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Review

ROLE OF HEME OXYGENASE-1 (HSP32) AND HSP90 IN GLIOBLASTOMA.

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ABSTRACT

Glioblastoma (GBM) is the most common and malignant primary brain tumor in adults. The current treatment regimes for glioblastoma demonstrated a low efficiency and offer a poor prognosis.Advancements in conventional treatment strategies have only yielded modest improvements in overall survival. The heat shockproteins, heme oxygenase-1 (HO-1) and Hsp90, serve these pivotal roles in tumor cells and have been identified as effective targets for developing therapeutics. This topic review summarizes the current preclinical and clinical evidences and rationale to define the potential of HO-1 and Hsp90 in GBM progression and chemoresistance.

1. Introduction

Infiltrative gliomas are the most common primary intracranial neoplasms, accounting for 40% of all primary and 78% of all malignant central nervous system tumours (1).The term “astrocytoma” is normally referred to “gliomas” by researchers worldwide.Glial cells have fundamental roles by providing essential support such as immune protection, mechanical support, nutrients and oxygen supply to neuronal cells. These cells also assist neurons to mediate complex processes as neurotransmission, signal transduction and providing more structures for the migration of neurons into their respective networks during development (2).

Particular attention is focused on grade IV astrocytoma, synonymous of common name Glioblastoma Multiforme or simply Glioblastoma (GBM) in the current World Health Organization (WHO) scheme. The term GBM was introduced by Mallory in 1914 (3) and commonly accepted in the surgical neuropathology lexicon by Bailey and Cushing in 1926 (4). Distinguished features of GBM are microvascular proliferation (MVP), loosely defined to include endothelial hypertrophy, endothelial hyperplasia and glomeruloid vessels, and/or necrosis (5). Glioblastoma may arise through two distinct pathways of neoplastic progression. Tumours progressing from lower-grade (II or III) astrocytic tumours are termed secondary or type 1 GBMs, display both well-differentiated and poorly differentiated foci. Secondary GBMs occurring in younger patients (fifth to sixth decade), with time to progression from months to decades. In contrast, primary type 2 GBMs develop in older individuals (sixth to seventh decade), have short clinical histories and arise *de novo* without any evidence of a lower-grade precursor. Genetic features of primary GBMs are relatively high frequencies of Epithelial Growth Factor Receptor (EGFR) amplification, Phosphatase and tensin homolog (PTEN) deletion and cyclin-dependent kinase Inhibitor 2A(CDKN2A) loss, while secondary GBMs often contain TP53 mutations (6). GBMs comprise a morphologically highly heterogeneous neoplasm, as designation *multiforme* implies. In other words, the cellular composition can vary widely and mixed histologic features are typical (5). Three GBM variants are recognized as distinct pathologies in the current WHO classification: conventional GBM, giant cell GBM (GC-GBM) and gliosarcoma (GC).

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2. Conventional GBM

Its cellular composition is heterogeneous and may include fibrillary, gemistocytic, and/or occasional giant cells (GCs). Neoplastic fibrillary astrocytes contain enlarged, irregularly shaped, hyperchromatic nuclei and variable glia fibrillary acidic protein (GFAP)-immunoreactive processes that form a loose, fibrillary matrix. Gemistocytes were first described by Franz Nissl as *glia* (*gemaestete glia*) with voluminous cytoplasm (7). Gemistocytic astrocytes contain full, glazed, eosinophilic GFAP-immunoreactive cytoplasm. Although gemistocytes may be found in all grades of astrocytoma, gemistocytic astrocytoma is recognized as a distinct variant only of diffuse astrocytoma. Gemistocytic astrocytoma is defined by WHO as an astrocytoma composed of more than 20% gemistocytes (5).Different reports have defined the percentage of gemystocytes present in a tumor (*gemistocytic index)* and correlated it with patient outcomes. Gemistocytic astrocytoma with greater than 5% gemistocytes have been reported to progress more rapidly to GBM (8).A rare form of this kind of tumor is Granular cell astrocytomas composed of large cells with a lot of glanular acid-Schiff-positive cytoplasm (5, 9-12). Most granular cell astrocytomas are GFAP positive and may show non-specifically cytoplasmic epithelial membrane antigen but they do not have a cytokeratin immunoreactivity (9, 10). These tumours may also have TP53 mutations, high-frequency loss of heterozygosis at 9p, 10q and 17p, and less frequent loss of heterozygosis at 1p and 19q(11). Furthermore, Brat at al. found that these tumours were more aggressive than non-granular cell astrocytoma of the same grade (9).

