Relaxin Enhances S100A4 and Promotes Growth of Human Thyroid Carcinoma Cell Xenografts

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Abstract

Relaxin increases cell motility and in vitro invasiveness in human thyroid carcinoma cells but the underlying molecular mechanisms of this action are largely unknown. In the present study, we show that relaxin transcriptionally upregulates the calcium-binding protein S100A4 (metastasin) and increases the cytosolic 10-kDa monomer and the 20-kDa dimer form of S100A4 in human thyroid carcinoma cells. The relaxin-induced increase in cell motility was blocked completely when S100A4 expression was diminished using an S100A4 small interfering RNA knockdown approach. We have shown previously the expression of the insulin-like family member relaxin in human thyroid carcinoma tissues but not in benign thyroid tissues. Human thyroid carcinoma tissues expressing relaxin also stained positive for S100A4. In nude mouse experiments, human thyroid carcinoma cell transfectants with constitutive expression of relaxin generated large and fast-growing tumors with significantly increased numbers of proliferating cells. We provide evidence in our cell model that the relaxin target protein S100A4 secreted by the thyroid carcinoma transfectants may not only enhance tumor cell motility but also promote xenograft angiogenesis as determined by the higher density of tumor microvessels and the angiogenic potential of S100A4 in in vitro tube formation assays. In conclusion, we have identified S100A4 as a major mediator of the actions of relaxin in thyroid carcinoma cell motility and in vivo thyroid tumor angiogenesis.

Mol Cancer Res; 8(4); 494–506. ©2010 AACR.

Introduction

Human thyroid carcinoma is the most common cancer of the endocrine system (1) and has been showing a marked increase in incidence in Europe (2-4), Canada (5), and the United States (6). In most Western countries, thyroid cancer is more common in women than in men (3:1; ref. 7) and is ranked seventh place of all cancers affecting women (1). Although the prognosis of well-differentiated thyroid carcinomas [follicular thyroid carcinoma (FTC) and papillary thyroid carcinoma (PTC)] is better than in most other cancers, the frequent and significant increase in incidence in Europe (2-4), Canada (5), and the United States (6). In most Western countries, thyroid cancer is more common in women than in men (3:1; ref. 7) and is ranked seventh place of all cancers affecting women (1). Although the prognosis of well-differentiated thyroid carcinomas [follicular thyroid carcinoma (FTC) and papillary thyroid carcinoma (PTC)] is better than in most other cancers, the frequent and early development of metastases significantly reduces survival rates particularly in patients with FTC and undifferentiated thyroid cancer (UTC; refs. 8, 9).

We have previously shown that the polypeptide hormone relaxin is expressed exclusively in human thyroid carcinoma but not in benign thyroid conditions (10). Relaxin (RLN2) confers increased carcinoma cell motility and in vitro invasiveness in human thyroid carcinoma cells and this process is mediated by the relaxin receptor, relaxin family peptide receptor 1 (RXFP1; ref. 10). Furthermore, we showed that in human thyroid carcinoma cells, the enhanced migration through elastin matrices was mediated as a result of relaxin-induced increase in the production and secretion of elastinolytic cathepsin-L (10).

The small calcium-binding protein S100A4, also known as metastasin, has been associated with metastasis and poor prognosis in patients with carcinomas of the breast (11-14), stomach (15), prostate gland (16), esophagus (17), non-small cell lung cancer (18), colon (19, 20), and the thyroid (21, 22). S100 family proteins are encoded in a cluster of genes on chromosome 1q21 and have been implicated in a variety of cellular events such as growth, signaling, apoptosis, motility, and angiogenesis (23-25). S100A4 was originally known as Fibroblast-specific protein 1 and identifies fibroblasts or epithelial cells in epithelial-mesenchymal transition (26). In wounding experiments, activated stromal fibroblasts of the cornea were also shown to express S100A4 (27). The identified intracellular binding partners of S100A4, nonmuscle myosin, p53, and liprin-β1 (28-32) are thought to be causally involved in the motility enhancing and antiapoptotic effects of S100A4. Extracellular S100A4 is believed to aid in tumor metastasis by inducing...
the secretion of matrix metalloproteinases (23), promoting angiogenesis through the stimulation of endothelial cell motility (24), and by increasing the formation of plasmin through binding to the endothelial cell surface receptor Annexin II (33). S100A4 was shown not to be tumorigenic per se but significantly increased the metastasis of tumor cells in transgenic mouse models (11, 24). Recently, S100A4 gene expression was described as a marker for poor prognosis in thyroid carcinoma (34) and as an independent prognostic factor for lymph node metastasis in papillary microcarcinomas (22, 35). In mouse models of anaplastic thyroid carcinoma, S100A4 was shown to mediate tumor growth and metastasis (21).

In this study, we show that S100A4 production is significantly enhanced by relaxin in human thyroid carcinoma cells. The motility-enhancing effect of relaxin is mediated by and dependent on S100A4 in human thyroid carcinoma cells. The proangiogenic activity of S100A4 may contribute to the increased angiogenic potential of relaxin as determined by in vitro angiogenesis assay and increased microvessel densities in xenografts of human FTC cells with constitutive expression of relaxin.

