Suppression of Rho B Expression in Invasive Carcinoma from Head and Neck Cancer Patients

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ABSTRACT

Purpose: In contrast to Ras small GTPases, which contribute to human malignancy when overexpressed or constitutively activated, convincing evidence for the involvement of Ras homologous (Rho) GTPases in human cancer is still missing. In cell culture and animal models, RhoB antagonizes malignant transformation, but no data are available regarding the expression of RhoB in human tumors. In this study, we have analyzed the status of the RhoB protein and the closely related family member RhoA in human head and neck squamous cell carcinomas.

Experimental Design: Protein immunoeexpression was quantitated by image analysis in the context of tumor invasion and differentiation. To account for possible individual variations, expression levels of RhoB and RhoA were evaluated in the tumor and its adjacent nonneoplastic tissue. Potential gene deletions or mutations were assessed by PCR and RT-PCR.

Results: RhoB expression is readily detected in normal epithelium, carcinomas in situ, and well-differentiated tumors, but it becomes weak to undetectable as tumors become deeply invasive and poorly differentiated. In contrast, Ki67 (proliferation marker) and RhoA protein levels increase with tumor progression. Furthermore, whereas in nonneoplastic keratinocytes RhoB is localized mainly in the nucleus, in carcinomas RhoB is predominantly located in the cytoplasm. RhoB gene deletions or mutations were not found.

Conclusions: These results give additional support to the notion that RhoB may play a tumor suppressive role in squamous cell carcinomas of the head and neck. The lack of RhoB expression in deeply invasive carcinoma argues against inhibition of RhoB farnesylation as a mediator of farnesyltransferase inhibitors’ antitumor activity.

INTRODUCTION

The Rho4 subfamily of low molecular weight GTP-binding proteins appears to control many aspects of cellular functions, including adhesion, morphology, motility, and cell cycle progression (1). Although 12 family members have been identified thus far, only 3 of these, RhoA, Rac1, and Cdc42, have been studied in detail (1). Whereas RhoA interferes with stress-fiber formation, and Rac1 and Cdc42 regulate formation of lamellipodia and filopodia, respectively, the function of RhoB remains unknown (2).

Despite the high homology of the different Rho isoforms, RhoA, RhoB, and RhoC, their physiological function appears to be distinct (3, 4). RhoB differs from other Rho proteins in its COOH-terminal isoprenylation and intracellular localization (5). Moreover, RhoB mRNA, but not that of RhoA, RhoC, Rac1, and Cdc42, is rapidly induced by UV irradiation and DNA damaging agents, indicating a role for RhoB in the early response of eukaryotic cells to genotoxic stress (6, 7). The induction of RhoB upon treatment of cells with DNA damaging agents either blocks DNA replication or causes apoptosis (8). Thus, it seems that RhoB plays a regulatory role sensing the level of cytotoxicity in the cell and quickly triggering subsequent protective responses, such as cell cycle arrest, through induction of the cell cycle kinase inhibitor p21WAF1/CIP1 or apoptosis (8–10).

Recent evidence suggests that RhoB may act as a tumor suppressor. First, as mentioned above, RhoB induction is an early response of eukaryotic cells to genotoxic stress (7). Second, overexpression of RhoB in human cancer cells results in inhibition of signal transduction pathways involved in oncogenesis and tumor survival, as well as induction of apoptosis (11). Third, when overexpressed in Ras-transformed cells, RhoB induces phenotypic reversal, cell growth inhibition, and activation of the cell cycle kinase inhibitor p21WAF1/CIP1 (12). Finally, overexpressing RhoB in human cancer cells antagonizes their anchorage-dependent and -independent cell growth and suppresses their growth in nude mice (11). Taken together, these results suggest that RhoB interferes with tumorigenesis, and thus it is of great interest to determine the genetic and functional status of RhoB in human primary tumors.