3. Giant cell GBM (GC-GBM)

Giant cell GBM constitutes approximately 5% of GBMs and it is recognized as a distinct pathology in the WHO 2000 classification (5). The tumor cells are considerably enlarged and bizarre, often appearing multinucleated. Giant cell GBMs are typically well-circumscribed masses that appear in younger patients.

Whereas EGFR amplification and CDKN2A deletion are rare in comparison to conventional GBMs, the molecular genetic features include relatively high frequencies of TP53 mutations (75%-90%) and PTEN deletion (5%-30%); thus, GC-GBMs contain clinical and molecular genetic characteristics of primary and secondary GBMs and occupy and intermediate position between these two (5, 13, 14). Fujita et al. and Maeda et al have described a potential molecular mechanism for GC formation (15, 16). Investigators have demonstrated that the molecular alteration causing collapse of cytoplasmic rift in cultured multinucleated GCs is loss of Aurora-B kinase function. It is demonstrated that Aurora-B kinase is overexpressed in astrocytomas in general, with mRNA and protein levels correlating with WHO grade (17).

4. Gliosarcoma (GS)

Gliosarcoma constitutes approximately 2% of GBMs and is equally recognized ad a distinct pathology in the WHO 2000 classification(5). Main features of these tumours are circumscribed, biphasic growth pattern with clearly identifiable glial and metaplastic mesenchymal components. The glial component of GS may show any previously described cytological characteristic and is typically immunoreactive for GFAP. The mesenchymal component may show a high variety of morphologic appearances with differentiation along fibroblastic, cartilagineous, osseous, smooth and striated muscle, and adipose lines (5). In addition to sarcomatous differentiation, an epithelial metaplasia may also occur in GS or conventional GBM, including cases of keratinizing squamous or glandular differentiation. These features allow establishing a differential diagnosis of metastatic carcinoma.

The metaplastic component in GS is neoplastic and frequently allows cytogenetic and molecular abnormalities similar to those found in the glial component. GSs are genetically similar to primary GBMs with TP53 mutations, PTEN and CDKN2A deletions demonstrated for 20-40% of tumours. An exception is the relative infrequency of EGFR amplification in these tumours (5, 18).

5. Other variants of GBM

It is possible to identify additional variants of GBM that have been published in WHO 2000 classification scheme. These include small cell astrocytomas (SCAs), glioblastoma with oligodendroglial features (GBM-O) and GBM with primary neuronal feature (primitive neuroectodermal tumor [PNET]-like)(5). These variant tumours demonstrated a significant morphologic junction with other recognized pathologies: to make and accurate histophatologic and molecular genetic characterization, it is important an identification of molecular genetic alterations and its association with responsiveness to therapy and an improved prognosis.Small cell astrocytoma is a variant of GBM with an important overlap with anaplastic oligodendroglioma (AO) carrying out a classical hematoxylin-eosin-stained sections(19, 20).The histopathologic features of this tumor are its bland nuclear cytology and bizarre mitotic activity. It is composed of a large number of astrocytes with small, uniformly oval nuclei with mild hyperchromasia and minimal distinguishable cytoplasm. Furthermore, SCA can show cortical infiltration and secondary structures, including perineuronal satellitosis.

6. Management and therapy

To date no current treatment is curative and it consists in maximal surgical resection, radiotherapy and concomitant adjuvant chemotherapy with temozolomide (21, 22).The use of radiotherapy is now widespread and, in particular, the addition to surgery increases the survival of patients (23, 24); nevertheless the responsiveness of GBM to radiotherapy varies.