**Materials and Methods**

**Thyroid Carcinoma Cell Lines and Transfectants**

**Cell Culture.** The human thyroid carcinoma cell lines (36) FTC133, FTC236, FTC238, 8505-C, C643, and ML-1 were propagated in DMEM/Ham’s F12 medium (Invitrogen). B-CPAP was cultivated in RPMI (Invitrogen) and supplemented with 1.125 g/L sodium carbonate (Merck) and 10% FCS (Biowest, Germany). TT2609-C02 was cultivated in RPMI supplemented with 1.125 g/L sodium carbonate, 20% FCS, 1% sodium pyruvate (Sigma-Aldrich), and 1% insulin-transferrin-selenium (Invitrogen). Stable FTC133-pIRES-EGFP-RLN2 and FTC133-pIRES-EGFP vector control transfecants were previously described (10). C643-pcDNA4HisMax (C643-HisMax) and C643-S100A4 in pcDNA4HisMax (C643-S100A4) stable transfecants were selected and cultured with 100 μg/mL Zeocin (Invitrogen). All cells were incubated in a humidified 5% CO₂ atmosphere at 37°C and passaged every 3 to 4 d using 0.5% Trypsin/EDTA (Invitrogen).

**Relaxin Treatments.** For incubations with recombinant human Relaxin2 (rhRLN2), thyroid carcinoma cells were cultured in medium containing 1% FCS for 24 h before treatment with 100 ng/mL rhRLN2 (Phoenix Pharmaceuticals) in 1% FCS for 24, 48, and 72 h. Medium containing rhRLN2 was changed every day. Cells were harvested in Trizol for RNA extraction or in 2x reducing Laemmli buffer for protein extraction.

**Human Thyroid Tissues**

The human thyroid tissues of normal thyroid, thyroid adenoma, goiter, and thyroid carcinoma were collected from patients at the Department of Surgery, University of Halle-Wittenberg, by surgical resection for clinical indications. This study was approved by the Ethical committee of the Martin-Luther-University, Faculty of Medicine. All patients gave written consent. For immunolocalization of S100A4 protein, paraformaldehyde-fixed and paraffin-embedded patient tissues were used as described (Table 2 in ref. 10). For Western blot analysis (Fig. 2B), a total of 66 human thyroid tissues were used, including 8 goiter tissues, 8 adenoma, 20 follicular carcinoma tissues (FTC), 19 papillary carcinoma tissues (PTC), and 11 undifferentiated carcinoma tissues [anaplastic (UTC)]. For transcriptional analysis (Fig. 2A), 58 human thyroid tissues were used, including 11 goiter, 13 adenoma, 11 follicular (FTC), 12 papillary (PTC), and 11 anaplastic (UTC) thyroid cancer tissues. Four human primary PTC tissue samples with defined metastasis staging were chosen for each group in Fig. 2C.

**Detection and Localization of S100A4 in Human Thyroid Carcinoma Cell Lines and Tissues**

**RNA Extraction and Reverse Transcription-PCR.** Total RNA of thyroid carcinoma cell lines and thyroid tissues was extracted using the Trizol reagent (WKS) and the amount of isolated total RNA was measured by spectrophotometry (UV-1602, Shimadzu) at 260 and 280 nm. Total RNA (1 μg) was used for cDNA synthesis using the SuperScript reverse transcriptase II and 100 ng/μL random primer 50 (Invitrogen). For the amplification of S100A4, RXFP1, RLN2, and 18S, specific oligonucleotide primers (Metabion; Table 1) were used. The 18S RNA content served as standard for semiquantitative PCR analysis. The conditions for reverse transcription-PCR (RT-PCR) were previously described (10). The number of cycles and the annealing temperature were specific for each primer (see Table 1). PCR products were separated on a 2% agarose gel. A Kodak software (Kodak Digital Science 1D v.3.0.2., Eastman Kodak) was used to perform densitometric quantification of the specific PCR products using 18S PCR products as internal standard (for Fig. 4C).

