ASPIRIN, A NON-STERoidal ANTI-INFLAMMATory DRUG INDUCES CYTOTOXICITY AND ARRESTS ANGIoGENESIS IN LN229 GLlOMA CELLS

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ABSTRACT:

Glioblastoma is a devastating cancer of the brain with fatal prognosis. It is a highly vascularised cancer due to the presence of angiogenic factors. In the present study, we investigated the anticancer effect of aspirin in LN229 glioma cell line by inhibiting critical angiogenic markers HIF-1α and VEGF. A dose dependent treatment of aspirin revealed a decrease in the markers HIF-1α and VEGF, which demonstrates that aspirin might arrest angiogenic ability of the LN229 cells. This study features the promising effect of aspirin for a potential therapeutic option in future.

Keywords: Glioblastoma, aspirin, HIF-1α, VEGF, angiogenesis

INTRODUCTION:

Glioblastoma (GBM) is the most devastating and damaging tumour of the brain which makes significance by nearly unavoidable predisposal to relapse even after rigorous current treatments and contributes a fatal prognosis. It is the most malignant and common sort of primary brain tumour (Anjum et al., 2017). GBM displays diffuse metastasis, rapid cellular proliferation and extensive angiogenesis and is the most aggressive kind of adult brain tumours (Zhang et al., 2018). Angiogenesis is the establishment of new blood vessels from the pre-existing ones which arises in various physiological and pathological conditions like cancer and wound healing (Jiang et al., 2013). Tumour progression depends on a series of sequential processes which include, tumour initiation, angiogenesis, growth and metastasis, occurring in a dynamic and complex dynamic microenvironment and they are controlled by a number of factors and signal transduction cascades (Weis & Cheresh, 2011). In normal cells, it is more precisely controlled by a sequence of angiogenic inhibitors and stimulators. When the cancer cells start to grow, the balance between the inhibitors and stimulators is tipped, to an “angiogenic switch” (Ribatti, 2009).

Glioblastoma has been described as one of the highly vascularized tumours because of the presence of a variety of pro-angiogenic factors. Dense vascular regions in glioblastoma is significantly higher than that in lower histological grade tumours (Wesseling, Ruiter, & Burger, 1997). A elevation in vascularization markedly worsens prognosis (Lebelt et al., 2008). These intense vascularization could be responsible for the generation of peri-tumoural edema which is one of the critical pathological characterization of GBM (Onishi, Ichikawa, Kurozumi, & Date, 2011).

Development of novel therapies for glioblastoma includes anti-angiogenic therapy with several other treatment targets are currently being studied (Seystahl, Wick, & Weller, 2016). Aspirin, which is well known for its analgesic, anti-inflammatory and cardioprotective effects, also reduces the incidence of the epithelial cancers. This inverse correlation with cancer is currently discovered for the cancers of the colon, prostate, breast, skin and the lung (Algra & Rothwell, 2012; Gamba et al., 2013; Sahasrabuddhe et al., 2012; Thun, Jacobs, & Patrono, 2012; Veitonmäki, Tammela, Auvinen, & Murtola, 2013).

In the present study, we have demonstrated that aspirin is effective in mitigating the angiogenic markers like HIF-1α and VEGF in LN229 glioma cells. We determined that aspirin inhibited angiogenesis and tumour growth through suppression of HIF-1α which in turn led to the down-regulation of VEGF. This study ascertains the use of aspirin as an effective anticancer agent for the treatment of human glioma through inhibition of angiogenesis in LN229 glioma cell line.
MATERIALS AND METHODS

Chemicals and reagents.
Acetylsalicylic acid was purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). Dulbecco’s modified Eagle’s medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), fetal bovine serum (FBS), penicillin-streptomycin and ampicillin were purchased from Invitrogen (Carlsbad, California, USA).

Cell culture
The LN229 human glioma cell lines were purchased from National Center for Cell Science, Pune, India and were cultured in DMEM medium supplemented with 10% FBS, 1% penicillin-streptomycin and ampicillin solution and were incubated at 37°C in a humidified atmosphere of 5% CO2.

MTT assay
The MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) has been utilized to determine cell viability. LN229 cells (5 × 10^4) were seeded in a 96 well plate for initial attachment and cultured under the standard conditions of cell culture for 24hrs. Later, these cells were treated with different concentrations of aspirin (0.5 - 10mM) at various time points (24, 48 and 72). After incubation, the media was discarded and washed with PBS and then freshly prepared MTT solution (1 mg/ml) was added and incubated for 3h. The formazan salt formed was dissolved with 100μL of DMSO and the absorbance obtained was read at 590 nm using microplate reader (Biotek, USA).

RNA Extraction and cDNA Synthesis
Total RNA was isolated from cells by Trizol reagent,*** according to the manufacturer instructions. cDNA was synthesized from 1μg of RNA using IScript cDNA Synthesis Kit, †††† following the manufacturer instructions. PCR amplifications were performed as follows: VEGF: forward 5'-CACGAACGAGTCCCTAGAGC-3', reverse 5'-ATGGTGATGCGGTTTTCTTC-3'; denaturation step at 94°C for 5 min, followed by 30 cycles at 94°C for 30s, 55°C for 30s and 72°C for 30s; HIF-1α, forward 5′-GTGGATTACCACAGCTGA-3′, reverse 5′-GCTCAGTTAACTTGATCCA-3′; denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 15s and annealing for 32s at 60°C. The PCR products were then analyzed in a 1% agarose gel and stained with ethidium bromide. The size of the amplification products was confirmed with a 100 bp ladder commercial molecular weight marker. Relative mRNA expression levels were obtained by normalizing to the GAPDH expression. All the experiments were repeated three times.

