Abstract

Glioblastoma multiforme (GBM) is the highly aggressive and most common primary brain tumor in adults with a dismal survival of one year post diagnosis. Malignant gliomas display extensive infiltration into the surrounding brain tissue making them resistant to the existing therapeutic strategies. Monocytes are recruited to the primary tumor site where they differentiate into tumor associated macrophages (TAMs) and secrete inflammatory cytokines such as Tumor Necrosis Factor-α (TNF-α), Interleukin-1β (IL-1β), VEGF and other chemokines that positively regulate invasion and angiogenesis aided by the secretion of high levels of proteolytic enzymes such as Matrix Metalloproteinases (MMPs). TNF-α is a pleotrophic cytokine that has been implicated in activating pathways involving PI3K/Akt and NF-κB. The constitutively high levels of activated Akt and NF-κB correlate with the aggressive nature and chemoresistance of gliomas. Identification and development of small-molecule inhibitors of these pathways is predicted as a better treatment strategy for cancer.

PI3K/Akt signaling pathway is important in a broad range of cellular functions in response to extra cellular signals. A number of components of this pathway have been mutated or deregulated in a wide variety of human cancers including Glioblastoma multiforme (GBM), highlighting the role of this pathway in cellular transformation. TNF-α-activated Akt pathway enhances cell growth and tumor progression. Among the numerous downstream targets of Akt, mTOR is an evolutionarily conserved 298KDa checkpoint kinase that integrates many cellular signals to control cell growth, proliferation, protein synthesis and cytoskeletal reorganization. mTOR form two types of multi-protein complexes. mTOR complex 1 or mTORC1 (with Raptor) has been widely studied in functions of cell growth and protein synthesis. The signaling mediated by mTOR, specially mTORC1, has been studied in some cell systems and its downstream targets are reportedly activated by TNF-α. The lesser known mTOR complex 2 or mTORC2 with Rictor and its response to TNF-α, an important secretory component of TAMs in gliomas, was the focus of the present study.

The study was conducted using human glioma cell lines – LN18 and LN229 and primary cells derived from tumor samples of GBM patients. Initial studies revealed that TNF-α resulted in the upregulation of Rictor in glioma cell lines used – LN18 and LN229. It is reported that S6Kinase (target of mTORC1-mediated pathway) can negatively regulate
mTORC2 protein complex by phosphorylation of Rictor at T1135. Exposure to TNF-α resulted in reduction of pRictor (T1135) indicating that this negative regulation might not be effective in glioma cell lines when treated with TNF-α. To examine the role of Rictor and its down stream targets in TNF-α-mediated signaling, siRNA was used to downregulate the expression of Rictor. Rictor ablation surprisingly did not affect the phosphorylation of PKC-α – a down stream target but the expression of total PKC-α was upregulated. In the same experiments there was increase in the nuclear expression of p21waf1/cip1. While the earlier studies from the group showed that TNF-α-induced p21waf1/cip1 inhibited the proliferation of glioma cells, the high levels of p21waf1/cip1 induced on Rictor silencing (higher than that induced by TNF-α) was not effective in inhibiting proliferation. Though p21waf1/cip1 is reported to be under the regulation of PKC-α, ASO studies revealed that p21waf1/cip1 was independent of PKC-α in the glioma cell lines used. Thus no role for Rictor in proliferation of glioma cells was identified.

Activation of Akt involves conformational change and phosphorylation on two residues – T308 and S473. mTORC2 has been speculated as one of the PDK2 kinase that phosphorylate Akt at S473. Rictor ablation reduced the ability of mTORC2 to phosphorylate Akt at S473 but had no effect on phosphorylation of T308. Since Akt is an important in cell survival, it was expected that Rictor ablation might reduce the viability of cells. However, knockdown of Rictor expression did not affect the viability of either LN18 or LN229 cells. Hence it was revealed that Rictor did not have a role in maintaining cell viability.