For quantitation, 1 μL of the reverse transcriptase reaction mixture was added to 25 μL reaction mixture consisting of 1× Advantage2 reaction buffer, 1.5 units of Taq polymerase (Clontech), 0.2x SYBR Green (Biozym), 200 μM/L of each deoxynucleotide triphosphate, and 0.5 μM/L of each primers listed (Table 1). A negative control without template was included. Assays were done in triplicates in a Rotor-Gene 2000 (LT). Initial denaturation at 95°C for 300 s was followed by 40 cycles with denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and elongation at 72°C for 20 s. To verify the single PCR products, melting curves were generated and amplifiers were cloned and sequenced bidirectionally. The fluorescence intensity of the double-strand specific SYBR Green, reflecting the amount of formed PCR product, was read after each elongation step at 82°C. Relative quantitation of gene expression was done with the software Rotor-Gene version 4.6 in comparative quantitation mode. SDs were determined by t test.
Protein Extraction. For extraction of total proteins, cells and tissues were lysed in 2× reducing Laemmli buffer [125 mmol/L Tris-HCl (pH 6.8; Fluka), 4% SDS (Sigma), 20% glycerol (Merck), 10% mercaptoethanol (Sigma), and 2% bromphenol blue (SERVA)] containing a protease inhibitor cocktail (Roche). For protein fractionation, cells were lysed in hypotonic buffer and cytoplasmic and nuclear proteins were extracted separately (see Supplementary Data). For the detection of secreted S100A4, FTC133-RLN2 and FTC133-EGFP transfectants were cultured in medium with 1% fetal bovine serum for 72 h. Culture supernatants were centrifuged at 12,000 rpm for 10 min to pellet the remaining cells and were concentrated in a vacuum centrifuge (Concentrator 5301, Eppendorf). Protein concentrations were determined with the Bradford assay (Bio-Rad). Supernatants were mixed with 2× reducing Laemmli-buffer (1:1 vol/vol) and 50 to 80 μg of each probe were used for Western blot analysis.

Western Blot Analysis. For the detection of S100A4 and S100A6, 40 μL of protein extracts were separated on a 12% SDS gel and blotted onto a polyvinylidene difluoride membrane (GE Healthcare). After blocking in 0.1% PBS-T with 3% bovine serum albumin (Sigma), membranes were incubated overnight at 4°C with a 1:500 dilution of either rabbit polyclonal S100A4 Ab-8 antibody or S100A6 antibody (both NeoMarkers). After washing, membranes were incubated for 1 h at room temperature with the horseradish peroxidase–conjugated goat anti-rabbit IgG secondary antibody (Santa Cruz) diluted 1:20,000. Specific staining was visualized with the enhanced chemiluminescence kit (Pierce). For the detection of Xpress tag, membranes were probed overnight at 4°C with the monoclonal Anti-Xpress Antibody (Invitrogen) diluted 1:1,000. The horseradish peroxidase–conjugated goat-anti mouse secondary antibody (Santa Cruz) was used at 1:20,000. Cytoplasmic and nuclear protein fractions were verified with mouse monoclonal antibodies directed against α-tubulin (cytosolic; Invitrogen) at 1:5,000 and Histone H3 (nuclear; Chemicon) at 1:500, respectively. β-Actin (Sigma) and α-tubulin (Abcam) were used as loading control.

Immunohistochemistry. Paraformaldehyde-fixed and paraffin-embedded human tissue sections (5 μm) of patients with normal thyroid; goiter; adenoma; and follicular, papillary, and undifferentiated thyroid carcinoma (Table 2 in ref. 10) were used for the cellular localization of S100A4. Sections were treated with citrate buffer (pH 6.0) for antigen retrieval and blocked in TBST containing 1% bovine serum albumin and 5% goat normal serum. The rabbit polyclonal antiserum to S100A4 (Ab-8, NeoMarkers) was applied at 1:200 overnight at 4°C. Negative controls were performed using a rabbit nonimmune serum at 1:200. An AP-conjugated goat anti-rabbit immunoglobulin secondary antibody (Dianova) at 1:300 was used for 1 h at room temperature and visualization of specific binding was achieved using an AP-substrate kit (HistoMark Red, Kirkegaard & Perry Laboratories). Sections were counterstained with hematoxylin and mounted in aqueous mounting medium for bright-field microscopy. For the staining of S100A4 and α smooth muscle actin on consecutive sections, a mouse monoclonal anti-α smooth muscle actin antibody was introduced (Sigma, 1:500). Both stainings were detected with the peroxidase detection method using horseradish peroxidase–conjugated goat anti-rabbit immunoglobulin and goat anti-mouse immunoglobulin (both Dianova), respectively, at 1:300 and the 3,3′-diaminobenzidine substrate (Pierce, Thermo Scientific). For the immunodetection of CD31 and Ki67 on sections of xenograft tumor tissues, a rabbit anti-CD31 polyclonal antiserum (Abcam) at 1:200 and a rabbit monoclonal antibody SP6 (Abcam) was used according to the manufacturer’s instruction. A rabbit isotype control IgG (DAKO) used at equivalent concentrations served as negative control.

S100A4 in Thyroid Carcinoma Cell Motility

Establishing Stable Transfectants of C643 Cells with Expression of Full-Size S100A4. PCR amplification of full-size human S100A4 was done on the cDNA of FTC-133 cells with the following primers encoding an NH2-terminal BamHI site followed by an Xpress tag and forward human S100A4 coding sequence [FXpress-FX1S100A4-BamHI
(5′-atg gat cca tgg gtc tgr acg atg acg ata aga ccc gcc ctc tgg aga aa-3′) and RhsuS100A4-Sall (5′-atg tcg act cat ttc tct ggc tgt cta tca-3′). The single PCR product was excised from a low-melting-point agarose gel, resin purified (Promega) and ligated in pGEM-T (Promega). Constructs were confirmed by sequence analysis from both sites, excised with BamH1 and Sall, resin purified, and cloned into pcDNA4 HisMaxC (Invitrogen). C643 cells were transfected with the empty vector and the pcDNA4HisMaxC-S100A4 vector (Supplementary Fig. S3) using the Lipofectamine 2000 kit according to the manufacturer’s protocol (Invitrogen). Stable transfectants were selected with 100 μg/mL Zeocin. Positive transfectants were identified by Western analysis with a mouse monoclonal anti-Xpress antibody (Invitrogen) and a rabbit polyclonal antibody to S100A4 (Abnova) as described for Western analysis.

