Extracellular RNA Liberates Tumor Necrosis Factor-α to Promote Tumor Cell Trafficking and Progression

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Abstract

Extracellular RNA (eRNA) released from injured cells promotes tissue permeability, thrombosis, and inflammation in vitro and in vivo, and RNase1 pretreatment can reduce all these effects. In this study, we investigated the role of the eRNA/RNase1 system in tumor progression and metastasis. Under quiescent and stimulatory conditions, tumor cells released much higher levels of endogenous extracellular RNA (eRNA) than nontumor cells. In glioblastomas, eRNA was detected at higher levels in tumors than nontumor tissue. eRNA induced tumor cells to adhere to and migrate through human cerebral microvascular endothelial cells (HCMEC/D3), in a manner requiring activation of VEGF signaling. In addition, eRNA liberated TNF-α from macrophages in a manner requiring activation of the TNF-α–converting enzyme TACE. Accordingly, supernatants derived from eRNA-treated macrophages enhanced tumor cell adhesion to HCMEC/D3. TNF-α release evoked by eRNA relied upon signaling activation of mitogen-activated protein kinases and the NF-κB pathway. In subcutaneous xenograft models of human cancer, administration of RNase1 but not DNase decreased tumor volume and weight. Taken together, these results suggest that eRNA released from tumor cells has the capacity to promote tumor cell invasion through endothelial barriers by both direct and indirect mechanisms, including through a mechanism involving TNF-α release from tumor-infiltrating monocytes/macrophages. Our findings establish a crucial role for eRNA in driving tumor progression, and they suggest applications for extracellular RNase1 as an antiinvasive regimen for cancer treatment. Cancer Res; 73(16); 5080–9. ©2013 AACR.

Introduction

Already in 1947, the presence of nucleic acids in normal human plasma was described (1–3). A few years later, RNA-proteolipid complexes were detected in the circulation of patients with cancer and were proposed to represent a specific secretory product of cancer cells that could mediate host–tumor interactions (4). Specific types of RNA in serum of patients with cancer have been pointed out to be very useful in cancer diagnosis (5, 6).

Recently, endogenous extracellular RNA (eRNA) has been characterized as a potent cofactor for the contactphase of the intrinsic blood coagulation pathway and as a permeability-increasing factor in vitro and in vivo (7, 8). eRNA also provides proinflammatory activity by inducing the adhesion of leukocytes, which was shown in vivo by using the cremaster mice model as well as by increasing the adhesion of monocytes to endothelial cells in vitro. Mechanistically, eRNA promoted the liberation of TNF-α from monocytes by activation of TNF-α–converting enzyme (TACE; ref. 9). TACE is a member of the ADAM family of disintegrins and metalloproteases (ADAM) family and serves as sheddase to cleave the transmembrane form of tumor necrosis factor, thus generating the soluble form of active TNF-α (10). Permeability-increasing and proadhesive functions of eRNA are mediated by its interaction with VEGF leading to the activation of the VEGF-R2/neuropilin-1 (NRP-1) system (8, 11). Collectively, eRNA serves as an endogenous alarm signal to provoke defense reactions that may expand into disease states.

The natural counterpart of eRNA in the vascular system is circulating RNase1, which is predominantly expressed and released by endothelial cells (12–14). Immediate release of RNase1 from endothelial cell storage granules (designated Weibel-Palade bodies) is promoted by proinflammatory and prothrombotic agents including eRNA itself. The endogenous RNA/RNase system may be considered as a regulator of vascular homeostasis thereby influencing several defense mechanisms (15).

Several malignant conditions are accompanied by an increase of both, eRNA and eDNA in the circulation (16, 17). Apoptosis or necrosis of tumor cells or even an active release of nucleic acids were suggested as mechanisms to explain the presence of nucleic acids in plasma from patients with cancer.
The presence of procoagulatory eRNA in the circulation of patients with cancer might also contribute to the prothrombotic state of tumor patients (20). However, until now, there is no information about the role of eRNA during tumor growth and metastasis. In this study, it is shown that eRNA induced the adhesion and transmigration of tumor cells to and across endothelial cells. Furthermore, eRNA enhanced the release of TNF-α from macrophages, which was mediated by activation of TACE. Finally, the involvement of eRNA during tumor growth in vivo was confirmed. After injecting tumor cells into mice, tumor growth was significantly reduced in the presence of RNase1. The presented data provide further mechanistic insights into activities of eRNA during tumor development and suggest the administration of RNase1 as a new antitumor regimen.

