Review

The Potential of RECK Inducers as Antitumor Agents for Glioma

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Abstract. *Reversion-inducing cysteine-rich protein with Kazal motifs (RECK), a tumor and metastasis suppressor gene, is critical for the regulation of the invasive and metastatic activities of tumor cells. RECK is down-regulated in some malignancies and its expression is positively correlated with survival of patients with cancer. Patients with malignant glioma have poor prognosis. Since RECK expression decreases as the tumor stage progresses from less invasive grade II glioma to invasive glioblastoma multiforme, up-regulation of RECK by natural or synthetic agents might be a valuable therapeutic option for glioma. Histone deacetylase inhibitors and non-steroidal anti-inflammatory drugs have been widely used clinically and demonstrated to increase RECK expression in cancer cells, thus they might be used as RECK inducers. In this article, the functions of RECK and the role of RECK in glioma are reviewed, with emphasis on the potential application of RECK inducers in the treatment of glioma.*

Malignant glioma is the most common primary brain tumor, consisting of grade 3 anaplastic astrocytoma and grade 4 glioblastoma multiforme (GBM) (1). It is histologically heterogeneous and invasive, and patients with malignant glioma have poor prognosis, with the survival depending on the histological grade of the tumor (1-4). The median survival time of patients with GBM is 12-15 months and that

of patients with anaplastic astrocytoma 2-5 years (2-4). The therapies for malignant glioma, including surgery, radiotherapy, and chemotherapy, have not been successful (1, 5). Malignant gliomas cannot be resected completely because of their infiltrative nature, although surgical debulking can reduce the mass effect and provide tissues for diagnosis (4). Postoperative radiotherapy can increase the survival of the patients; however, the majority of the patients have local tumor recurrence (4). Brachytherapy and stereotactic radiosurgery have been used to treat glioma, but there is no clear evidence that they can improve the survival of patients (6, 7). In recent years, concomitant temozolomide and radiotherapy, and biodegradable polymers containing carmustine (Gliadel Wafers; MGI Pharma, Inc., Bloomington, MN, USA) have been demonstrated to improve the survival of patients with glioma, and most patients are able to tolerate these treatments because of their limited systemic toxicities (2, 4, 8, 9). However, the prognosis of patients with malignant glioma is still poor, thus, it is mandatory to develop more effective treatment strategies for these tumor types.

Reversion-inducing cysteine-rich protein with Kazal motifs (RECK). *RECK* gene, encoding a glycosylphosphatidylinositol-anchored glycoprotein of about 110 kDa with multiple serine protease inhibitor-like motifs, is considered a tumor and metastasis suppressor gene (10). *RECK* was first identified as a cDNA clone inducing morphological reversion in NIH3T3 cells transformed by the v-*K-ras* oncogene (11). RECK is expressed ubiquitously in normal human tissues, and is essential for normal tissue development, morphogenesis, and remodeling; stabilization of tissue architecture; cell migration; and dynamic cell–cell interaction (10, 12). In addition, RECK expression plays a role in processes such as angiogenesis, chondrogenesis, and myogenesis (13-16). RECK-deficient mice exhibit reduced extracellular matrix

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(ECM) integrity, such as reduced type I collagen, disrupted basement membranes, cellular disarray, increased tissue fragility, and halted vascular development (10, 16). Furthermore, because of the role of RECK in the regulation of various ECM proteins, altered expression of RECK has been found to be involved in various human disorders, such as rheumatoid arthritis and asthma (17, 18). In contrast to normal tissues, RECK is undetectable in some tumor-derived cell lines and down-regulated in some malignancies such as pancreatic, breast, lung, colorectal, prostate, and gastric cancer, cholangiocarcinoma, ameloblastic tumor, middle ear squamous cell cancer, and osteosarcoma (10, 19-29). RECK expression is also positively correlated with the survival of patients with prostate, lung, pancreatic, breast, stomach, colorectal, hepatocellular cancer, cholangiocarcinoma, neuroblastoma, and osteosarcoma (10, 22, 23, 25, 26, 28-34). Restoration of expression of RECK in tumor cells suppresses tumor angiogenesis, invasion, and metastasis in animal models, and its residual expression level in tumor tissues often correlates with better prognosis (10, 11, 16). All these data suggest that RECK has tumor-suppressing effects.