Proliferation Assay. To determine changes in cell proliferation, MTT assays were done on C643-S100A4 transfectants and their respective controls (empty vector transfectants and parental cells). Cells were plated at a density of 0.25, 0.5, and 1 × 10⁴ cells per well in 100 μL culture medium and cultured for 24 h before performing the assay according to the manufacturer’s instruction (Easy-for-you; Biomedica).

S100A4 siRNA Experiments. SiRNA knockdown of S100A4 in FTC133 and FTC238 thyroid carcinoma cells was done by transient transfections using S100A4 small interfering RNA (siRNA; antisense 5′-AG CUU GAA CUU GUC ACC CTC-3′; Ambion). Cells (8 × 10⁵) were seeded in six-well culture plates. S100A4 siRNA and nonsilencing siRNA control (30, 50, and 100 nmol/L; Ambion) were complexed with SilentFect reagent (Bio-Rad) or Lipofectamine 2000 (Invitrogen) in serum-free OptiMem medium. Stable transfectants were selected with 100 μg/mL Zeocin. Positive transfectants were identified by Western analysis with a mouse monoclonal anti-Xpress antibody (Invitrogen) and a rabbit polyclonal antibody to S100A4 (Abnova) as described for Western analysis.

Motility Assay. To assess cell motility following S100A4 siRNA knockdown, a 24-well Transwell chamber (Greiner) assay was used as previously described (10). Briefly, FTC133 (1 × 10⁴ cells) transfected with siRNA for S100A4 or siRNA control (both at 30 nmol/L) were plated in 200 μL medium on top of a 8-μm pore filter insert (upper chamber). The lower chamber contained 500 μL culture medium containing 1% FCS. After 24 h, cells that had migrated to the underside of the filter were stained and counted as previously described (10). To investigate the effect of RLN2 on the motility of C643 transfectants with expression of S100A4, rhRLN2 at 100 ng/mL was added to the lower chamber and cell motility of stable C643-S100A4 and C643-mock stable transfectants was assayed as described above. All experiments were done at least in triplicates and were expressed as mean ± SEM.

The Role of Relaxin in Tumor Growth and Angiogenesis

Tumor Growth in Nude Mice. Male athymic nude mice (NMRI), 10 to 15 g body weight were obtained from the Central Animal Laboratory of the Medical School and were bred under pathogen-free conditions under a 12/12-h light/dark schedule and provided with a standardized diet. The nude mouse experiments were approved by the Animal Ethics Committee of the Martin-Luther University Halle-Wittenberg. Five mice at 3 wk of age were used for FTC133-EGFP and for each of the two FTC133-RLN2 transfectants (clones 4 and 10). Tumor cells were injected s.c. at 2 × 10⁷ cells/mL in medium without fetal bovine serum containing 1 mmol/L sodium pyruvate. Injection sites were inspected daily; tumors were measured twice weekly; and tumor volumes were determined according to caliper measurements and a formula for determining the volume of an ellipsoid. Mice were sacrificed when the tumor size exceeded 10% of body size. Primary tumors were collected and mice were dissected and visually inspected for metastases. Tumor tissues were cryopreserved and fixed in 4% paraformaldehyde for paraffin embedding. H&E staining and immunodetection of Ki67 and CD31 were done as described above. For quantitative assessment of in vivo proliferation, three tumors for each cell clone were assessed. Ki67-positive cells were counted in three sections of each xenograft tissue and the number of Ki67-positive cells per square millimeter area was determined.

Tube Formation In vitro Assay. Human umbilical vein endothelial cells (HUVEC) were purchased from BD Biosciences and PromoCell, grown on culture flasks coated with 2% gelatin (Sigma), and propagated in EGM-2 endothelial cell growth medium (Lonza). For this in vitro angiogenesis assay, HUVEC were used at passages 6 to 8. Growth factor-reduced Matrigel was purchased from BD Biosciences and used according to the manufacturer’s instructions. To assess tube formation, 300 μL Matrigel was used per well of 24-well plates. Following polymerization of the Matrigel, 5 × 10⁴/well HUVEC cells were seeded on top of the Matrigel in a total volume of 500 μL medium without and with the following peptides: 1 and 0.5 μg/mL human recombinant S100A4 (rhS100A4; Abnova), 100 ng/mL rhRLN2 (Phoenix), and 10 nmol/L vascular endothelial growth factor (BD Biosciences). Capillary-like tube formation was documented after 18 h with an inverted microscope (Zeiss, Axiocert 100). The capillary tube area was quantified using the Axiovision 4.2 software and shown as percent of the total area.

