Epigenetic Reactivation of RANK in Glioblastoma Cells by Curcumin: Involvement of STAT3 Inhibition

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DNA methylation plays an essential role in carcinogenesis. Promoter hypermethylation can result in transcriptional silencing of specific genes, such as tumor suppressors. Thus far, few reports have investigated the effect of curcumin, an active component of the perennial herb *Curcuma longa*, on DNA methylation. In the present study, we evaluated the effects of curcumin on receptor activator of NF- κ B (RANK) gene expression in human glioblastoma cells. Incubation of cells with therapeutic concentrations of curcumin resulted in a significant elevation of RANK expression at both the mRNA and protein levels in two glioblastoma cell lines. We further confirmed that this elevation was associated with promoter demethylation through methylation-specific polymerase chain reaction (PCR) and bisulfite sequencing PCR. Additionally, we demonstrated that knockdown of STAT3, an oncogenic transcription factor, is sufficient to induce RANK promoter demethylation along with RANK reactivation. These results demonstrated that curcumin induced RANK gene reactivation through epigenetic modification in human glioblastoma cells, and that STAT3 is involved in RANK promoter hypermethylation and epigenetic silencing, thus allowing for further applications of curcumin epigenetic therapy in glioma and therapeutic implications of STAT3 in human glioblastoma.

Introduction

RECEPTOR ACTIVATOR of NF- κ B (RANK), also known as tumor necrosis factor (TNF) receptor superfamily member 11A, is an essential mediator of osteoclast and lymph node development (Hanada *et al.*, 2011; Maeda *et al.*, 2012). It is activated by binding to its ligand, RANKL, and regulates the interaction between T-cells and dendritic cells (Roberts *et al.*, 2012). RANK lacks the death domain and binds to specific members of the TNF receptor-associated factor family for transduction of stimulation signals to NF- κ B and c-Jun N-terminal kinase activation (Hanada *et al.*, 2011). RANK/RANKL signaling also mediates survival signaling through activation of the Akt or EGFR pathway and through upregulation of survival proteins (Georgakis *et al.*, 2006; Yi *et al.*, 2008). Nevertheless, several observations indicate that, similar to other members of the TNF superfamily, RANK/RANKL also possesses antitumorigenic and proapoptotic properties (Bharti and Aggarwal, 2004; Papanastasiou *et al.*, 2012; von dem Kneesebeck *et al.*, 2012).

The initiation and progression of cancer are controlled by both genetic and epigenetic events. Epigenetic alterations in tumor suppressor genes are potentially reversible, allowing

the utilization of epigenetic targets as an effective and valuable approach to cancer chemotherapy (Yoo and Jones, 2006). Epigenetic silencing mechanisms of tumor suppressor genes include alterations in CpG island methylation patterns and histone modifications, both of which regulate gene expression without altering the underlying DNA sequences. Aberrant DNA methylation patterns have been proven to be associated with a large number of human malignancies. In particular, many tumor suppressor genes are inactivated by promoter CpG island hypermethylation (Yoo and Jones, 2006). Therefore, evaluation of the CpG island methylator phenotype is helpful for clinical decision making, and targeting DNA methylation is useful for cancer treatment (Hardy and Tollefsbol, 2011; Liao *et al.*, 2012). Indeed, 5-aza-2'-deoxycytidine (DAC), an FDA-approved DNA methyltransferase inhibitor, results in the abrogation of tumorigenicity *in vivo* (Tsujioka *et al.*, 2012). STAT3, a well-characterized oncogenic transcription factor, promotes oncogenesis by upregulating a number of genes that promote proliferation or inhibit apoptosis. Although the underlying machinery remains unclear, STAT3 is responsible for hypermethylation of CpG islands in certain tumor suppressor genes by directly inducing the expression of and interacting with DNA methyltransferase 1 (DNMT1),

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providing evidence for epigenetic regulation of STAT3 in cancer cells (Mathews *et al.*, 2010; Lee *et al.*, 2012).