Materials and Methods

Cell culture

Human cerebral microvascular endothelial cells (HCMEC/D3) were kindly supplied and characterized by P.O. Couraud (Cochin Institute, Paris, France) and cultivated as described (21). Human monocyteic leukemia cell line THP1, the fibrosarcoma cell line HT1080, the human colon adenocarcinoma cell line HT29, and the mouse fibrosarcoma cell line T241 were purchased from American Type Culture Collection (ATCC) and cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) and 2 mmol/lL-glutamine as described by the supplier. Cell lines were last tested and authenticated by the company multiplexion (DKFZ) in April 2013. The glioblastoma cell line G55 was kindly provided by M. Westphal and K. Lamszus (Department of Neurosurgery, University Hospital Hamburg-Eppendorf, Hamburg, Germany; refs. 22, 23) and U87 by the ATCC (HTB-14). Cultures were grown from stocks frozen at low passage within 6 months of purchase. Cell authentication was conducted at ATCC by short-tandem repeat profiling, cell morphology monitoring, and karyotyping.

THP1 cells were differentiated by adding phorbol 12-myristate 13-acetate (5 ng/mL; Sigma-Aldrich) for 3 days (24, 25). Bovine aortic endothelial cells (BAEC) were prepared and cultured as described (26). Before the experiments, cells were washed once with PBS, serum starved, and incubated for the indicated time periods in cell culture medium without FCS containing different agents.

The following agents were used for stimulating cells: TNF-α from R&D, RNase inhibitor from Epicentre Biotechnologies, recombinant bovine RNase1 and DNase from Fermentas, Bay11-7082 (Bay) from ENZO Life Sciences, and SB203580, from Millipore, TAPI from ENZO Life Sciences, poly:IC, R848, and CD107 from InvivoGen, or antibodies against VEGF and neuropilin-1 (NP-1) from R&D system, TNF-α from Biozol, ribosomal RNA from Acris, CD14 from Sigma, CD68 from Dianova, KI67 from Dako, or MAP-2 from Sigma at the indicated concentrations.

Isolation of RNA and DNA

Total cellular RNA and DNA were isolated from confluent cultures of smooth muscle mouse cells (MvSMC) using an extraction kit (Sigma-Aldrich) or the DNAzol reagent (Invitrogen). Cells were washed once with PBS and extraction was carried out according to the manufacturer’s instructions. mRNA was isolated from total cellular RNA derived from MvSMC or HT29 cells using the dynabeads mRNA purification kit from Life Technologies. Total RNA from cell supernatants was isolated using an extraction kit from Biozym. Quality of total RNA, DNA, and of hydrolyzed RNA was confirmed by electrophoresis on a 1% agarose gel followed by ethidium bromide staining. Total RNA was quantified using a Gene-Quant-photometer (Amersham Pharmacia) or the Quant-iT Technology (Invitrogen).

Adhesion assay

HCMEC/D3 were grown to confluence in 96-well microtiter plates and stimulated for 24 hours in FCS-free M199 medium as described in the legends of the corresponding figures. Tumor cells were labeled for 1 hour at 37 °C with 2’-β-(carboxyethyl) 5,6-carboxy-fluorescein acetoxymethyl ester (BCECF-AM; Calbiochem) followed by trypsinization and washing twice with PBS containing Ca2+ and Mg2+ ions (DPBS). Cells were diluted to 105 cells/mL, and endothelial cells were incubated with 100 μL-labeled cell suspension/well for 1 hour at 37°C. Fluorescence was measured at λex=485/λem=530 (RFU1). After 2 times washing, fluorescence was measured again (RFU2). Percentage of adherent cells was calculated as % adherent cells = RFU2/RFU1 × 100.