Statistical Analysis

All experiments were done at least in triplicates and data were expressed as mean ± SEM. Kodak software (Kodak Digital Science 1D v.3.0.2.) was used to perform semiquantitative densitometry of specific PCR and Western products. The t test and one-way ANOVA software was used to calculate for statistical significance with P ≤ 0.05 being considered significant. A reference to the statistical method used is made in each figure legend where applicable. Data of the thyroid carcinoma tissues from patients were depicting in a box and whisker diagram.
Results

Monomeric and Dimeric S100A4 Is Detected in Human Thyroid Carcinoma Cell Lines and Human Thyroid Carcinoma Tissues

The human follicular (FTC133, FTC-236, and FTC-238, ML-1), papillary (B-CPAP), and anaplastic (8505-C, C-643, BHT-101) thyroid carcinoma cell lines all expressed transcripts for S100A4 as determined by RT-PCR (Fig. 1A). These thyroid cancer cell lines also expressed transcripts for RLN2 (10) and RXFP1 (Fig. 1A), with the exception of FTC133 cells that were devoid of RLN2. Western analysis was used to determine the presence of S100A4 in total protein lysates. Expression of the 10-kDa monomorphic form of S100A4 was detected in FTC133, in ML-1, and in B-CPAP (Fig. 1B). The other human thyroid carcinoma cell lines investigated (FTC236, FTC238, B-CPAP, 8505-C, and C643) expressed the 20-kDa dimeric form of S100A4 as shown here for 8505-C and B-CPAP (Fig. 1C). S100A4 siRNA knockdown experiments showed that the SDS-resistant 20-kDa S100A4 protein band was indeed downregulated following 72 h of siRNA treatment as shown here for FTC238 thyroid carcinoma cells (Fig. 1D).

S100A4 gene activity and protein content were increased in thyroid carcinoma tissues (PTC, FTC, and UTC) compared with goiter and adenoma tissues, and highest mean values were detected in undifferentiated thyroid carcinoma tissue samples (Fig. 2A and B) with statistical significance detected for the RNA values only (Supplementary Fig. S1). S100A4 protein was expressed in thyroid carcinoma cells of all histologic types investigated, predominantly localized within the cytoplasm with only sporadic nuclear staining (Fig. 3D-F). Further, primary PTC tumor tissues with known lymph node metastases, although only a few tissues was investigated, showed a trend to higher S100A4 concentrations than PTC without nodal or distant metastases, suggesting a role for S100A4 in the early phases of invasiveness in thyroid cancer (Fig. 2C). In thyroid tissue specimens, we predominantly detected the monomeric form of S100A4. Thyrocytes derived from human normal thyroid tissues (Fig. 3A), goiter tissues (Fig. 3B), and human thyroid adenoma tissues (Fig. 3C) were devoid of S100A4 protein. However, smooth muscle cells in the walls of arterial and venous blood vessels, identified by their positive immunoreaction to α-smooth muscle actin (Fig. 3J), stained intensely for immunoreactive S100A4 (Fig. 3B and C).

FIGURE 1. Human thyroid carcinoma cell lines express S100A4 protein in monomeric or dimeric form. RT-PCR analysis showed transcripts for RXFP1, RLN2, and S100A4 in human thyroid carcinoma cell lines. 18S transcripts were used as a PCR loading control (A). S100A4 protein, 10-kDa monomer and 20-kDa dimer, was detected by Western blot in different human thyroid carcinoma cell lines using a rabbit polyclonal antibody (B and C). β-Actin and α-tubulin served as internal standard. The 10-kDa monomer of the S100A4 protein was detected in FTC133, B-CPAP, and ML-1 (B). Other thyroid carcinoma cell lines displayed the 20-kDa dimeric S100A4 protein. A representative Western blot is shown for UTC 8505-C and PTC B-CPAP (C). Transient transfection assays were done on FTC238 cells using S100A4 siRNA. The marked reduction of the 20 kDa S100A4 after 72 h showed the specificity of the 20-kDa S100A4 protein band (D).
RLN2 Increased the Expression of S100A4 in Human Thyroid Carcinoma Cell Lines and S100A4 siRNA Treatment Inhibited RLN2-Induced Enhanced Motility

All stable transfectant clones of FTC133 with expression of relaxin (FTC133-RLN2) displayed a marked upregulation of S100A4 mRNA (data not shown) and protein (Fig. 4A) when compared with FTC133-EGFP mock transfectants or untransfected FTC133. Both the human follicular and anaplastic thyroid cancer cell lines FTC133 and C-643, respectively, responded to treatment with recombinant human RLN2 (rhRLN2) with an increase in S100A4 mRNA and protein as shown for S100A4 protein in FTC133 (Fig. 4B) and S100A4 transcripts in C643 (Fig. 4C).