To address the question of whether RhoB expression is...
altered in human tumorigenesis, we analyzed RhoB levels in head and neck squamous cell carcinomas. Our results show that RhoB expression is prevalent in carcinomas \textit{in situ} and well-differentiated tumors. This expression becomes weak to undetectable as the tumors become deeply invasive and more undifferentiated. Furthermore, whereas RhoB was localized mainly in the nucleus in nonneoplastic epithelial cells, in carcinomas RhoB was predominantly located in the cytoplasmic compartment.

**MATERIALS AND METHODS**

**Selection of Cases.** Human head and neck squamous cell carcinomas were selected from the archives of the Pathology Department at the H. Lee Moffitt Cancer Center and Research Institute (Tampa, FL) to evaluate protein expression in the context of the different histopathological stages in the progression from normal epithelium to invasive cancer. The selected cases had to fulfill all of the following criteria: (a) presence of areas of “nonneoplastic” squamous epithelium “adjacent” to the tumor and lacking histopathological features of dysplasia or malignancy; (b) presence of areas of dysplasia and/or \textit{in situ} carcinoma; (c) presence of superficial and infiltrating components; (d) presence of at least two different degrees of differentiation; (e) specimens were properly processed (no delayed fixation or poor handling) and free of necrosis; (f) patients did not receive any previous treatment (with the exception of a diagnostic biopsy); and (g) patients were initially diagnosed, treated, and followed-up at the Moffitt Cancer Center to ensure collection of clinical data, adherence to Institutional Review Board requirements, and patient confidentiality. The diagnosis of squamous cell carcinoma was made by a head and neck pathologist using standard criteria that included intracellular bridges, keratinization, keratin pearl formation, and various degrees of cytological atypia. Degree of differentiation was assigned as follows: (a) well differentiated: large cells with obvious keratinization, intercellular bridges, or keratin pearl formation; (b) moderately differentiated: heterogeneous population of cells of moderate size with only focal keratinization, poorly preserved intercellular bridges, and a higher degree of cytological atypia; and (c) poorly differentiated: anaplastic population lacking keratinization and intercellular bridges and with minimal resemblance to squamous epithelium. Desmoplasmic stromal infiltration required desmoplastic reaction associated with ragged tumor borders. The superficial component of the invasive carcinoma was defined as that region of the tumor in continuity with the overlying squamous epithelium and that invaded the underlying stroma up to a distance of \(~300~\mu m\). Within this distance, there is a high probability of angiolymphatic invasion. Deep invasion was defined as those areas of the tumor invading to a distance \(>300~\mu m\) and/or with tumor tongues separated from the main mass. Clinicopathological staging was assigned following the recommendations of the American Joint Committee on Cancer (13).

**Immunohistochemical Analysis of RhoB, RhoA, SP1, and Ki67.** Five-\(\mu m\) sections from formalin-fixed, paraffin-embedded tissues were cut and placed on poly-L-lysine-coated slides. Slides were subjected to deparaffinization in xylene and hydration through a series of decreasing alcohol concentrations, following standard procedures. Endogenous peroxidase activity was quenched with a 3% solution of \(H_2O_2\) for 20 min at \(37^\circ C\), and the slides were washed in deionized water for 5 min. Antigen retrieval was performed by placing the slides in a clear plastic container with a vented top containing citrate buffer [0.1 M citric acid (4.5 ml), 0.1 M sodium citrate (21.5 ml), and deionized water (225 ml)] in a microwave oven set on high, twice for 5 min each. The slides were then allowed to cool for 10–20 min, rinsed in deionized water, placed in PBS for 5 min, and drained. Blocking serum was applied, and the slides were incubated in a humid chamber for 20 min at room temperature. After blotting, the following primary antibodies were applied at room temperature: polyclonal anti-RhoB (Santa Cruz Biotechnology); anti-RhoA (Santa Cruz Biotechnology); anti-SP1 (Santa Cruz Biotechnology); or anti-Ki67 (Dako Corporation, Carpinteria CA). After 1 h, slides were rinsed with PBS and placed in PBS for 5 min. For detection, the Vectastain ABC Kit, Rabbit IgG, Elite series (Vector Laboratories, Inc., Burlingame, CA) was used, following the manufacturer’s specifications. The biotinylated secondary antibody was applied for 20 min at room temperature in a humid chamber. At the end of this incubation, the slides were rinsed and placed in PBS for 5 min, followed by the addition of the avidin-biotin complex. The slides were incubated in a humid chamber for 30 min at room temperature and then rinsed and placed in PBS for 5 min. 3,3′-diaminobenzidine, prepared according to the manufacturer’s specifications, was applied to the slides and color development monitored. When maximal intensity was reached (~5 min), the slides were rinsed in water and counterstained with modified Mayer’s hematoxylin for 30 s. The slides were finally washed in running water for 10 min, dehydrated, cleared, and mounted with resinous mounting medium.