Transmigration assay

HCMEC/D3 were cultured to confluence on collagen-coated porous polycarbonate filter inserts (pore diameter = 6 μm) placed into 24-well plates. At the start of the experiment, medium from top and bottom chamber was removed. Different agents diluted in DPBS were added into the abluminal compartment and 5 × 105 tumor cells were added into the luminal compartments. After 24 hours, HT1080 cells in the abluminal chamber were counted in a CASYII cell counter (Schaerfe Systems). For control, it was confirmed that the cell number of tumor cells was not changed during the time of transmigration.

RNase activity

RNase activity was determined according to the method of Zimmermann and colleagues (27) with minor modifications as described previously (15). Protein concentrations were determined using the BCA kit from Pierce.

TNF-α-ELISA

Macrophages were treated for different time periods with agents as indicated in the corresponding figure legends. After centrifugation of cells, supernatants were removed and concentrated using centricon tubes (Millipore) with a cutoff of 10 kDa; protein concentration was determined using the BCA kit from Thermo Fisher Scientific. TNF-α-ELISA was conducted using the commercially available kit from ebioscience.
subcutaneously into the left flanks of 8-week-old nude mice using a 25-gauge needle syringe. Isotonic saline (100 µL; control), RNase (42 µg/kg), or DNase (42 µg/kg) were intravenously injected 3 times a week starting at day 7 in 3 experimental groups of 5 animals each. Tumor growth was quantified by caliper measurements every third or fourth day, whereby tumor volume was calculated according to the formula: \( V = \frac{1}{2} \times \text{length} \times \text{width} \times \text{height} \). Mice were sacrificed at day 24, tumors were taken out, weighted, and embedded into tissue TEK (Sakura) for cryosectioning. All procedures were carried out with approval of the appropriate authority for animal protection (Regierungspräsidium Karlsruhe, #35-9185.81/G-77/06).

For in vivo experiments using the T241 fibrosarcoma cells, 5-week-old female NuNMRI mice (Taconic; 9185.81/G-77/06) were included and anesthetized with isoflurane (Forene; Abbott Scandinavia; induction 4.5%; maintenance 2.5%–3%) during all manipulations. All mice were inoculated subcutaneously into the left flank with \( 0.5 \times 10^6 \) T241 fibrosarcoma cells in a total volume of 100 µL PBS. The mice received an intraperitoneal injection of either DNase or RNase at 100 µg/kg body weight 10 minutes before injection of tumor cells. DNase or RNase were then administered by intraperitoneal injection at 100 µg/kg body weight every third day for the duration of the study. The tumors were allowed to grow for 18 days, when several tumors were reaching the maximum allowed size. One mouse was lost in the RNase1-treated group before the end of the experiment due to a ruptured tumor. At the last day of the experiment, blood samples were taken by heart puncture from all mice before euthanization. Tumors were removed and measured with a caliper before saturation in 30% sucrose overnight, and subsequent cryopreservation in isopentane/dry-ice was done. Tumor volume was calculated using the formula \( V = \frac{1}{2} \times \text{length} \times \text{width} \times \text{height} \). The frozen tissue was stored in \(-80^\circ\text{C}\) until further processing. This animal work was approved by the Uppsala University board of animal experimentation (reg. nr 314/11) and thus conducted according to the United Kingdom Coordinating Committee on Cancer Research guidelines for the welfare of animals in experimental neoplasia (28).

**Hematoxylin and eosin staining and immunohistochemistry of tumor xenografts**

Cryosections of HT29 tumors were fixed with 4% PFA/PBS for 10 minutes and washed 5 minutes with H2O. Nuclei were stained with Mayer’s hematoxylin (Sigma) for 2 minutes, washed 5 minutes in tap water and afterwards in normal H2O. Cytoplasm staining was conducted with eosin Y (Sigma) for 1 minute. After washing with H2O and successive dehydration, the slides were mounted with DPX mounting medium (Fluka). Tissue morphology and necrotic area stated in percentage was visually analyzed by microscopy (Olympus IX71).

