl-3-cyclopentene-1,2-dione and others have been identified as AGE chromophores formed from carbohydrates upon thermal food processing which impair the proliferation of human mammary carcinoma cells in vitro (57). Although these and our studies indicate the beneficial effect of AGEs in tumorigenicity, it does not exclude the importance of other factors that are directly or indirectly accompanied by an altered AGE level in human.

In summary, we demonstrated that the AGE-related plasma fluorescence is a simple predictor for the outcome of NSCLC patients after curative surgery. The worst prognosis was associated with patients having a low level in the AGE-related fluorescence, which might be based on the tumor growth-inhibiting effect of fluorescent AGEs as suggested by the results of our experimental studies. Since extent and prognosis of NSCLC patients are insufficiently assessed by the current TNM classification, additional analyses of the individual plasma fluorescence may support the patients’ assessment significantly.

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DISCLOSURE

The authors declare that they have no competing interests as defined by Molecular Medicine or other interests that might be perceived to influence the results and discussion reported in this paper.

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