To determine the biological function of S100A4 for the motility of human thyroid cancer cells, we performed specific siRNA knockdown of S100A4 in the human thyroid carcinoma cell line FTC133 followed by motility assays. Expression of the S100A4 monomeric band in FTC133 was reduced to almost undetectable levels 72 hours after siRNA transfection when compared with nonsilencing siRNA controls (Fig. 5A). The siRNA knockdown was specific for S100A4 because the expression of the structurally related calcium-binding EF-hand protein S100A6 was unaffected (Supplementary Fig. S2). Next, we investigated the motility of these S100A4 siRNA treated thyroid cancer cells in filter-based motility assays in the presence and absence of rhRLN2. We had previously shown that RLN2 increased the cellular motility and invasiveness of human thyroid carcinoma cells (10). The S100A4 siRNA knockdown in FTC133 cells resulted in slightly reduced motility compared with FTC133 cells treated with nonsilencing siRNA (Fig. 5B). Further, S100A4 siRNA–treated FTC133 exposed to rhRLN2 (100 ng/mL) for 24 and 48 hours were no longer able to respond with increased motility (Fig. 5B), suggesting that S100A4 mediated this induced migratory response downstream of RLN2-RXFP1 signaling. To investigate whether exogenous S100A4 can bypass the RLN2-RXFP1–induced effect on motility increase, we blocked RLN2-RXFP1 signaling through RXFP1 siRNA knockdown in parental FTC133 cells (Supplementary Fig. S5). As expected, RXFP1 knockdown prevented the RLN2-induced motility increase, but exogenous rhS100A4 was able to rescue the RXFP1 phenotype and increased motility (Fig. 5C).

S100A4 Enhanced Cell Motility of C643-S100A4 Anaplastic Thyroid Transfectants

We generated C643-S100A4 stable transfectants that expressed Xpress-tagged human S100A4 (see Supplementary Fig. S3; Fig. 6A) to determine the effect of a further increase in S100A4 on the motility of human UTC cell line C643. All stable C643-S100A4 transfectants showed increased cell motility when compared with C643-HisMax controls or C643 parental cells (Fig. 6B). Expression of S100A4 did not increase cell proliferation in C643-S100A4 transfectants (Fig. 6C). Thus, increasing the cellular level of S100A4 protein in C643 further enhanced the motility of these anaplastic thyroid cancer cells. Although rhRLN2 treatment (100 ng/mL) caused a significant increase in motility of C643 and C643-HisMax mock cells, the C643-S100A4 transfectants failed to respond to exogenous rhRLN2 with higher motility rates (Fig. 6D), indicating that the increased levels of S100A4 coincided...
with maximal migratory potential of the C643-S100A4 transfectants.

**RLN2 Enhances Growth and Vascularization of Tumor Xenografts in Nude Mice**

Xenotransplant experiments were done to determine the ability of FTC133-RLN2 stable transfectants (10) to generate tumors in nude mice. Both FTC133-RLN2 clones tested generated fast-growing large xenografts, whereas enhanced green fluorescent protein (EGFP) clones only generated small dense nodular tumors (Fig. 7A). All mice carrying the FTC133-RLN2 clone 4 had to be sacrificed 3.5 weeks after s.c. injection and mice inoculated with FTC133-RLN2 clone 10 were sacrificed after 5 weeks (Fig. 7A) when the tumor exceeded 10% of their body weight. Histologic analysis revealed loosely arranged tumor cells in RLN2 xenografts but a dense tumor cell mass in EGFP xenografts (Fig. 7B, a and b). The proliferation marker Ki67 was immunolocalized at the perimeter of xenotransplant tissues showing multiple FTC133-RLN2 transfectants undergoing mitotic division (Fig. 7B, c). By contrast, FTC133-EGFP showed only isolated Ki67-positive cells (Fig. 7B, d). Quantification of Ki67-positive cells showed increased proliferation of FTC133-RLN2 cells *in vivo* (Fig. 7C).

On gross appearance, FTC133-RLN2 tumors showed the presence of multiple small blood vessels and this increased vascularization was confirmed by the detection of immunoreactive CD31 in the endothelial lining of small blood vessels (Supplementary Fig. S4A and B). When the primary antibodies were replaced by nonimmune serum, no specific immunostaining was detected (Supplementary Fig. S4, inserts). Quantitative assessment of microvessel density in consecutive tumor sections revealed a significantly higher number of blood vessels in FTC133-RLN2 tumors compared with FTC133-EGFP tumors.