For RNA staining, cryosections of HT29 tumors were fixed in ice-cold methanol for 10 minutes, followed by 1-hour incubation with RNASelect green fluorescent cell stain (Life Technologies). Thereafter, the slides were washed with PBST and nuclei were stained with Hoechst dye (2 µg/mL; Sigma-Aldrich) for 30 minutes in the dark. After washing with PBST, slides were mounted in fluorescent mounting medium (Invitrogen). Specimens were inspected using a fluorescent photomicroscope (Leica).

**Immunofluorescence staining of human glioblastoma tissue**

Paraffin-embedded tumor slides derived from patients with glioblastoma were obtained from patients undergoing surgery in accordance with a protocol approved by the institutional review board (Institute of Neuropathology, UKGM, Justus-Liebig-University, Giessen). After deparaffinization, tissue sections were stained using the histostain-plus kit from invitrogen. Antibodies used were against glial fibrillary acidic protein (GFAP) from DAKO, CD68 from Dianova, CD14 from Sigma-Aldrich, ribosomal RNA (rRNA) from Novus, TNF-α from Santa Cruz, and Ki67 from DAKO.

Cryosections from glioblastoma were fixed with acetone:methanol (1:1), followed by blocking with PBS containing 0.1 triton × 100 (weight/volume) and 1% donkey serum. Sections were incubated with RNA-select (diluted 1:500 in PBS) or with anti TNF-α for 1 hour at room temperature followed by incubation with corresponding secondary antibody labeled with Alexa Fluor 555 (Invitrogen). Nuclei were stained with Hoechst dye (2 µg/mL; Sigma-Aldrich) for 30 minutes in the dark. After washing with PBS, slides were mounted in fluorescent mounting medium (Invitrogen). Specimens were inspected using a fluorescent photomicroscope (Leica).

**Statistical analysis**

Sigma Stat 3.5 (Systat Software) was used for statistical analysis. Results between groups and treatments were compared with one-way ANOVA followed by a multiple pairwise comparison test (Dunn test) or by Wilcoxon rank-sum test, when appropriate. Results were expressed as mean ± SE. Results were considered as statistically different at \( P < 0.05 \).

**Results**

**Extracellular RNA-mediated adhesion and transmigration of tumor cells in vitro**

Microvascular endothelial cells (HCMEC) were treated with eRNA or TNF-α for different time periods and the adhesion of different tumor cell lines to HCMEC was analyzed. eRNA significantly enhanced the adhesion of the fibrosarcoma cell line HT1080 as well as of colon carcinoma cells HT29 in a concentration-dependent manner after 24 hours (Fig. 1A), whereas treatments for shorter periods of time were not effective (data not shown). Same results were obtained using ribosomal RNA, but not mRNA derived from nontumor or tumor cells, respectively (Fig. 1B). To rule out direct effects of eRNA on cell survival, we tested the viability of tumor cells and found no significant difference between eRNA-treated and untreated cells in our assay (Supplementary Table S1). Also, eRNA did not decrease the viability of endothelial cells as shown previously (9). DNA as well as RNase-predigested RNA did not influence the basal adhesion of tumor cells, excluding the possibility that single nucleotides might have an effect on cell adhesion (Fig. 1C). Furthermore, eRNA (but not DNA or RNase-predigested RNA) increased the transmigration of...
of tumor cells to endothelial cells, a significant reduction of adherent tumor cells was observed as well (Fig. 2E and F). Adhesion of tumor cells to unstimulated endothelial cells was also increased after pretreatment of tumor cells with eRNA, which was slightly increased when both, endothelial and tumor cells were pretreated in this way. However, in the presence of anti-ICAM-1 antibodies or RNase1, tumor cell adhesion to

HT1080 and HT29 cells across tight monolayers of endothelial cells. A similar increase in transmigration was found for TNF-α–treated monolayers (Fig. 1D). For control, eRNA itself did not provide any chemotactic activity toward tumor cells, as the number of transmigrating cells across cell-free filter inserts was not altered (Supplementary Table S1). Partly degraded RNA containing shorter fragments of total cellular RNA did not change tumor cell adhesion (Supplementary Fig. S1), indicating that a critical size and structure of RNA is needed for this process.