**Image Analysis.** The Optimas 6.5 (Media Cybernetics, Inc.) software was used to quantitate protein expression. Images were stored as TIFF images using identical magnifications \((\times 400)\) and camera settings. The ROI (normal squamous epithelium, dysplasia, carcinoma \textit{in situ}, superficial, and deep components of the tumor and different degrees of differentiation) were defined, and the total area and the number of nuclei in a given ROI were determined. Thresholds were set to discriminate between the brown color of RhoB-positive nuclei and the negative (blue) nuclei. A RhoB index was generated as the percentage of positive areas versus total area.

**Statistical Analysis.** The Wilcoxon-Rank Sum test was used to compare paired samples.

**PCR and RT-PCR Analysis.** DNA and RNA samples were obtained from the H. Lee Moffitt Cancer Center Tissue Procurement Laboratory. cDNA was synthesized from 1.0 \(\mu g\) of total cellular RNA by reverse transcription with Sensiscript reverse transcriptase (Qiagen, Valencia, CA) using random hexamers as primers. The histone H3.3A sense (\(5′-CCACTGAACCTGATGC-3′\)) and antisense (\(5′-CGTGCTAGCTGGATGCTT-3′\)) primers correspond to sequences on two different exons. They were used as positive controls for the reverse transcription reaction and genomic DNA contamination (generation of an additional large PCR fragment). The following RhoB sense (\(5′-ATGGCGGCC-CATCCGCCGAAAGC\)) and antisense (\(5′-TCATAGCCACCTTG-CAGCAGTTG\)) primers were used. These primers amplify the entire RhoB gene from nucleotides 1 to 591. Of note, RhoB is a
591 bp intronless gene (3, 4), thus, RhoB primers will not allow the distinction of mRNA from genomic DNA. The PCR mixture contained 50 mM Tris (pH 9.0), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 100 ng of each primer, 0.056 μM TaqStart Antibody (Clontech Laboratories, Palo Alto, CA), and 1 unit Taq polymerase (Life Technologies, Inc.). After an initial denaturation at 94°C for 5 min, amplification was carried out for 30 cycles at 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min, followed by a final elongation step at 94°C for 2 min, 55°C for 2 min, and 72°C for 10 min. The PCR products were resolved on a 2% agarose gel and stained with ethidium bromide.

RESULTS
RhoB Expression Is Decreased in Invasive Carcinomas of the Head and Neck. To test whether RhoB expression is altered in tumorigenesis, we evaluated the status and subcellular localization of RhoB protein in head and neck cancer patient specimens across the different histopathological stages from normal epithelium to invasive squamous cell carcinoma. Sections from formalin-fixed, paraffin-embedded tissues were immunostained with antibodies to RhoB and the expression index of RhoB, was determined using image analysis, selectively excluding nontumoral components such as stroma, vessels, and inflammation. The number of nuclei that were positive for RhoB staining was calculated by setting the software threshold to overlap the brown color of 3,3’-diaminobenzidine, whereas that of negative nuclei was calculated by setting the threshold over the blue color of the hematoxylin counterstain (14). To quantify cytoplasmic staining, the area showing cytoplasmic expression for a given marker was calculated as a function of the total cytoplasmic area of tumor cells contained within the region of interest. Images corresponding to the different tumor areas are
Suppression of RhoB Expression in Invasive Carcinoma