Curcumin is an active component of the perennial herb *Curcuma longa* (turmeric). Extensive research has indicated that this polyphenol possesses several biological activities beneficial to both cancer treatment and prevention. For example, curcumin suppresses both constitutive and induced STAT3 activation in malignant cancer cells (Saydmohammed *et al.*, 2010). Moreover, curcumin also modulates gene expression through its activity as an epigenetic agent. In particular, curcumin induces DNA hypomethylation through inhibition of DNMT1, as well as regulation of the acetylation state of histones by inhibiting histone acetyl transferases. Both of these properties can significantly alter the epigenome of cancer cells by reactivating prometastatic genes and proto-oncogenes. (Ravindran *et al.*, 2009; Hardy and Tollefsbol, 2011). Therefore, in the present study, we evaluated whether curcumin reactivates RANK expression via promoter demethylation in human glioblastoma cells. Additionally, we investigated the role of STAT3 in curcumin-induced epigenetic reactivation of RANK in human glioblastoma cells.

Materials and Methods

Chemicals, reagents, and cell culture

Curcumin, DAC, dimethyl sulfoxide (DMSO), and mouse monoclonal IgG antibodies against β -actin were purchased from Sigma (St. Louis, MO). Curcumin diluted in DMSO was prepared as a stock solution of 50 mM and stored at -20°C until use. M.SssI and BstUI were purchased from New England Biolabs (Beverly, MA). Human STAT3 small interfering RNA (siRNA) and scrambled control siRNA were obtained from GenePharm (Shanghai, China). The human neuroblastoma cell lines U251 and U87 were obtained from the Chinese Academy of Medical Sciences (Beijing, China). The cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Valencia, CA) supplemented with 10% fetal bovine serum (Invitrogen) in a humidified incubator with 5% CO_2 at 37°C , and were passaged twice weekly.

Cell transfection

U251 cells were cultured in six-well plates (2×10^5 cells per well) for 24 h and then transfected with STAT3 siRNA (30 nM) or control siRNA (30 nM) using LipofectamineTM RNAiMAX Reagent (Invitrogen) according to the manufacturer's instructions. The target sequences of the STAT3 siRNA and the control siRNA oligonucleotides were AAC TTCAGACCCGTCACAAA and CACCAGAACCATGGC CAAC, respectively.

RNA preparation, reverse transcription–polymerase chain reaction, and real-time reverse transcription–polymerase chain reaction

Total RNA was extracted using an E.Z.N.A. Total RNA Kit (Omega Bio-Tek, Norcross, GA), and was reverse-transcribed using a PrimeScript II 1st Strand cDNA Synthesis Kit (Takara, Shiga, Japan). For reverse transcription–polymerase chain reaction (RT-PCR), Premix Ex Taq polymerase (Takara) was used according to the manufacturer's protocols. PCR was performed for 35 cycles under the following conditions: 95°C for 5 min, 98°C for 20 s, 60°C for 40 s, 72°C for 45 s, and

72°C for 7 min. The products were electrophoresed on a 1.5% agarose gel. Real-time RT-PCR was performed using an SYBR Premix Ex Taq Kit (Takara) and an ABI Vii7 detection system (Applied Biosystems, Foster City, CA). The reaction conditions were 95°C for 30 s, 95°C for 5 s, and 60°C for 34 s (40 cycles). β -Actin was used as an internal control.

Western blotting

Western blotting was performed as described previously (Saydmohammed *et al.*, 2010). Briefly, whole-cell extracts were prepared using ProteoJETTM Mammalian Cell Lysis Reagent (Fermentas, Burlington, Canada) supplemented with protease and phosphatase inhibitors (Fermentas) according to the manufacturer's instructions. Protein (20–40 μg) was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis in 8%–10% gels and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). The blots were blocked for 1 h at room temperature with 5% bovine serum albumin (Sigma) in Tris-buffered saline/0.1% Tween-20. Next, the blots were probed with anti-RANK (Cell Signaling Technology, Inc., Danvers, MA), anti-STAT3 (Epitomics, Burlingame, CA), anti-phospho-STAT3 (pY705; Epitomics), or β -actin antibodies overnight at 4°C . The blots were then incubated with corresponding horseradish peroxidase-conjugated secondary antibodies (1:3,000 dilution) for 1 h at room temperature. After additional washes, signals were detected using SuperSignal ECL (Pierce, Rockford, IL).