In previous studies, we showed that eRNA-induced vascular hyperpermeability or monocyte adhesion was promoted via the VEGF/VEGF-R2/neuropilin1 (NRP-1)-mediated signaling pathway (9, 11). Accordingly, eRNA-induced tumor cell adhesion and transmigration were prevented by neutralizing antibodies against VEGF or the extracellular domain of NRP-1 (Fig. 2A–D) as well as by neutralizing antibodies against ICAM-1 (Fig. 2E and F). In the presence of RNase1 during the adhesion of tumor cells to endothelial cells, a significant reduction of

Figure 1. eRNA-mediated adhesion and transmigration of tumor cells. HCMEC were treated with different concentrations of eRNA or ribosomal 18S RNA or mRNA (50 μg/mL) derived from MVSMC (mRNA1) or HT29 cells (mRNA2) as indicated for 24 hours, and the adhesion of HT1080 and HT29 cells (A) or HT1080 cells (B) was determined. C, HCMEC were either left untreated (Control) or treated with TNF-α (10 ng/mL), eRNA (50 μg/mL), RNase1-predigested RNA, or DNA (50 μg/mL) for 24 hours and HT1080 or HT29 cell adhesion was determined. D, HT1080 or HT29 cells were loaded in the top compartment of collagen-coated Transwell filter inserts coated with tight monolayers of HCMEC. The bottom compartment contained either PBS (control) or PBS with TNF-α (10 ng/mL), eRNA (50 μg/mL), RNase1-predigested RNA, or DNA (50 μg/mL). After 24 hours, transmigrated cells in the bottom compartment were counted using the CASYII cell counter. All values are expressed as mean ± SE (n = 9); *, P < 0.05 versus buffer-treated Control.

Figure 2. VEGF dependence of eRNA-mediated adhesion in vitro. Adhesion of tumor cells HT1080 (A) or HT29 (B) to HCMEC was determined; these were either treated with buffer alone (filled bars) or with eRNA (50 μg/mL, hatched bars) both in the absence (−) or presence of anti-VEGF-, anti-VEGF-R2, or anti-NRP-1 antibodies (each 5 μg/mL) for 24 hours. Transmigration of tumor cells HT1080 (C) or HT29 (D) was studied in collagen-coated Transwell filter inserts coated with tight monolayers of HCMEC. The bottom compartments contained either buffer (filled bars) or eRNA (50 μg/mL, hatched bars). Transmigrated cells were quantitated in the lower compartment after 24 hours either in the absence (−) or presence of anti-VEGF-, anti-VEGF-R2, or anti-NRP-1 antibodies (each 5 μg/mL). Adhesion of tumor cells HT1080 (E) or HT29 (F) to HCMEC was determined; these were treated either with buffer alone (filled bars) or eRNA (50 μg/mL, hatched bars), both in the absence (−) or presence of anti-ICAM-1 antibodies (5 μg/mL), or RNase1 (1 μg/mL) for 24 hours. Values represent mean ± SE (n = 7–10); *, P < 0.05 versus untreated control value.
endothelial cells (both preactivated by eRNA) was decreased (Supplementary Fig. S2A and S2B).

**Release of extracellular RNA from tumor cells**

To identify the source of eRNA during tumor growth, the concentration of eRNA in supernatants derived from different tumor cell lines was determined, whereby the human colon adenocarcinoma cell line HT29, cell lines derived from patients with glioblastoma like G55 and U-87, or HT1080 fibrosarcoma cells exhibited an elevated release of eRNA as compared with nontumor cells such as endothelial cells. The release of active RNase in all cells tested was equivalent (Fig. 3A and B). eRNA released from tumor cells predominantly contained ribosomal RNA as shown by agarose gel electrophoresis (Fig. 3C). During hypoxia, an elevated release of eRNA up to 16-fold from tumor cells was observed (Fig. 3D). In accordance with these in vitro results, human glioblastoma tissue contained appreciable levels of eRNA compared with nontumor tissue derived from the same patient, as was shown by immunohistochemistry (Fig. 3E and F).