The antitumor effects of RECK have been associated with its inhibitory effects on matrix metalloproteinases (MMPs) (35). The family of MMPs consists of multiple human zinc-dependent endopeptidases that can degrade cell proteineous components of the ECM (36, 37). The ECM is important for creating the cellular environment required during development and morphogenesis; MMPs cleave ECM components, such as collagen, laminin, and fibronectin, as well as non-matrix components, such as growth factors and cell surface receptors (36, 38). In malignant tumors, the remodeling of basement membrane and degradation of ECM are critical steps in tumor development, invasion and metastasis (36). The cancer cells and/or adjacent stromal cells secrete MMPs to degrade the ECM, and facilitate tumor invasion and progression (39, 40). RECK negatively regulates MMP family members, including MMP-2, MMP-9, and membrane type-1 MMP (10). Down-regulation of *RECK* gene expression is strongly associated with high expression of MMP-2 and MMP-9 in various types of cancers (10, 23, 39, 41). RECK inhibits MMP activity through direct suppression of its protease activity, regulation of cellular release, as well as possible sequestration at the cell surface (42). In addition, RECK has also been found to regulate MMP-9, not only post-transcriptionally, but also at the gene expression level (43). Generally, the expressions of RECK and MMPs are inversely correlated (44).

RECK has also been found to be a suppressor of tumor angiogenesis. Angiogenesis is a process by which new blood vessels are formed from the existing vasculature and it plays critical role in tumor growth and metastasis (45, 46). Angiogenesis is stimulated by cytokines and growth factors, such as vascular endothelial growth factor and

thrombospondin (38, 47, 48). These factors act on specific receptors on the endothelial cells, causing endothelial cell proliferation and production of proteolytic enzymes to destroy the matrix, and activate endothelial cell migration and invasion into tissues (49). As stated earlier, RECK affects the expression and activity of MMPs, which are involved in angiogenesis in malignant tumors (38, 50). In addition, angiogenesis in several kinds of cancer cell is suppressed by restored expression of RECK (33). Furthermore, tissue inhibitors of metalloproteinases (TIMPs) inhibit endothelial cell migration through increased RECK expression (51). These data indicate that RECK plays an important role in tumor angiogenesis.

Regulation of RECK. The regulation of RECK is interesting. The *RECK* gene is a common negative target for oncogenic signals that act on the specificity protein 1 (SP1)-binding site of the *RECK* promoter (12). *RECK* is down-regulated upon cell transformation by trio related transforming gene in ATL tumor cells (TGAT), human epidermal growth factor receptor 2 (HER-2/neu), and rat sarcoma (RAS) oncoproteins (52-55). TGAT oncoprotein inhibits RECK through 15 amino acids on its *C*-terminal (55). HER-2/neu induces the binding of SP proteins and histone deacetylase 1 to the *RECK* promoter to repress RECK, and activates the extracellular signal-regulated kinase (ERK) signaling pathway (54). RAS inhibits RECK expression *via* histone deacetylation and promoter methylation mechanisms, and acts through inhibition of the SP1 promoter site of the *RECK* gene (52, 53, 56). Activation of RAS signaling, including the rapidly growing fibrosarcoma (RAF)/mitogen-activated protein kinase kinase (MEK)/ERK and MEK kinase (MEKK)/MEK/c-jun-N-terminal kinase (JNK) pathways, can then up-regulate microRNA-21 and suppress RECK expression in tumor cells (52, 54, 57).

Although the main functions of RECK are considered to occur through the inhibition of MMPs as described above, there are reports revealing that RECK might act *via* some mechanisms other than through MMPs (50, 52, 54, 55). The relationship between RECK and neurogenic locus notch homolog protein (NOTCH) signaling has been noted in neural precursor cells, with RECK specifically targeting NOTCH signaling (15). In addition, the upstream regulator of RECK, RAS, can increase the level of NOTCH-1 (58). The *NOTCH* genes encode heterodimeric transmembrane receptors that can be activated by interacting with a family of its ligands (59). They are important in a variety of cellular processes, including proliferation, differentiation, survival, and apoptosis (60). Upon activation, NOTCH is cleaved, releasing intracellular NOTCH (ICN), which translocates into the nucleus (60). ICN associates with transcriptional factors to regulate the expression of target genes; thus, it plays an important role in development and cell growth (60). As for cancer, NOTCH signaling is linked to epigenetic

silencing and cell-cycle control during tumorigenesis in *Drosophila* (61), and dysregulated expression of NOTCH1 has been noted in some tumors, including glioma, lung, colon and pancreatic cancer, and hematopoietic malignancies (60, 62-66). Furthermore, down-regulation of NOTCH-1 reduces pancreatic cell invasion, whereas NOTCH-1 overexpression leads to increased tumor cell invasion (60). These information suggests that RECK might function through NOTCH signaling in cancer cells (67).