Statistical analysis
Data are expressed as averages ± SD. Statistical analyses were conducted using Graphpad prism software. Differences were considered to be significant for values of p<0.05.

RESULTS:

Cytotoxic effect of aspirin in glioblastoma cells
Initially, this study analysed the anti-proliferative effect of aspirin on LN229 cells with different concentrations and at different time points (24, 48 and 72 h). The MTT results (Fig. 1) showed that, aspirin affected the proliferation of LN229 cells significantly in a concentration and time dependent manner. However, the strong inhibition was found after 24h time point with the IC50 value of 3mM.
Aspirin inhibits HIF-1α and VEGF expression in glioma cells

Angiogenesis is an intricate process that involves protein expression of several pro-angiogenic factors, including VEGF and HIF-1α. To understand the critical role of HIF-1α and VEGF in angiogenesis of GBM, this study first detected HIF-1α and VEGF protein expressions after cisplatin and aspirin alone and in combination treatment for 24 hours. VEGF-A and HIF-1α expressions were normalized to β-actin expression by band intensity. VEGF-A and HIF-1α expression were found to be significantly decreased in the glioma cell lines treated with cisplatin alone or in combination with aspirin. The reduced expressions of these vital proteins indicated that aspirin reversed the expression and transcription of VEGF induced by HIF-1α, which confirms it to be a potential inhibitor of angiogenesis and thereby can be suggested to apply for reducing neovascularity.

DISCUSSION:

Extensive vascularisation, invasiveness and molecular heterogeneity are the characteristic features of glioblastoma (Liu, Fang, Sun, & Liu, 2016). Targeting angiogenesis is another promising feature
in the anticancer research in conjunction with standard therapies, therefore the current study explores that aspirin inhibit the angiogenic response of glioma. GBM remains one of the most vascularized cancers, and its poor survival principally results from its invasive properties. To dissect the role of aspirin in glioma cells, we first investigated the cytotoxic effect of aspirin by MTT assay. Cytotoxic levels of aspirin were assessed and later we analysed the dose dependent effects of the aspirin in inhibiting angiogenesis. Therefore the present study features the ability of aspirin to arrest the expression of hypoxia inducible factor-1α (HIF-1α) and VEGF.

The most critical to gliomagenesis is HIF-1, which is an important activator of angiogenesis and invasion via its up-regulation of target genes vital for these functions. Up-regulation of the HIF-1 signaling pathway is a common characteristic of gliomas and thus explains the intense vascular hyperplasia found in glioblastoma multiforme. Through several regulatory mechanisms, HIF functions as a delicate sensor making a cell to respond quickly to the changes in oxygen levels in the environment. It plays a role as a central activator of angiogenesis by activating the production of VEGF and several other factors that could initiate endothelial cell proliferation, migration and invasion (Kaur et al., 2005).

The present study has observed an increase in the VEGF and HIF-1α protein expression in LN229 cells and upon aspirin treated groups marked reduction of both the proteins was found (Fig 2). This is consistent with the recent studies that aspirin inhibits angiogenesis in ovarian cancer, hepatocarcinoma and sarcoma models (Huang, Lichtenberger, Taylor, & Bottsford-miller, n.d.; Zhao, Wang, Wang, Wu, & Zhang, 2016).

Cancer cells produce stimulators of angiogenesis, such as vascular epithelial growth factors (VEGF), which play a pivotal role in endothelial cell proliferation and differentiation, survival and new vessel sprouting (Ferrara, 2002). VEGF is a growth factor which has been known to play a vital role in angiogenesis. VEGF is the most thoroughly studied pro-angiogenic molecule in GBM and is more likely the critical mediator of neovascularization in several tumours(Kuo et al., 2001). Preclinical work confirms that inhibition of VEGF could block new tumour vessel generation and arrests malignant glioma progression. Members of the VEGF family are VEGF-B, VEGF-D, VEGF-C, and PIGF, but VEGF-A has the best ascertained role in pathologic angiogenesis (Norden, Drappatz, & Wen, 2008).

Preclinical work has confirmed that blocking VEGF expression impedes new tumour vessel formation and arrests malignant glioma progression (Norden et al., 2008). VEGF-A is mainly induced by tissue hypoxia through the HIF-1 pathway. Shortly, hypoxic conditions progress to dissociation of von Hippel Landau protein from HIF1, reducing its proteosomal-mediated degradation and thereby allows binding to the promoter region of numerous pro-angiogenic factors, like VEGF, which in turn activates survival, angiogenic and vasculogenic pathways (Kaur, Brat, Calkins, & Meir, 2003).

While further studies are needed, we believe that our work will provide a framework to explore that aspirin could be arresting the new blood vessel formation.

**CONFLICTS OF INTEREST:**
The author declares no conflict of interest.

**REFERENCES:**