DNA methylation analysis

For methylation-specific PCR (MSP), genomic DNA was isolated from drug-treated cells using the MiniBEST Universal Genomic DNA Extraction Kit (Takara), according to the manufacturer's instructions. The extracted genomic DNAs were subjected to bisulfite modification. Bisulfite conversion was carried out using the EZNA one-step DNA methylation kit (Omega Bio-Tek, Norcross, GA), following the manufacturer's instructions. The converted DNA was amplified with the primer sets listed in Table 1 to determine promoter hypermethylation. For bisulfite sequencing PCR (BSP), the promoter region spanning 17 CpG sites (-146 to $+10$) was amplified using sodium bisulfite-modified genomic DNA as a template. PCR products were then cloned into the pUC18-T vector. Plasmid DNA from at least six colonies per treatment was prepared using Tianpure Mini Plasmid Kit (TianGen, Beijing) and sequenced (Sangon, Shanghai).

In vitro methylation assay

The inhibitory effect of curcumin on DNMT1 was assessed by an *in vitro* methylation assay based on the CpG methyl transferase M.SssI enzyme. A 301-bp fragment ($+77$ to $+378$ relative to the translation start site) was amplified from cDNA of the human RANK gene. The PCR product was purified using a MiniBEST DNA Fragment Purification Kit (Takara) and subsequently used as substrate DNA for the *in vitro* methylation assay. The methylation reaction contained ~ 400 ng substrate DNA and 4 U M.SssI methylase in a final volume of 50 μL . Curcumin (10 μM , 20 μM , or 40 μM) or DAC (20 μM), along with 160 μM S-adenosyl methionine (NEB) and NEB buffer 2 (NEB), was added. Reactions were incubated at 37°C for 6 h. Next, the enzyme was deactivated

TABLE 1. PRIMERS USED FOR MSP, BSP, RT-PCR, AND REAL-TIME RT-PCR

Gene	Primer	Sequence (5'-3')	Product (bp)
RANK	MSP methylation forward	TGGTTGGTATCGTCGGGTTTC	124
	MSP methylation reverse	CTCTATAACCCAACGAACGCG	
	MSP unmethylation forward	TATTTGGTTGGTATTGTTGGGTTT	129
	MSP unmethylation reverse	CCTCTATAACCCAACAAACACAACACTAC	
	BSP forward	TAGGGGAGTTTGGGTATTATTTGG	156
	BSP reverse	ACCTCAACAACCTCTATAACCCAAC	
	^a RT-PCR forward	GGCTTTCAGATCGCTCCT	301
	^a RT-PCR reverse	GCACTCGCAGTCCTGGCTC	
	real-time RT-PCR forward	TCAACAAGGACACAGTGTGC	181
	real-time RT-PCR reverse	TTTGGTGGTTTCTAGCTGG	
β-Actin	real-time RT-PCR forward	AGCGAGCATCCCCAAAGTT	285
	real-time RT-PCR reverse	GGGCACGAAGGCTCATCATT	

^aFor *in vitro* methylation assay.

BSP, bisulfite sequencing PCR; MSP, methylation-specific PCR; PCR, polymerase chain reaction.

at 65°C for 20 min, and DNA was purified using a MiniBEST DNA Fragment Purification Kit (Takara). The purified DNA was incubated with 30-U *Bst*UI for 4 h at 60°C and analyzed on a 1.5% agarose gel.