There is another family of metalloproteinases, the disintegrins and metalloproteinases or adamalysins (ADAMs), which contain extracellular disintegrin, metalloproteinase, cysteine-rich, epidermal growth factor-like domains, and transmembrane and cytoplasmic regions (37). Some ADAMs (ADAM-10, 28 and 33) have been found to be present in the nervous system and exert effects on neuronal migration (15, 37), and RECK directly interacts with ADAM-10 in neural precursor cells (15, 37). Because ADAMs are considered to be related to cancer progression, the relationship between ADAMs and RECK in cancer cells is interesting and deserves further investigation (37). In addition, transforming growth factor-β (TGF-β) has been implicated in many aspects of cancer progression, including proliferation, infiltrative growth, angiogenesis, and immune suppression (68). TGF-β can stimulate tumor invasion by regulating the activity of MMP, which is also closely related to the function of RECK (69). Moreover, TGF-β signaling in activated pancreatic stellate cells promotes ECM accumulation *via* preservation of the protease-inhibitory activity of RECK (70). Thus RECK might interact with TGF-β and affect the invasiveness and tumor growth of cancer cells.

As a whole, the information about the regulation of RECK is limited and the pathways mentioned above might represent only part of the regulatory mechanisms. More studies are necessary to understand the regulatory mechanisms of RECK.

Role of RECK in glioma. One important characteristic of malignant glioma is invasiveness, which involves tumor cell–ECM interactions and the activities of MMPs (40). No expression of MMP-2 and MMP-9 is present in the normal brain tissue, whereas in glioma, positive staining for these MMPs is significantly elevated, progressively increasing with the degree of malignancy (71). On the other hand, RECK negatively regulates MMP-2, MMP-9, and membrane type-1 MMP (16, 44). RECK expression decreases as the tumor stage progresses from less invasive grade II to invasive GBM (1, 35). Thus, MMPs, especially MMP-2 and MMP-9, are closely related to glioma invasiveness, and RECK, as a potent inhibitor of MMP-2 and MMP-9, is involved in the suppression of the invasiveness of glioma cells (44). Cell migration requires actin polymerization for the formation of motility-associated processes, such as lamellipodia (35). Lamellipodia associated with MMPs mediates proteolysis of

ECM constituents, including fibronectin, laminins, and collagens, in tumor cells and transformed cells (72). In glioma cells, RECK has been found to reduce cell motility and invasion through the regulation of actin cytoskeleton rearrangements and stabilization of focal adhesion (35). Overexpression of RECK inhibits the invasive process through rearrangement of actin filaments, promoting a decrease in migratory ability (35). In addition, RECK expression is increased by 1-50 mM valproic acid (di-n-propylacetic acid, VPA), a mood stabilizer and an antiepileptic drug, in T98G glioma cells in a concentration-dependent manner (73). VPA induces cytotoxicity, apoptosis, suppression of invasiveness and MMP-2 activity in T98G cells, while *RECK* siRNA markedly reverses these effects (73). These data suggest that RECK expression and activity of MMPs play a role in VPA-induced cytotoxicity, apoptosis, and suppression of invasiveness in T98G cells (73).