Expression of RhoB in Squamous Cell Carcinoma Across Different Degrees of Differentiation. The results from Fig. 1 suggest that RhoB localizes in the nucleus in normal tissue and in the cytoplasm in tumors. To determine whether RhoB expression and localization is affected by the differentiation state of the tumors, RhoB expression was analyzed in tumors with different degrees of differentiation. Fig. 2 shows RhoB expression in two different tumors (Fig. 2, A and B from case 2 in Table 1; Fig. 2, C and D from case 18 in Table 1). Fig. 2A shows predominantly nuclear expression in overlying nonneoplastic epithelium and predominantly cytoplasmic RhoB expression in adjacent superficially invasive well-differentiated squamous cell carcinoma. The well-differentiated areas in both tumors (Fig. 2, A and C) show higher RhoB expression levels than the poorly differentiated areas in the same tumors (Fig. 2, B and D, respectively). The analysis in all 18 cases shows that RhoB levels progressively decrease from well-differentiated areas (average RhoB index: 32), to moderately differentiated areas (average RhoB index: 12), to poorly differentiated areas (average RhoB index: 3.2). In well-differentiated areas, if decreased RhoB intensity is observed, it is restricted to deeply infiltrating areas. RhoB expression status was evaluated in 18 head and neck squamous cell carcinomas from different anatomical locations (Table 1). Cases were tabulated according to disease stage at presentation. Table 1 also lists the gender, age, status of the patient, RhoB indexes, and statistical analysis. A marked reduction in the staining intensity of RhoB was observed on all 18 cases studies. When compared with nonneoplastic tissue, carcinoma in situ shows a reduction of ~15%, the superficial component of the invasive carcinoma a reduction of 60%, and the deep component of the invasive carcinoma a reduction of 97%. Specifically, no significant differences in RhoB protein levels were found between nonneoplastic epithelium and carcinoma in situ, to superficially invasive carcinoma, to deeply infiltrating carcinoma. The specificity of the nuclear staining of nonneoplastic epithelium with the RhoB antibody (Fig. 1A) was ascertained by abolishing the staining in the presence of the immunizing specific peptide (Fig. 1B). RhoB nuclear expression was maintained in carcinoma in situ (Fig. 1C); however, in areas of transition of carcinoma in situ to invasive carcinoma, RhoB was progressively found in a cytoplasmic location in a higher number of tumor cells (Fig. 1D). Finally, in invasive carcinoma, RhoB could no longer be detected in the nucleus and its level in the cytoplasm was variable, and RhoB was often absent from the most deeply invasive areas of the tumor (Fig. 1, E and F).

RhoB’s amino acid sequence is 86% identical to that of the closely related family member RhoA. Therefore, we investigated whether the expression of RhoA was also altered in head and neck tumors. Sections from formalin-fixed, paraffin-embedded tissues were immunostained with antibodies to RhoB and to RhoA to evaluate the tissue expression of another Rho protein, Sp1, to evaluate the tissue status of a marker known to localize to the nucleus and Ki67 to evaluate cell proliferation. As shown
Table 1  Quantitation of RhoB expression in head and neck squamous cell carcinoma

The Optimas 6.5 Image Analysis software was used to quantitate nuclear RhoB expression in 18 squamous cell carcinomas containing superficial invasive component (SIC) and deeply invasive component (DIC) areas of the tumor, as well as in adjacent areas of carcinoma in situ (CIS) and nonneoplastic squamous epithelium. RhoB quantitation was also performed independently in well-differentiated (WD), moderately-differentiated (MD), and poorly-differentiated (PD) areas of the tumors. The intensity of RhoB staining is expressed as a RhoB index. The average and SD for each of the grouped areas are depicted at the bottom of the table. A Wilcoxon Rank-Sum test analysis showed significant differences (P < 0.0001) between nonneoplastic epithelium, SIC, and DIC, between CIS, SIC, and DIC, and between SIC and DIC. No significant differences were found between nonneoplastic epithelium and CIS. Significant differences (P < 0.0001) were also observed among the three degrees of differentiation.