**FIGURE 3.** S100A4 is localized in the cytosol of human neoplastic thyrocytes and is absent in thyrocytes of normal thyroid and in benign thyroid disease. Representative immunostaining for S100A4 in human thyroid tissues. Thyrocytes in normal thyroid tissues (A), in goiter tissues (B), and in thyroid adenoma tissues (C) were devoid of S100A4. Strongly positive S100A4 staining was detected in vascular smooth muscle cells as shown here within goiter (B) and thyroid adenoma tissues (C). A consecutive section of (B) was used to show the immunodetection of α-smooth muscle actin (αSMA) in identical cells staining positive for S100A4 (insert J). In thyroid carcinoma tissues, S100A4 protein was detected in neoplastic thyroid cells and localized predominantly in the cytosol of carcinoma cells of anaplastic (D), papillary (E), and follicular (F) thyroid carcinoma tissues. Arrows, representative S100A4-positive cells. Carcinoma tissues treated with nonimmune serum were devoid of staining (G-I). Magnification, ×800 for B and J; all others, ×400.
Detailed inspection of the carcasses did not reveal local or distant metastases in any of the nude mice with xenotransplants. Secreted S100A4 Stimulates In vitro Angiogenesis of HUVEC

To investigate a potential angiogenetic role for extracellular S100A4 in the xenograft tissues, we tested FTC133-EGFP and FTC133-RLN2 transfectants for their ability to secrete S100A4 into the culture medium. Concentrated supernatants from FTC133-EGFP and FTC133-RLN2 transfectants contained the secreted 10-kDa S100A4 monomer as shown for FTC133-EGFP and FTC133-RLN2 clone 10 (Fig. 8B). To provide further evidence for a role of S100A4 in increased vascularization and tumor growth, we used recombinant human S100A4, rhRLN2, and HUVEC endothelial cells in an in vitro angiogenesis assay. The rhS100A4 was capable of promoting the
formation of capillary-like tubes on Matrigel (Fig. 8C). Similar angiogenetic potential was observed with rhRLN2 (Fig. 8C). The extent of capillary-like tube formation induced by S100A4 and RLN2 was comparable with the known angiogenic factor vascular endothelial growth factor A used as a positive control (Fig. 8C).

Discussion

The polypeptide hormone H2-relaxin (RNL2) is increased in human carcinoma and is associated with increased migratory capacity of carcinoma cells of the breast (37, 38), prostate (39), and thyroid (10). We showed that RLN2 enhances cell motility and matrix invasion of human thyroid carcinoma cells and this action requires functional RXFP1 signaling (10). However, the cellular mechanisms mediating this increase in motility are not fully understood. Here, we show that RLN2 induces S100A4 gene activity and increases protein content in human thyroid carcinoma cells. We showed that RLN2 is exclusively expressed in thyroid cancer tissues but not in the normal thyroid gland or in tissues of benign thyroid disease (10). Employing the same human thyroid tissue samples used in our previous study (10), we show that noncarcinogenic follicular thyroid cells from normal thyroid tissues, goiter, and benign thyroid adenoma tissues were devoid of S100A4. By contrast, RLN2-immunopositive neoplastic thyrocytes of follicular, papillary, and anaplastic...
FIGURE 7. FTC133-RLN2 stable transfectants grow large tumors in nude mice. When injected s.c. into nude mice, FTC133-RLN2 clones 4 and 10 showed enhanced growth of xenografts compared with FTC133-EGFP vector controls (A). The xenografts derived from FTC133-RLN2 were highly vascularized on gross appearance. Xenografts from all transfectants were movable and contained within a collagen capsule. Tissue sections of FTC133-RLN2 (a and c) and FTC133-EGFP (b and d) were examined and H&E staining is shown (a and b). Proliferating tumor cells were immunolocalized with an anti-Ki67 antibody and found to be more numerous in FTC133-RLN2 (c) than in FTC133-EGFP xenografts (d). Arrow, representative Ki67-positive cells. Magnifications, ×400 (a and b) and ×200 (c and d; B). For quantitative assessment of in vivo proliferation, Ki67-positive cells were counted in three sections for each xenograft tumor. The diagram shows the number of Ki67-positive cells per square millimeter as mean ± SEM. One-way ANOVA was used for statistical analysis and a \( P < 0.05 \) was considered significant (*; C).
The presence of S100A4 was identified as a marker for poor prognosis in patients with breast cancer (13) and was associated with lymph node metastases in patients with PTC (35). In the present study, primary PTC with lymph node metastasis displayed higher levels of S100A4 protein. Despite the small sample size, this trend may suggest that the presence of strong S100A4 expression is a predictive parameter for early lymph node metastasis in primary PTC. Similar to a previous report on human PTC tissues (35), we found S100A4 to be mainly located within the cytosol with only scattered nuclear staining in single tumor cells of all three carcinoma subtypes studied. We detected the 10-kDa monomeric and/or the 20-kDa dimeric S100A4 form in human thyroid cancer tissues and cell lines. The formation of the SDS-resistant S100A4 dimer and S100A4 oligomers were previously described (43, 44) but the functional role of this strong SDS-resistant oligomer of S100A4 remains unknown.