MicroRNAs (miRs) are small RNAs with 19-23 nucleotides in length, which are found in all mammalian cells. The miRs are incorporated into the RNA-induced silencing complex and target the 3'-untranslated region of specific mRNAs by a seed sequence that is located near the 5' region of the miRNA. The results of miR binding are that the mRNA is silenced or degraded, resulting in reduced expression level of the protein encoded by the targeted mRNA (74). miR-21 has been found to regulate genes involved in various pathways, such as cell death, cell proliferation, stress response and metabolism (1). Elevated miR-21 expression is causally linked to proliferation, apoptosis, and migration of several cancer cell lines, and has been found in various cancer types, including breast, lung, colon, prostate, pancreas, ovary and stomach, and in chronic lymphatic leukemia and glioblastoma (1, 75, 76). In glioma, with progression from less invasive grade II glioma to invasive GBM, the *RECK* and TIMP levels decrease, whereas miR-21 expression increases (1). miR-21 is associated with glioma cell survival, migration, and invasiveness, and specific inhibition of miR-21 with antisense oligonucleotides leads to decreased migratory and invasion abilities, elevated RECK and reduced MMP activity in glioma cells (1). U87MG glioma cells transfected with either anti-miR-21 or control oligonucleotide were implanted into nude mice subcutaneously, and the tumors produced from miR-21-inhibited cells demonstrated significantly lower MMP activity than did control tumors (1). These data indicate that miR-21 contributes to glioma malignancy by the down-regulation of MMP inhibitors such as RECK, which leads to activation of MMPs, thus promoting invasiveness of glioma cells (1).

As a whole, the pathway involving miR-21, RECK and MMPs seems to play an important role in the invasiveness and tumor grade of gliomas. Treatment targeting this pathway might provide beneficial effects to patients with malignant glioma.

RECK inducers. From the above, it is seen that RECK can suppress the activity of MMPs, cause apoptosis, and inhibit migration, invasiveness and angiogenesis of cancer cells, including glioma cells. Thus, strategies inducing RECK expression might be used as antitumor therapy for glioma. From the literature, two categories of pharmaceutical agents induce RECK expression: histone deacetylase (HDAC) inhibitors and nonsteroidal anti-inflammatory drugs (NSAIDs).

A. *HDAC inhibitors:* HDAC inhibitors have been found to exert anti-metastatic and anti-angiogenic activities *in vitro* and *in vivo* (77). VPA inhibits the proliferation of and induces the differentiation of cells in different malignancies including leukemia, lymphoma, teratocarcinoma, neuroblastoma, medulloblastoma, and atypical teratoid/rhabdoid tumor; clinically, it has been used to treat leukemia and some types of solid tumors (78-83). VPA can also suppress glioma cell growth and proliferation and is able to cross the blood–brain barrier; thus it is considered a possible treatment agent for malignant glioma (78, 81). In the literature, there is a trend for longer survival of patients with glioblastomas and seizure who are treated with VPA as compared to patients not treated with VPA (84). Furthermore, patients with GBM receiving VPA gain more benefit from temozolomide/radiotherapy than patients receiving an enzyme-inducing antiepileptic drug or patients not receiving any antiepileptic drug (85). In pediatric patients with high grade glioma, or diffuse pontine glioma, receiving VPA as oral maintenance treatment after fractionated focal radiation and chemotherapy, the VPA treatment was found to be safe and have moderate antitumor efficacy (86).

The mechanisms underlying the antitumor effects of VPA are variable. VPA can increase the DNA-binding of activating protein-1 transcription factor, inhibit glycogen synthase kinase-3β, down-regulate protein kinase C activity, activate peroxisome proliferator-activated receptors, *etc.* (87, 88). In addition, VPA can inhibit histone deacetylase, which increases histone acetylation to modulate epigenomic and DNA methylation (88-90). VPA is a RECK inducer, and can cause cytotoxicity, apoptosis, and suppression of invasiveness of glioma cells. The induction of RECK expression might also contribute to the mild antitumor effects on gliomas by VPA noted clinically. Because VPA is often used to treat seizure in patients with glioma, and as it is a RECK-inducing agent, it might be a promising adjuvant therapeutic agent for treatment of glioma.

In addition to VPA, trichostatin A (TSA) and phenylbutyrate are also HDAC inhibitors (52, 73, 77, 91). Inhibition of HDAC by TSA has been shown to prevent tumorigenesis and metastasis (89). Treatment with 100 nM TSA for 48 h reduced MMP-2 mRNA and activity, and potently antagonized the inhibitory action of RAS on RECK in NIH3T3 cells (52, 89). In addition, treatment with 100 nM TSA for 48 h up-regulated *RECK* gene expression *via* transcriptional activation, and suppressed MMP-2 activity and invasiveness of CL-1 human lung cancer cells (52, 77, 91). Phenylbutyrate, a carboxylic acid HDAC inhibitor, inhibited the anaplastic thyroid cancer cell line ARO from penetrating a matrigel-coated transwell, with concomitant suppression of MMP-7 and stimulation of RECK, without affecting MMP-2 expression (73). These two agents might also be potential anticancer agents for glioma, however, more studies are needed to determine their effects and toxicities.