**Extracellular RNA-induced release of TNF-α from macrophages**

As previously shown, upon stimulation of monocytic cells with eRNA (but not DNA), the release of TNF-α was increased in a concentration- and time-dependent manner, involving the

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**Figure 3.** Release of eRNA from different cell types in vitro. eRNA (A) or RNase (B) activity was quantified from each supernatant of tumor cells HT29, G55, U87, HT1080, or endothelial cells BAEC and HCMEC, each cultured for 24 hours in serum-free media. C, eRNA isolated from supernatants of HT1080 cells was analyzed by agarose gel electrophoresis. D, tumor cells HT29, HT1080, U-87, and G55 were incubated in medium without FCS for 24 hours under normoxia and hypoxia and eRNA was quantified from each supernatant. All values are expressed as mean ± SE (n = 6); *, P < 0.05 versus normoxia. Cryosections from glioblastoma tissue (E) or control white matter (F) were stained with RNA-select (green) to visualize eRNA or DAPI (blue) for nuclear staining.
activation of TACE (9). In agreement with our previous results, eRNA-induced release of TNF-α from macrophages was mediated by TACE, as the liberation of TNF-α release was completely blocked in the presence of the metalloproteinase inhibitor GM6001 or by the specific TACE inhibitor TAPI. Furthermore, eRNA-induced TNF-α release was inhibited by blockade of mitogen-activated protein kinase (MAPK) p38, p44/42, as well as the Akt or the NF-κB–mediated pathways (Fig. 4A). To investigate any role of TLR3 and TLR7 as RNA-recognizing receptors in relation to the release of TNF-α, macrophages were treated with poly:IC to activate TLR3-mediated signaling as well as with the agonists R848 and CL075 to activate both, TLR7, and TLR8. All agents promoted only a very weak release of TNF-α from macrophages, not comparable with eRNA, excluding that eRNA-liberated TNF-α was mediated via activation of TLR3, TLR7, or TLR8, respectively (Fig. 4B). For control, DNA or RNase-predigested eRNA did not influence TNF-α release from macrophages. Furthermore, after stimulation with eRNA tumor cells did not release TNF-α (data not shown).

To investigate whether eRNA-induced release of TNF-α from macrophages may be related to promotion of tumor cell adhesion, endothelial cells were pretreated with supernatants derived from eRNA-treated macrophages. Following this treatment, adhesion of tumor cells almost reached the same extent as seen after direct stimulation of endothelial cells with TNF-α. Neutralizing antibodies against TNF-α completely blocked supernatant-stimulated tumor cell adhesion (Fig. 4C).

In accordance with these in vitro results, tumor tissue derived from human glioblastomas was found to contain proliferating cells (Ki67 expression) as well as macrophages, as shown by the expression of CD14 and CD68, together with eRNA and TNF-α. In fact, coexpression of RNA and TNF-α in this tumor tissue was shown by immunohistochemical analysis (Fig. 5).

**Influence of RNase1 treatment on tumor growth in vivo**

To investigate whether our in vitro results are operative in tumor progression in vivo, tumor cells were injected into mice and the influence of RNase1 or DNase treatment was...
investigated. Seven days after implanting HT29 cells subcutaneously into the flanks of nude mice, RNase1 or DNase was administered every third day before tumors were removed after 24 days. Administration of RNase1 (but not DNase) significantly reduced tumor volume as well as tumor weight (Fig. 6A and B). These results were confirmed in a second in vivo tumor model. Here, T241 fibrosarcoma cells were injected into NuNMRI mice that were subsequently treated with RNase1 or DNase every third day before tumors were removed at day 18. Similar to the HT29 model, the volume of tumors derived from RNase-treated mice was significantly reduced compared with the DNase-treated group (Fig. 6C). To confirm in vitro that the trafficking of mouse fibrosarcoma cells T241 was inducible by eRNA, an in vitro system with mouse endothelial cells was used. eRNA, but not DNA or RNase1-predigested RNA, significantly increased the adhesion and transmigration of T241 cells on and across mouse endothelial cells (Supplementary Fig. S3).