Furthermore, radiosensitizer (drug that makes tumor cells more sensitive to radiation therapy) such as newer chemotherapeutic agents (25), targeted molecular agents (26, 27) and antiangiogenic agents may increase the therapeutic effect of radiotherapy (15, 28). However, the useof radiotherapy for recurrent GBM is controversial.

Currently, there not exists a defined optimal chemotherapeutic regimen for GBM, even thought adjuvant chemotherapy appears to produce a significant benefit in more than 25% of patients (21, 29-34). The agents currently used include Temozolomide, nitrosoureas (eg. Carmustine), O(6)-methylguanine-DNA methyltransferase (MGMT)inhibitors (eg. O6-

benzylguanine), Cisplatin, Bevacizumab (alone or with Irinotecan for recurrent glioma).

New therapies include gene therapy, peptide and dendritic cell vaccines, synthetic chlototoxins, radiolabeled drugs and antibody (35-40).

From a chirurgical point of view and because GBM cannot be cured surgically, the surgical goals are to establish a pathologic diagnosis, to relieve any mass effect, to achieve a gross total resection to facilitate adjuvant therapy (41). The extent of surgery has been demonstrated in a number of studies to affect length survival; surgical options include gross total resection and subtotal resection: the first one has a better survival.

7. Heat Shock Proteins and cancer

In the mid 1950's the Nobel Prize Laureate Christian B. Anfinsen from his research on the folding of ribonuclease A (42), began to concentrate on the problem of the relationship between structure and function in enzymes. On the basis of studies on ribonuclease, he proposed that the information determining the tertiary structure of a protein resides in the chemistry of its amino acid sequence. He also elegantly showed that, after cleavage of disulfide bonds and disruption of tertiary structure, many proteins could spontaneously refold to their native forms (43). However, in the following years several studies demonstrated that this assertion is not valid for all synthetized proteins thus suggesting that some proteins may violate the Anfinsen's dogma (44). In fact, some highly specific 'steric chaperones' do convey unique structural (steric) information onto proteins, which cannot be folded spontaneously. This finding changed dramatically the way of studying protein synthesis and their maturation following the translation process. In addition, further studies demonstrated that impairment of the chaperone machinery might lead to various pathologies including cancer. We will review the role of two Hsps, which have recently offer new insights and potential therapeutic potential.

8. Role of Heme oxygenase-1 (Hsp 32)

Heme oxygenases (HO) catalyze the degradation of heme into biliverdin, carbon monoxide (CO) and ferric iron (45-51). Heme functions as the prosthetic group in hemoproteins, e.g., nitric oxide synthase, cyclooxygenases, soluble guanylate cyclase, cytochrome P450, peroxidase, and catalase and since HO is the sole physiological pathway of heme degradation. It consequently plays a critical role in the regulation of cellular heme-dependent enzyme levels (52-56). To date, two HO isoforms have been shown to be catalytically active in heme degradation and each is encoded by a different gene (46, 57). Heme oxygenase-1 (HO-1) is expressed at low levels under basal conditions and it is induced by polyphenols (58-64), statins (65), metals (66-69) and a variety of stimuli such as inflammation, oxidative stress, hyperoxia, hypoxia and trauma (50, 70-76). Such upregulation represents an intrinsic defence mechanism to maintain cellular homeostasis and enhance cell survival (77-80). In particular, HO-1 is considered to play a major role as an essential survival factor, protecting against chemotherapy-induced reactive oxygen species (ROS) increase (72, 81-85).

In particular, HO-1 overexpression is implicated in tumor genesis, growth and resistance to chemo- and radiotherapy in a lot of cancers (86-90). In 1996, Shibahara et al. demonstrated that expression level ofHO-1 mRNA is higher in brain tumours compared to the brain tissue (91). Furthermore, they showed that HO-1 mRNA expression is higher in human glioblastoma cell line T98G after a treatment with three types of Nitric Oxide (NO) donors: although this has not been shown any increase ofHO-2 mRNA expression (92).Researchers showed that macrophages play a key role in angiogenesis of malignant formations through the infiltration process (93-96). Leading an immunostaining on gliomas samples has been shown that the number of infiltrating macrophages and density of small blood vessels is higher in glioblastomas compared to astrocytomas or anaplastic astrocytomas. HO-1 is also associated with the activation of macrophages. In fact, the expression of mRNA coding for HO-1 correlates with macrophage infiltration. In addition, macrophages are positively stained with anti-HO-1 antibody: this result has led to the hypothesis of using HO-1 as a marker of macrophage infiltration and neovascularization in human gliomas (97).