B. *Nonsteroidal anti-inflammatory drugs (NSAIDs):* NSAIDs are known to exert anti-angiogenic and anti-metastatic activities both *in vitro* and *in vivo* (92). NSAIDs can up-regulate RECK expression in cancer cells (23, 77, 92). Treatment with 500 μM aspirin [a nonselective cyclo-oxygenase (COX) inhibitor] for 48 h or 100 μM NS-398 (a COX-2 inhibitor) for 48 h upregulated *RECK* mRNA and protein levels and suppressed MMP-2 activity in CL-1 human lung cancer cells (77, 92). These effects are considered to be independent of COX-2 inhibition, as prostaglandin E_2 and COX-2 overexpression failed to reverse the effects (77, 92). Treatment with 500 μM aspirin for 48 h can cause growth suppression, reduction of RAS signaling, including phosphorylation of v-akt murine thymoma (AKT)/ERK/c-JUN, elevation of RECK expression, inhibition of MMP-2 and MMP-9 activity, and suppression of invasiveness of M139 cholangiocarcinoma cells (23). In HeLa cells, aspirin also inhibits ERK and AKT activities by reducing phospho-ERK and phospho-AKT, and induces apoptosis (93). These data suggest that the induction of RECK by aspirin is mediated by the inhibition of the RAS signaling pathway, which is possibly due to the re-activation of transcription initiation at the SP1 promoter site of the *RECK* gene, but not through COX inhibition (23, 56, 77, 92). As a whole, NSAIDs such as aspirin and NS398 might be used as RECK inducers and potential adjuvant therapeutic agents for cancer. Because an inverse association between NSAIDs use and GBM has been noted, NSAIDs might also be effective in the inhibition of GBM development or progression (94). Certainly, whether the RECK expression is induced in gliomas should be affirmed.

Conclusion

The proliferation and invasiveness of cancer are related to many factors, including growth factors, oncogenes, and tumor suppressor genes. Strategies to modulate these factors might be used to treat cancer. Since *RECK* is a tumor and metastasis suppressor gene (11, 50), up-regulation of *RECK* could be a valuable therapeutic approach for therapy of cancer (12). Both natural and synthetic agents have been identified, which enhance RECK expression, including forced expression of RECK, use of mimetics, recombinant peptides, microRNA antagonists, and gene therapy (12). However, the results of most related clinical trials are disappointing, with no evidence

of therapeutic benefits for patients (41, 44). VPA, an HDAC inhibitor and also a RECK inducer, has been shown to exert antitumor effects on gliomas (78, 81, 84-86). Because VPA has been widely used for the treatment of patients with gliomas and seizures, and is often orally administered for several years clinically, it might be used as an adjuvant treatment agent in addition to other treatment strategies such as surgery, radiation and chemotherapy. Because it is difficult to carry out long-term experiments about the effects of VPA on glioma cells, most *in vitro* studies use concentrations of VPA in a higher dose range than what is used clinically (95). Therefore, suitable doses of VPA as a RECK inducer to be used clinically are unknown. Future investigations should include *in vitro* and *in vivo* studies about the effects of long-term and low concentrations of VPA on gliomas, since high concentrations of VPA may cause somnolence, hematotoxicity and hepatotoxicity (86, 95). Long-term daily use of adult-strength aspirin is associated with modest reduction in overall cancer incidence in populations in which colorectal, prostate, and breast cancer are common (96). Nevertheless, clinically, the concentrations of this drug, required to induce RECK expression must be precisely evaluated, as high doses of aspirin may have undesirable side-effects (23). As a whole, further efforts to explore the effects of the induction of *RECK* expression by HDAC inhibitors, NSAIDs, or other new pharmaceutical agents should be continued. In addition, more studies are necessary to explore the benefits and risks of using HDAC inhibitors and NSAIDs as inducers of *RECK* expression in the treatment of glioma.

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