Experiments to decipher the role of Rictor in glioma progression revealed that loss of Rictor expression resulted in enhanced MMP-9 expression, activity and invasion of glioma cells. Rictor expression had no impact on TNF-α-induced MMP-9 activity or invasive potential suggesting that TNF-α might over rule the negative regulation that Rictor has over MMP-9. Studies to unravel the molecular mechanism of increase in MMP-9 levels on Rictor ablation revealed that the reduced levels of phosphorylated Akt (S473) resulted in the activation of Raf-1 kinase. The activated Raf-1 kinase led to activation of ERK further leading to increase in MMP-9 expression and invasion. These results were also confirmed in primary cells derived from GBM patients. This is the first study to show that Rictor functions as a negative regulator of MMP-9 and invasion involving the activation of Raf-1 / MEK / ERK. In these studies loss of Rictor had no
effect on MMP-2. The finding is consistent with reports that different mechanisms regulate the expression of the gelatinases – MMP-9 and -2.

It was interesting to observe that all the protein targets upregulated on Rictor ablation (p21waf1/cip1, MMP-9, ERK) were also targets of TNF-α-mediated NF-κB pathway. It was therefore of interest to examine if Rictor had a role in regulation of NF-κB signaling. Experiments with siRNA to Rictor showed that Rictor ablation resulted in upregulation of p65 in the nucleus and increase in the DNA-binding ability of NF-κB. TNF-α-induced p65 translocation was comparable in the presence as well as absence of Rictor. Analysis of NF-κB targets showed that genes such as Cathepsin B important in adhesion and invasion were upregulated significantly on Rictor ablation. NF-κB can interact with many types of transcription factors including members of c-Fos and transcription members such as HDACs. The expression of c-Fos transcripts was upregulated with increase in p65 (in LN18 but not LN229) on Rictor ablation. HDAC 1 and 3 (in LN18) and HDAC 2 and 3 (in LN229) have been found to be downregulated allowing for acetylation of p65 and thereby indicating its activation.

Further experiments revealed showed that the activation of NF-κB on Rictor silencing was mediated by the upstream kinase – IKKβ. Rictor ablation resulted in upregulation of IKKβ but not IKKα and knockdown of IKKβ expression (using siRNA) reduced pp65 levels. Experiments to deduce the underlying molecular mechanism revealed that the inhibition of Raf-1 kinase (by GW5074) or ERK (by PD98059 and U0126) inhibited the expression of IKKβ, reduced the translocation of p65 into the nucleus and its DNA-binding activity. Thus Rictor ablation resulted in activation of Raf-1/ERK pathway which activated NF-κB pathway via IKKβ. This is the first study to show a novel mechanism of NF-κB regulation by Rictor / Raf-1 / ERK / IKKβ.

In conclusion, the findings provide evidence for a new role for Rictor as a negative regulator of MMP-9 and invasion via Raf-1 / MEK / ERK pathway. Rictor also regulates NF-κB transcription factor and few selected targets by modulating the expression of Raf-1 / ERK / IKKβ pathway. Thus the study focuses Rictor as a novel regulator of both Akt and NF-κB signaling via Raf-1 / MEK / ERK and IKKβ as the convergence point of the two pathways.

Cancer progression is largely dependent on the tumor microenvironment that is rich in cytokines produced by TAMs. A prominent cytokine - TNF-α promotes motility and invasion of the tumor cells via induction of matrix metalloproteinases. TNF-α has
conflicting roles in cancer – as both necrotic and growth promoting factor. In the present study, the results revealed TNF-α as a powerful modulator of invasion. In the presence of TNF-α the ability of Rictor to negatively regulate MMP-9-mediated invasion is overruled. Moreover, TNF-α activates numerous pathways that encourage tumor progression. In the light of this finding, it is important to include strategies in cancer therapy that do not just eliminate cancer cells but also target the microenvironment which continuously supports the tumor growth and invasion.