Inspection of the tumor tissue derived from our animal model revealed that treatment with RNase1 (but not DNase) further increased the area of necrosis, as documented by immunohistochemical analysis (Fig. 7A and B). Immunohistochemical inspection also showed the presence of eRNA in xenografts, which were removed from the animals at the end of the experiment. Tumor tissue-associated eRNA was strongly reduced in mice that were treated with RNase1 but not in those treated with DNA (Fig. 7C).

Discussion
This study shows, using in vitro and in vivo approaches, that extracellular RNA (eRNA) contributes to tumor progression and tumor cell adhesion to endothelial cells in a TNF-α-dependent manner. Administration of RNase1 (but not DNase) in experimental tumor models significantly reduced tumor volume and weight, indicating that eRNA influences mitogenic, permeability-increasing and proinflammatory factors relevant for tumor spread (8).

Tumor cells seem to release much higher levels of eRNA into their cell supernatants as compared with nontumor cells like endothelial cells; this process was promoted under hypoxia, a situation that is common to solid tumors. The released RNA mostly consisted of ribosomal RNA, but this does not exclude the presence of other types of RNA like tRNA or mRNA. Specifically, glioblastoma tumor tissue contained substantially increased levels of eRNA in situ as compared with nontumor tissue samples. Despite the presence of RNase1 in plasma, elevated levels of eRNA were previously recognized in patients with tumor (6), and it was postulated that eRNA in the circulation may become protected against degradation by binding to lipids, proteins, lipoproteins, phospholipids, or to DNA in nucleosomes (4, 29–32). Furthermore, eRNA is associated with microparticles (33), which was confirmed by recent experiments. Alternatively, binding to tumor cells might increase the stability and half-life of extracellular ribonucleic acids in vivo (34, 35). Together, there is no doubt...
The process of tumor cell adhesion, which involves the VEGF-induced signaling in tissues expressing high amounts of depletions of eRNA ribonucleic acids, as neutralizing antibodies against VEGF, mean/C6 minutes before injection of tumor cells and then administered every either DNase or RNase at 100 the tumor growth was followed by help of a caliper. Treatment with represent mean/tumors were removed and tumor weight (B) was calculated. Values varied days, tumor volume (A) was determined, and after 24 days, tumors were removed and tumor weight (B) was calculated. Values represent mean ± SE; *, P < 0.05. C, T241 fibrosarcoma cells were subcutaneously injected into NuNMRi mice (5 × 10^6 cells/mouse) and the tumor growth was followed by help of a caliper. Treatment with either DNase or RNase at 100 μg/kg body weight was carried out 10 minutes before injection of tumor cells and then administered every third day until the study was terminated at day 18. Values represent mean ± SE; *, P < 0.05.

Figure 6. Influence of RNase and DNase treatment on tumor growth in mice. HT29 colon carcinoma cells were injected subcutaneously into nude mice (3 × 10^6 per mouse) and treatment with RNase or DNase was started 7 days after tumor cell inoculation (3 times per week). After various days, tumor volume (A) was determined, and after 24 days, tumors were removed and tumor weight (B) was calculated. Values represent mean ± SE; *, P < 0.05. C, T241 fibrosarcoma cells were subcutaneously injected into NuNMRi mice (5 × 10^6 cells/mouse) and the tumor growth was followed by help of a caliper. Treatment with either DNase or RNase at 100 μg/kg body weight was carried out 10 minutes before injection of tumor cells and then administered every third day until the study was terminated at day 18. Values represent mean ± SE; *, P < 0.05.

about the presence of locally enriched eRNA, particularly in situations of tumor burden.