We identified S100A4 as a novel target molecule of RLN2-RXFP1 signaling. Recently, we showed S100A4 to be downregulated in MDA-MB-231-RLN2 transfecatants (45). In human thyroid carcinoma cells, RLN2 had the opposite effect and markedly upregulated S100A4 gene activity and protein levels, suggesting cell type-specific effects of RLN2 on S100A4 regulation. Several mechanisms have been previously described to be involved in the regulation of S100A4 gene expression. DNA methylation on the first intron of S100A4 was shown to control gene expression in mouse mammary carcinoma cell lines (46). In MDA-MB-435 human breast cancer cells, integrin αβ4-mediated activation of NFAT upregulates S100A4 gene activity and multiple NFAT consensus–binding sites at the S100A4 promoter were identified (43). Luciferase S100A4 promoter constructs showed the regulation of human S100A4 promoter through ErbB2 activation in a medulloblastoma cell line (47). The regulatory mechanisms in human thyroid carcinoma cells have not been identified yet. S100A4 may mediate some of the tumor-promoting effects of RLN2 in human thyroid carcinoma on motility and matrix invasion (10). S100A4 knockdown by siRNA interference reduced cell motility in FTC133 transfectants and exogenous relaxin was unable to increase cell motility in S100A4 siRNA–treated FTC133 cells. Indeed, stable transfectants of C643 thyroid carcinoma cells with constitutive expression of S100A4 showed enhanced cell motility and were unable to respond to relaxin with increased motility, suggesting that in this cell model, S100A4 protein levels are rate limiting for C643 motility. RXFP1 siRNA knockdown in FTC133 prevented RLN2-induced motility increase, but exogenous S100A4 was still able to elicit a promigratory response, suggesting S100A4 to act downstream of RLN2-RXFP1 signaling to increase cell motility.

FTC133-RLN2 transfectants were highly tumorigenic in nude mice, generating fast growing and large xenografts compared with mock clones. Despite high levels of RLN2 and S100A4, none of the mice harboring FTC133-RLN2 clones showed macroscopically visible metastases after 5 weeks of local tumor growth. S.c. injection of tumor cells...
has been suggested not to be permissive of metastatic growth but rather promote localized growth of xenografts. In addition, the rapid tumor growth observed with both FTC133-RLN2 clones may not have allowed enough time for the tumors to produce distant metastases in nude mice. A distinctive feature was the increased number of microvessels in the FTC133-RLN2 xenografts, suggesting that RLN2 and/or S100A4 secreted by thyroid tumor cells may enhance in vivo tumor growth by promoting tumor angiogenesis. S100A4 was shown to facilitate the invasiveness of endothelial cells in Matrigel by stimulating the production and secretion of extracellular matrix-degrading enzymes such as matrix metalloproteinase 13 (collagenase 3; refs. 23, 48, 49). Enhanced endothelial cell motility and angiogenesis are dependent on the presence of oligomeric extracellular S100A4 (24, 50). FTC133-xenografts contained high levels of S100A4 and our in vitro analysis revealed that S100A4 was secreted by FTC133-RLN2 transfectants and FTC133-EGFP vector controls, whereas RLN2 was secreted only by FTC133-RLN2 transfectants (10). Both rhRLN2 and rhS100A4 were able to induce capillary-like tube formation of HUVEC cells in vitro. Isolated HUVEC cells were reported to respond to relaxin with increased the production of nitric oxide synthase II and increased nitric oxide generation (51). Relaxin was also shown to induce vascular endothelial growth factor and procollagenase expression in human endometrial cells and to stimulate blood vessel formation in the primate endometrium (52, 53). Here, we show for the first time a direct angiogenic effect of RLN2 by promoting endothelial tube formation of HUVEC by RLN2. Thus, RLN2 may act angiogenic by a direct effect on endothelial cells and through increased S100A4 protein levels. The transmembrane receptor receptor for advanced glycation end-products was shown to be the receptor for S100 proteins (54-56), potentially including S100A4 (55-57). Receptor for advanced glycation endproducts protein is expressed in HUVEC cells (58, 59), implicating secreted S100A4 as a new paracrine executioner of RLN2 actions in thyroid carcinoma angiogenesis.

In conclusion, we identified S100A4 as novel target molecule for RLN2-RXFP1 signaling in human thyroid carcinoma cells. S100A4 mediated the motility-enhancing effects of relaxin and promoted angiogenesis in thyroid carcinoma xenograft tumors. Further studies are needed to clarify the role of the monomeric and dimeric form of S100A4 and the possible effect of cellular compartmentalization of S100A4 on thyroid carcinoma cell functions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Karthirn Hammje, Dana Henderson, and Jenna Gim for their excellent technical support.

Grant Support

Deutsche Forschungsgemeinschaft (DFG HO2319/5-1), Manitoba Medical Service Foundation, and Manitoba Health Research Council.

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Received 07/12/2009; revised 02/17/2010; accepted 02/19/2010; published OnlineFirst 03/23/2010.

References


www.aacrjournals.org Mol Cancer Res; 8(4) April 2010

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42. Treutiger CJ, Mullins GE, Johansson AS, et al. High mobility group 1 is a member of the metastasis-related Mts1 (S100A4) protein stimulate neuronal differentiation in cultures of rat hippocampal neurons. Mol Brain 2007;3:30.


# Molecular Cancer Research

## Relaxin Enhances S100A4 and Promotes Growth of Human Thyroid Carcinoma Cell Xenografts

Yvonne Radestock, Cornelia Willing, Astrid Kehlen, et al.


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