Statistical analysis

All data are given as the mean ± standard deviation (S.D.). Statistical analyses were conducted using two-tailed paired Student's *t*-tests. *p* < 0.05 was considered statistically significant.

Results

Curcumin reactivated RANK expression in glioblastoma cells

We first assessed the effect of curcumin on RANK expression. As shown in Figure 1, curcumin elevated the mRNA levels of RANK in both U87 (~1.5 to 2.6-fold, *p* < 0.05) and U251 (1.7 to 3.7-fold, *p* < 0.05) cells, and 30 μM curcumin treatment induced a higher level of RANK mRNA levels than that of 15 μM. Notably, curcumin also enhanced the RANK protein level, as shown in Figure 1D. These results suggest that curcumin-induced elevated RANK protein ex-

pression levels may correlate with enhanced RANK mRNA levels in glioblastoma cells. These results demonstrate that curcumin can increase RANK expression in both U87 and U251 cells.

Curcumin demethylated RANK CpG sites in glioblastoma cells

To determine whether curcumin-induced RANK elevation was caused by epigenetic modifications, we first evaluated the methylation status of the CpG sites within the RANK promoter with MSP in U251 and U87 cells. As shown in Figure 2A, the RANK promoters were hypermethylated in both U251 and U87 cells, which was confirmed by DAC treatment. To examine whether the above-described RANK reactivation is associated with promoter hypomethylation, we evaluated the promoter methylation level of RANK in curcumin-treated cells. Results showed complete conversion of promoter hypermethylation in curcumin-treated samples, with an absence of a methylation-specific band (Fig. 2A). We further validated these results by bisulfite sequencing of the selected promoter region of RANK in curcumin-treated and untreated U251 cells. Untreated cells showed a significant methylated signature; all 17 CpG sites were methylated,

FIG. 1. Effects of curcumin on RANK expression in glioblastoma cells. Cells were treated with various concentrations of curcumin for 4 days. Total RNA was prepared and quantified using specific primers by real-time RT-PCR (U87, A; U251, B) and RT-PCR (C). Cells were also harvested, and whole-cell extracts were prepared and probed for RANK (D). β-Actin was used as a loading control. All experiments were repeated at least three times with similar results. The values are means ± standard deviation (SD). **p* < 0.05 based on paired *t*-tests.

FIG. 2. Conversion of promoter methylation status of RANK in glioblastoma cells. **(A)** MSP analysis of U87 and U251 cells after treatment with vehicle, DAC (20 μ M), or curcumin (30 μ M) for 4 days. M, methylation-specific band; U, unmethylation-specific band. **(B, C)** Bisulfite sequencing for the RANK promoter region in curcumin-treated U251 cells. ▼, cytosine in a CpG site; ●, methylated cytosine residues; ○, unmethylated cytosine residues.

whereas treatment with curcumin led to hypomethylation of all 17 CpG sites, thus explaining the complete loss of the methylation band and confirmed the reversal of CpG methylation status by curcumin (Fig. 2B–C).

Curcumin inhibited the DNMT1 analog M.SssI in vitro

The CpG methyltransferase, M.SssI, methylates all cytosine residues (C^5) within the double-stranded dinucleotide recognition sequence, CpG. Inhibition of methylation activity by M.SssI is reflected by the presence of a 259-bp band after restriction digestion of a 301-bp template by methylation-sensitive *Bst*UI (CG/CG). Curcumin caused incomplete inhibition of M.SssI activity at 10 μ M and 20 μ M and complete inhibition at 40 μ M (Fig. 3). Due to methylation of the CG/CG site by active M.SssI, the template was left uncut by *Bst*UI, whereas the reaction lacking M.SssI showed active *Bst*UI.