We show here that eRNA (but not DNA or RNase-treated RNA) induced the adhesion and transmigration of tumor cells to and across monolayers of brain-derived microvascular endothelial cells. In particular, eRNA initiated VEGF-related signaling events by mobilizing the endogenous, extracellular bound cytokine and increased VEGF binding to NRP-1, followed by activation of the VEGF-receptor-2 (8, 11). This signaling pathway also lead to the expression of ICAM-1 on endothelial cells (9). In essence, eRNA-induced tumor cell adhesion follows a signaling route that seems to be superimposable to previously recognized inflammatory properties of ribonucleic acids, as neutralizing antibodies against VEGF, VEGF-R2, or NRP-1 abolished tumor cell adhesion. However, depletion of eRNA in vivo probably will not completely abolish VEGF-induced signaling in tissues expressing high amounts of VEGF. The process of tumor cell adhesion, which involves the interaction between endothelial cells and circulating tumor cells followed by their emigration into tissue, is the prerequisite for metastasis. Attachment of cancer cells to the "injured" endothelium, expressing E-selectin, intracellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1), seems to be initiated by multiple interactions between selectins and the oligosaccharide ligand sialyl Lewis X, expressed on circulating vascular and tumor cells (36). This is followed by subsequent firm adhesion of tumor cells via integrins (36–39), allowing them to emigrate into tissue. In this study, we characterized a new role of eRNA in this multistep process.

Endothelial ICAM-1 was found to be induced by eRNA (9), and is required for tumor cell adhesion, as neutralizing antibodies against ICAM-1 blocked this step. Tumor cell-expressed ICAM-1 may play a role in eRNA-induced tumor cell adhesion as well, as stimulation of both tumor cells and endothelial cells with eRNA further increased tumor cell adhesion in an ICAM-1–dependent manner. Moreover, eRNA itself seems to be involved in tumor cell binding to endothelial cells, as cancer cell adhesion was prevented by RNase1. Whether cell-bound RNA (34, 35) itself could strengthen cellular contacts in this regard requires further studies.

Malignant tumors are known to be infiltrated by leukocytes and macrophages, whereby the level of macrophages, which can comprise up to 50% of the total tumor mass, was reported to positively correlate with microvessel density, tumor stage, and angiogenesis (40, 41). These angiogenic properties of monocytes/macrophages rely on the release of several cytokines, chemokines, and growth factors (42). Our study also showed that eRNA, which is present in tumor tissue as revealed by immunohistochemical staining of glioblastoma, induced a massive release of TNF-α from macrophages, but not from tumor or endothelial cells (data not shown). In particular, eRNA provoked TNF-α liberation from macrophages in a TACE-dependent manner, which corresponded with the previously described release of TNF-α from monocytes under inflammatory conditions (9). TACE-mediated shedding events involved activation of the MAPK cascade (43) as well as activation of Akt- and NF-kB–dependent signaling pathways. TNF-α, liberated from macrophages, may increase the binding of tumor cells to endothelial cell monolayers, as evidenced by our in vitro data. These cell–cell adhesion events were also promoted after stimulation with supernatants derived from eRNA-treated macrophages in an equivalent manner similar to direct stimulation with TNF-α alone: In both situations, neutralizing antibodies against TNF-α inhibited cell adhesion. Together, these results indicate that eRNA released from tumor cells may induce the shedding of TNF-α from macrophages, resulting in the enhancement of tumor cell adhesion to the endothelium. Although it is unclear which signaling events will induce the "activation" and shedding function of TACE in an eRNA-dependent manner, involvement of Toll-like receptors (TLR) such as TLR3, TLR7, or TLR8, which were described to be activated by double- or single-stranded RNAs (44–46), were found not to be engaged in the described experimental system.
Once released by eRNA-dependent shedding reactions, TNF-α may activate the NF-κB signaling pathway, resulting in increased survival and proliferation of (tumor) cells (47, 48), associated with induction of angiogenic factors or adhesion molecules on endothelial cells (42). Our in vivo findings showed an increase of the necrotic areas in growing tumors after application of RNase1. In contrast, DNase administration leads to a reduction of necrotic areas. Furthermore, HT29 tumors contained high amounts of free eRNA, which was strongly associated with induction of angiogenic factors or adhesion molecules on endothelial cells (42). Our findings in vivo showed an increase of necrotic areas in growing tumors after application of RNase1 but not by DNase indicative for the fact that RNase1 strongly counteracted the tumorigenic activities of eRNA and may thus serve as a new antitumor regimen.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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