![Rho B expression in squamous cell carcinomas from 18 head and neck cancer patients](image)

![Rho B expression in squamous cell carcinomas from 18 head and neck cancer patients](image)

**Fig. 3** Rho B expression in squamous cell carcinomas from 18 head and neck cancer patients. The average from Table 1 of RhoB index in nonneoplastic squamous epithelium (78.5 + 11.5), carcinoma in situ (CIS; 66.5 + 9), superficially invasive carcinoma (SIC; 30.1 + 16), and deeply invasive carcinoma (DIC; 2.2 + 1.1) is graphically represented.

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Average 78.5  66.5  30.1  2.2  32  12  3.2  11.5  9.0  16.0  1.1  15.8  10.4  1.7

* NED, no evidence of disease; AED, alive with evidence of disease; DOD, dead of disease.

The low level or lack in RhoB protein levels in head and neck tumors could be attributable to either deletions or decrease expression of the RhoB gene. Therefore, we analyzed RhoB genomic DNA and RNA from 12 head and neck tumor samples that were previously examined for RhoB gene deletions. How-ever, this result does not exclude the possible occurrence of small deletions or point mutations, which cannot be detected by the PCR method used. Next, we analyzed RhoB RNA from these samples by RT-PCR using the same set of primers. Total RNA was reverse transcribed as described in “Materials and
Methods," and the resulting cDNA was PCR amplified using primers specific for either the housekeeping gene, histone H3.3A, or RhoB. The histone H3.3A primers correspond to sequences in two different exons, thus, if genomic DNA contaminates a sample, an additional larger PCR fragment will be generated. However, RhoB primers will not allow the distinction of mRNA from genomic DNA because RhoB is an intronless gene. The RT-PCR experiments, using Sensiscript, for the reverse transcriptase reaction show that the PCR amplification with RhoB primers of tumor RNA without the addition of reverse transcriptase did not yield any bands (Fig. 5). In addition, RT-PCR reactions using histone primers yield similar levels of a single band in all of the tumor samples examined (data not shown). However, the level of RhoB RNA was low to

Fig. 4 Expression of RhoB (A and B), RhoA (C and D), Sp1 (E and F), and Ki67 (G and H) in nonneoplastic epithelium and deep regions of invasive carcinoma. Sections from formalin-fixed, paraffin-embedded tissues were processed and immunostained with anti-RhoB, RhoA, Sp1, and Ki67 antibodies as described in “Materials and Methods.” RhoB is strongly expressed in the nuclei of cells from lower layers of squamous epithelium (index: 76.05) and with low intensity and only in rare cells in carcinoma (index: 17.22). RhoA is expressed in the cytoplasm of the lower two-thirds of the nonneoplastic epithelium (index: 43.23) and with increased intensity in the majority of carcinoma cells (index: 87.16). Sp1 is strongly expressed in the nuclei of cells from all layers in squamous epithelium (index: 96.75) and in the majority of carcinoma cells (index: 78.89). Ki67 is expressed in the nuclei of proliferating layers of nonneoplastic squamous epithelium and in the majority of the carcinoma cells (index: 71.03).
genetic alterations are most likely not involved. The data also suggest that any mutations. However, it could be argued that in some tumors a mutation or deletion of the gene could be masked by contamination with normal tissue. The best way to avoid this problem would be to isolate our genetic material using laser capture microdissection of the areas of interest. However, for our analysis we used archived paraffin blocks, which unfortunately are not amenable to these procedures. Future prospective studies will include the collection of the paraffin blocks under conditions that would conserve the RNA. Taken together, our data suggest that the absence of RhoB may have contributed to tumor progression and aggressiveness.