Lu et al. demonstrated a correlation between HO-1 and human gliomas, in particular using HO-1 as possible novel therapeutic target. They showed the relationship between Nuclear factor E2-Related Factor 2 (Nrf2) and osteopontin-stimulated HO-1 expression: briefly, Nrf2 activation is essential for osteopontin-stimulated HO-1 expression, based on the evidence that Nrf2 small interfering RNA (siRNA) inhibits the enhancement of osteopontin-induced migration; moreover, osteopontin stimulated Nrf2 accumulation in nucleus and increased Nrf2-DNA binding activity. Taken all together, these results propose that Nrf2 activation is required for osteopontin-HO-1 expression and cell migration in human glioma. This study presents a novel mechanism of osteopontin-directed migration and HO-1 up-regulation in human glioma cells by activation of Protein kinase B (Akt),Extracellular signal–Regulated Kinases (ERK) and Nrf2-dependent pathway (98). Pan et al. worked on the involvement of Nrf2-Antioxidant Responsive Element (ARE) pathway in regulation of apoptosis in human glioblastoma cell U251: they showed that after increasing expression of Nrf2, the apoptosis was reduced with an up-regulation of expression of HO-1, Bcl-2/Bax and a decreased expression and activity of Caspases 3 and 9. The apoptosis rate was enhanced decreasing Nrf2 expression accompanied with a down-regulated expression of HO-1, Bcl-2/Bax and an increased expression and activity of Caspases 3 and 9 (99).

There are conflicting reports about the role of HO-1 plays in tumor initiation and progression, ever since it has been demonstrated that can play a role as tumor-promoter or inhibitor on tumor progression (86, 100).

Gandini et al. produce strong evidence of HO-1 overexpression in human gliomas compared with non-malignant samples. Moreover, this expression was associated with a worse prognosis in patients with grade II and III astrocytoma. They suggested that the enzyme could be involved in tumor proliferation and point to a pro-tumoral role of HO-1 in glioma progression. (101)

Furthermore, some studies demonstrated that HO-1 has a cytoprotective role in glioma and showed evidence that the enzyme could be a potential therapeutic target in this cancer type. (98, 102) Further, a lot of groups showed that a decrease (102, 103) or an increase (104, 105) in HO-1 is necessary for the anti-cancer effects of many compounds on human glioma cells (Table 1).

Table 1: Heat Shock Protein 32 (HSP 32) in Glioblastoma Multiforme (GBM)

Expression of HSP32 mRNA	Activation and infiltration of macrophages
Nrf2 activation	Osteopontin-HO-1 expression
Osteopontin-HO-1 expression	Inducible cell migration and invasion
Increasing expression of Nrf2	Reduced apoptosis with HO-1 and Bcl-2/Bax upregulated expression, decreased expression and activity of Caspases 3 and 9
Cytoprotective role	Potential therapeutic target