Curcumin induced RANK expression through STAT3 suppression

STAT3 is crucial for activation of the RANK/RANKL pathway (Mori *et al.*, 2011). Activated STAT3 binds to

FIG. 3. Inhibition of purified recombinant DNA methyltransferase activity by curcumin. Equal amounts of purified M.SssI methylase were incubated with an unmethylated DNA fragment. The addition of curcumin resulted in a detectable decrease in DNA methyltransferase activity, as visualized by the appearance of small *Bst*UI restriction fragments. Increasing amounts of curcumin progressively inhibited methyltransferase activity.

DNMT and promotes methylation-associated silencing of tumor suppressor genes (Zhang *et al.*, 2006). We therefore investigated the role of STAT3 in mediating curcumin-induced RANK expression. We observed a correlation between curcumin-induced deactivation of active STAT3, downregulation of STAT3 expression, and RANK upregulation (Fig. 4A). Next, we tested whether knockdown of STAT3 could result in upregulation of RANK in U251 cells. After STAT3-specific siRNA transfection, STAT3 suppression was evaluated by Western blotting. As shown in Figure 4A, STAT3 expression was successfully inhibited, and RANK was upregulated in STAT3-suppressed cells, suggesting that knockdown of STAT3 is sufficient for induction of RANK

FIG. 4. Effect of STAT3 downregulation on RANK gene silencing. **(A)** Promoter methylation status in U251 cells transfected with control siRNA or STAT3-specific siRNA. **(B)** Protein expression of p-STAT3, STAT3, and RANK in U251 cells. Cells were treated with the indicated concentrations of curcumin for 4 days or transfected with siRNA for the indicated times. ●, methylated cytosine residues; ○, unmethylated cytosine residues.

expression. Notably, the promoter hypermethylation status of RANK in si-STAT3-transfected U251 cells was also converted. However, only 12 of 17 CpG sites were demethylated, which was lower than those induced by curcumin (Fig. 4B). Nevertheless, our results suggest that STAT3 is involved in curcumin-induced RANK reactivation. These findings support a link between the epigenetic restoration of RANK expression and changes in the STAT3 pathway in curcumin-treated U251 cells.

Discussion

A recent study proposed that RANK is silenced by promoter hypermethylation in human gliomas (von dem Kneesebeck *et al.*, 2012). Our data demonstrate that curcumin demethylates the CpG sites within the RANK promoter sequence and transcriptionally upregulates RANK gene expression in glioblastoma cell lines. Our data further indicate that STAT3 suppression is sufficient for reactivation of RANK expression, suggesting a role for STAT3 in curcumin-induced epigenetic activation of RANK.

First, we found that curcumin induced an increase in RANK mRNA and protein levels in both the U251 and U87 cell lines. RANK and RANKL are essential regulators of osteoclastogenesis (Maeda *et al.*, 2012), the inflammatory response (Mori *et al.*, 2011), and neutrophil migration (Riegel *et al.*, 2012). Inactivation of RANK sensitizes mammary gland epithelial cells to DNA damage-induced cell death and decreases the incidence and onset of progestin-driven breast cancer in the mammary epithelium (Schramek *et al.*, 2010). These investigations highlight an oncogenic role for RANK/RANKL signaling in various diseases. However, evidence has also established that RANK/RANKL possesses proapoptotic activity in other cellular contexts (Bharti and Aggarwal, 2004). Notably, overexpression of RANK in cultured glioblastoma cells leads to elevated apoptotic activity (von dem Kneesebeck *et al.*, 2012). Therefore, based on our data, it seems that elevated RANK expression may contribute to curcumin-induced apoptosis in glioblastoma cells. Moreover, von dem Kneesebeck *et al.* (2012) have also demonstrated that the RANK expression level was correlated with the malignancy of gliomas. However, there having been no report concerning with the effects of RANK upregulation on the growth of glioblastomas *in vivo*; therefore, further investigation was required to delineate the exact role of RANK to the *in vivo* growth of glioblastomas.