The absence of RhoB in advanced tumors was not because of deletion or mutation of RhoB gene, but rather it appears that RhoB may have been either transcriptionally down-regulated or its mRNA became unstable. In addition to its low abundance in advanced tumors, RhoB mislocalized to the cytoplasm. Indeed, in nonneoplastic epithelium, RhoB was present predominantly in the nucleus but, as the tumors progress to invasive carcinoma, RhoB was mostly present in the cytoplasm at levels varying from low to null in the most deeply invasive areas of the tumor. Consistent with our results, showing a nuclear presence of RhoB, is the association of RhoB with the transcription factor DB1 and the nuclear membrane, and its presence in an intranuclear laminar region (16). Thus, it seems that in normal cells, RhoB would translocate to the nucleus where it performs its function but, as cells progress toward a malignant phenotype, RhoB loses this ability to translocate to the nucleus and remains mainly in the cytoplasm. RhoB does not possess a nuclear localization signal, and it is unclear how RhoB is transported to the nucleus.

In contrast to RhoB, RhoA was predominantly cytoplasmic and its level increased by a 2-fold in 80% of carcinomas. This is in agreement with a previously reported study in which RhoA expression was shown to be up-regulated in colon and lung tumors and in breast tumors progressing from WHO grade I to grade III (17). Furthermore, the analysis of expression of RhoB and RhoA in 60 human cancer cell lines of the National Cancer Institute showed that RhoA was expressed in all cell lines, whereas RhoB protein was barely detectable. Thus, despite their high sequence homology, RhoA and RhoB appear to play distinct roles in the cell. In addition to differences in cellular localization, RhoB has several features that distinguish it from RhoA. Most notably, RhoB, but not RhoA, is inducible by a variety of stimuli, including growth factors, UV, and DNA-damaging agents (7, 18), and unlike RhoA, both RhoB mRNA and protein are labile with half-lives of ~20 min and 2 h, respectively. RhoB turnover occurs via ubiquitin-mediated destruction by the 26 S proteasome (19). The transcriptional induction and the short half-lives of RhoB, together with its posttranslational modifications (isoprenylation) and GTP/GDP binding allow the regulation of RhoB function at several levels and suggest that RhoB may be a component of a regulatory pathway(s).

Whereas in normal epithelium and in most of the well-differentiated areas, RhoB was predominantly nuclear, in moderately and poorly differentiated regions of the tumors, RhoB localized to the cytoplasm and its expression decreased dramat-
ically. These results suggest that RhoB function may be involved in cellular differentiation. Interestingly, RhoB was previously cloned as a gene induced by Bone Morphogenetic Proteins during the early differentiation of neural crest cells, suggesting a role of RhoB in differentiation in response to Bone Morphogenetic Proteins signaling (20). Moreover, transforming growth factor β was shown to exert a stabilizing influence on RhoB, thereby resulting in its accumulation (19).

Mounting evidence points to a role of RhoB in tumor inhibition. Indeed, accumulation of geranylgeranylated RhoB, after treatment of cancer cells with farnesyltransferase inhibitors, has been suggested to be the event mediating the antitumor effect of farnesyltransferase inhibitors (10). In addition, under in vitro conditions, overexpression of RhoB results in inhibition of transformation, phenotypic reversion, and cell growth inhibition (11–12). The data described in this study demonstrating a decrease of RhoB expression during tumor progression and the lack of expression of RhoB in deeply infiltrating carcinoma gives additional support for the tumor-suppressive activity of RhoB.

To summarize, our findings indicate that RhoB expression and localization become altered during tumor progression. On the basis of these studies, our results support the view that a decrease in RhoB expression is a critical event in malignant transformation and/or progression of human cancer cells. The molecular basis for this decrease in RhoB expression remains to be elucidated.

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Suppression of Rho B Expression in Invasive Carcinoma from Head and Neck Cancer Patients

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