9. Role of Hsp 90 and Glioblastoma

Heat-shock-protein 90 (HSP90) is a molecular chaperone conserved and abundant in eukaryotic cell where this protein is an ubiquitous protein highly expressed in the cytosol, both in normal and stress conditions (106). This chaperones performs its "canonical" functions inside cells by forming cytosolic chaperoning machines (107) and is involved in the correct folding and conformational maturation of newly translated proteins, so called HSP90 client proteins, many of which are deregulated in glioblastomas (108, 109). Hsp90 is also involved in signal transduction and other key pathways critical for malignancy in several cancers (110-112) and glioblastomas specifically (113). Hsp90 can accumulate in cancer cells and is implicated in the carcinogenesis process for many reasons (114). This molecule favours tumorigenicity and is crucial to cancer cell growth and survival (115) by inhibiting programmed cell death and senescence (116). Hsp90 client proteins involved in its activities are many and varied and among them are included core mediators of glioblastoma's cellular growth like Akt kinase (117), endothelial nitric oxide synthase (eNOS), (109, 118) epidermal growth factor receptor (EGFR) and transforming growth factor-beta (TGF-beta) (119). Hsp90 has interactions, for example, with a protein complex in which there is a mutant epidermal growth factor receptor, EGFRvIII, that is expressed in most glioblastomas and is associated with poor prognosis promoting an aggressive growth (120). Among the client protein of Hsp90 there are client kinases such as Protein Kinase C (PKC) that has been directly implicated in the proliferation of glioblastoma. PKC also activates Protein Kinase D family (PRKD), which consists of PRKD1, -2 and, -3 that could play a major role in glioblastoma growth (121). PKD2 regulates glioblastoma cell migration and invasion (122). Hsp90 also influences tumor neoangiogenesis because it stabilizes proteins important for the metabolism of endothelial cells such as Vascular Endothelial Growth Factor (108) and nitric oxide synthase (123).

Indeed it is suggested that Hsp90 regulating the stability of PRKD2, is implicated in the formation of new blood vessel involving up-regulation and secretion of VEGF-A (108).

Recent works have claimed within glioblastomas the existence of small population of stem cell (124, 125), which are able to enable them to self-renew and give rise to the heterogeneous mass that characterizes these brain tumours (117). This set of stem cell appears also to be responsible of chemotherapy resistance and higher clonogenic capacity. The origin of these stem cells within neoplastic mass is not very clear; in fact they may arise from endogenous stem cells or from glioma cells that have undergone a block in differentiation (109). The aldehyde dehydrogenase (ALDH) is considered a marker of stem cell in many cancers and in particular in those of glioblastomas, were the same stem cells showed also an high expression of Hsp90 which proves its implication in many crucial mechanism of cell survival (126). Heat Shock Protein 90 is involved in a cytoprotective mechanism against cellular stressors such as DNA damage (127). For this reason several studies (128) have focused on alternative therapies for potentiating chemotherapy and X-ray irradiation using for example, Hsp90-inhibitors as antitumor agents in glioblastoma's therapy. It is showed that Geldanamycin interacts with the HSP90ATP-binding site to interfere in its interaction with client proteins (129) and 17-allylamino-17-demethoxygeldanamycin, an analogous of Geldanamycin, inhibits growth and invasion of GBM tumor(117, 130). HSP90 inhibitors promote the degradation of HSP90-dependent oncoproteins finally leading to cell cycle arrest and cell death.

Given the high proliferative capacity of glioblastoma cells and their resistance to current therapy it is necessary to introduce a multitargeted strategy that may be more effective. The HSP90 inhibition might be a good therapeutic strategy given that HSP90 client protein are protein known to deregulated in the neoplastic process of Glioblastoma(117) (Table 2).

Table 2: Heat Shock Protein 90 (Hsp 90) in Glioblastoma Multiforme (GBM)

"Canonical" function	Chaperoning machine involved in correct folding and conformational maturation of protein
Accumulation in cancer cells	Stimulation of tumor genesis, cancer cell growth by inhibiting programmed cell death
Interaction with EGFRvIII (mutant EGFR)	Associated with poor prognosis, promoting and aggressive growth
Influence on neoangiogenesis	Stabilization of VEGF, NOS, PRKD2 with formation of new blood vessels
Cytoprotective mechanism	Potential therapeutic target: promote the degradation of HSP90-dependent oncoproteins finally leading to cell cycle arrest and cell death

10. Conclusion

The treatment of glioblastoma remains an open challenge for modern medicine. Despite the discovery of new chemotherapic agents, no drug showed improvement in survival in phase III clinical trials. However, the increased knowledge of molecular characterization of various glioblastomas and patient clinical management has led to an increase in survival over the past 10 years. Furthermore, identification of new molecular targets (i.e. Hsps) involved in glioblastoma progression and the advent of immune-oncology will further improve the prognosis of these patients.

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