A recent report demonstrated that CpG islands within the RANK gene are frequently methylated in gliomas (von dem Kneesebeck *et al.*, 2012). Our results show that curcumin-induced RANK elevation is associated with an alteration in its promoter methylation status. Curcumin converted hypermethylation to hypomethylation by epigenetic modification. In human cancer cells, certain enzymes, such as DNMT1, are responsible for the maintenance of the methylation of tumor suppressor genes. The hypomethylation activity of curcumin was mediated mainly by covalently blocking the catalytic thiolate (C1226) of DNMT1 (Liu *et al.*, 2009). Consistent with previous studies, our results confirmed this activity through an *in vitro* methylation assay. However, Du *et al.* (2012) demonstrated that curcumin can decrease the mRNA and protein levels of DNMT1 in breast cancer cells, while Shu *et al.* (2011) discovered that curcumin

treatment exerted limited effects on the expression of epigenetic-modifying proteins, including DNMT1, at the protein level in prostate LNCaP cells. Therefore, the effects of curcumin on DNMT1 expression in glioblastoma cells call for further investigation. Moreover, establishment of the abnormal DNA methylation profile may be mediated through histone modification, particularly lysine acetylation (Cedar and Bergman, 2009). Curcumin, which inhibits CBP/p300, attenuates the acetylation state of histones by inhibiting histone acetyl transferases (HATs) (Reuter *et al.*, 2011). Hence, in addition to directly blocking DNMT1 activity, curcumin may also modify the methylation status of the RANK promoter through histone deacetylation.

Another important finding in this study is that STAT3 suppression, induced by both curcumin treatment and siRNA-specific transfection, induces promoter demethylation and reactivation of RANK. Although STAT3 appears to act as a transcription activator, transcriptional repression by STAT3 has also been reported, providing evidence that oncogenic STAT3 promotes epigenetic gene silencing (Zhang *et al.*, 2005). STAT3 also induces transcriptional expression of DNMT1 and cooperates directly with DNMT1, fostering epigenetic silencing of the tumor suppressor genes (Zhang *et al.*, 2005; Zhang *et al.*, 2006). Notably, in the present study, the suppression of STAT3 by siRNA transfection was sufficient for RANK gene promoter demethylation and reactivation. Given the fact that treatment of U251 cells with curcumin increased RANK gene expression at both the mRNA and protein levels, which was associated with a decrease in STAT3 activity, as well as a decrease in methylation of the RANK gene promoter in this cell line, STAT3 deactivation may contribute to the curcumin-induced demethylation effect of RANK. Additionally, Lee *et al.* (2012) reported that acetylated STAT3 facilitates the STAT3-DNMT1 interaction in tumors and is crucial for methylation of tumor suppressor gene promoters. Both phosphorylation and acetylation of STAT3 are crucial for STAT3-mediated regulation of oncogenic genes or tumor suppressor genes. Since curcumin also inhibits the acetylation of nonhistone proteins through HAT inhibition (Reuter *et al.*, 2011), examining the role of STAT3 deacetylation in curcumin-induced epigenetic reactivation of RANK is warranted. However, in the present study, fewer CpG sites (12/17) were demethylated in the si-STAT3-siRNA-transfected cells compared with CpG sites after curcumin treatment. This is likely due to the fact that curcumin induced more potent demethylation effects through multiple pathways than si-STAT3 treatment.

Aberrant hypermethylation at normally unmethylated CpG islands plays a critical role in tumorigenesis, and DNA methylation inhibitors have been used clinically (Yoo and Jones, 2006). Our findings not only identify a potential underlying mechanism for curcumin-induced RANK gene reactivation in human glioblastoma, but also suggest the importance of STAT3 inhibition involving RANK promoter hypermethylation and epigenetic silencing, thus paving the way for further applications of curcumin epigenetic therapy in glioma. Moreover, the involvement of STAT3 in epigenetic silencing of the RANK gene may also have therapeutic implications in human glioblastoma. However, *in vivo* study is warranted to elucidate the effects of curcumin-induced upregulation of RANK on the growth of glioblastomas.

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Disclosure Statement

No competing financial interests exist.

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