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Original Article

Bee venom induces apoptosis and suppresses matrix metaloprotease-2 expression in human glioblastoma cells

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ABSTRACT

Glioblastoma is the most common malignant brain tumor representing with poor prognosis, therapy resistance and high metastasis rate. Increased expression and activity of matrix metalloproteinase-2, a member of matrix metalloproteinase family proteins, has been reported in many cancers including glioblastoma. Inhibition of matrix metalloproteinase-2 expression has resulted in reduced aggression of glioblastoma tumors in several reports. In the present study, we evaluated effect of bee venom on expression and activity of matrix metalloproteinase-2 as well as potential toxicity and apoptogenic properties of bee venom on glioblastoma cells. Human A172 glioblastoma cells were treated with increasing concentrations of bee venom. Then, cell viability, apoptosis, matrix metalloproteinase-2 expression, and matrix metalloproteinase-2 activity were measured using MMT assay, propidium iodide staining, real time-PCR, and zymography, respectively. The IC_{50} value of bee venom was 28.5 μg/ml in which it leads to decrease of cell viability and induction of apoptosis. Incubation with bee venom also decreased the expression of matrix metalloproteinase-2 in this cell line ($p < 0.05$). In zymography, there was a reverse correlation between bee venom concentration and total matrix metalloproteinase-2 activity. Induction of apoptosis as well as inhibition of matrix metalloproteinase-2 activity and expression can be suggested as molecular mechanisms involved in cytotoxic and antimetastatic effects of bee venom against glioblastoma cells.

Introduction

Glioblastoma is recognized as the most common malignant primary brain tumor with a particularly poor prognosis. Despite multiple therapeutic strategies such as surgery, radiotherapy, and chemotherapy, no effective treatment has been identified for glioblastoma (Haar et al., 2012; Naik et al., 2013). Moreover, resistance of brain tumors to available drugs has become a clinical challenge (Haar et al., 2012). Therefore, development of new natural therapeutic strategies is necessary. In different experiments, it was illustrated that the glioma cells show ability to produce and secrete various matrix metalloproteinases (MMP) enzymes (Rooprai and McCormick, 1996; Forsyth et al., 1999). It has been proposed that extracellular matrix degradation, triggered by MMP-2 activation via interaction with tissue inhibitor of metalloproteinase-2, is essential for invasion of glioma cells (Fillmore et al., 2001). Additionally, extracellular matrix degradation, especially by MMP-2, releases growth factors and provides more free spaces to vascular extension. Growth factors released by MMP such as vascular endothelial growth factor, fibroblast growth factor-2 and transforming growth factor beta may exert a significant effects in induction of angiogenesis (Egeblad and Werb, 2002). Therefore, protocols aiming to target MMP-2 activity may become a promising therapeutic strategy for treatment of glioma (Abe et al., 1994; Deryugina et al., 1997; Senota et al., 1998).

Bee venom (BV, apitoxin) is one of a natural biological complex compound with many different therapeutic effects including neuroprotective, anti-allergic, and anti-angiogenesis properties (Huh

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et al., 2010; Kim et al., 2011; Shin et al., 2014). The two main components of BV are melittin and phospholipase A_2. It has been reported that melittin has proapoptotic effect and shows anti-tumor activity (Oršolić, 2012). The BV has also been utilized in treatment of variety inflammatory conditions such as rheumatoid arthritis (Kwon et al., 2001; Park et al., 2004). These effects are shown to be mediated by inhibition of nuclear factor kappa-light-chain-enhancer of activated B cells, mitogen-activated protein kinase and Ca^{2+}/calmodulin signaling pathways (Cho et al., 2010; Park et al., 2010). Studies on glioblastoma cell lines revealed that disturbance of Ca^{2+}/calmodulin signaling pathway could result in tumor cells apoptosis through inhibition of DNA synthesis (Tsuruo et al., 1982; Oršolić, 2009). Also, it has been previously reported that BV induces cell cycle arrest in human cervical cancer cells (Ip et al., 2008). In the present study, antiproliferative and apoptogenic properties of venom of honey bee on human A172 glioma cancer cells were investigated. Also, because of critical role of MMP (especially MMP-2) in invasion of glioblastoma (Lu et al., 2004), the possible inhibitory effects of BV on MMP-2 expression and activity were evaluated.

Material and methods

Materials

Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT), propidium iodide (PI), Triton X-100 and gelatin were purchased from Sigma (St. Louis, USA). RPMI-1640 media and fetal bovine serum (FBS) and penicillin-streptomycin solution were from Gibco (Life Technologies, Carlsbad, USA). The venom of honey bee (persica, worker bees) was purchased from Royan Zahr (Isfahan, Iran). Total RNA extraction kit, agarose gel, green viewer dye and the entire solvents and flasks were prepared from Parstous co (Iran). Human A172 glioblastoma and normal murine L929 fibroblast cell lines were obtained from Pasteur Institute, Iran.

Cell culture and treatment: The A172 and L929 cells were cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin and incubated in 37 °C and 5% CO_2. The BV stock solution (15 mM) was prepared in phosphate buffer saline, pH 7.2. For cell viability assay, the cells were seeded in 96-well culture plates (1×10^4 cells/well). Then, the RPMI media was changed by fresh one containing varying concentrations (5–160 μg/ml) of BV or reference drug (cisplatin at 70 μg/ml). The cells were incubated for 24 or 48 h in 37 °C and 5% CO_2. For apoptosis assay, the cells were cultured in 6 well plates (1×10^5 cells per well) and treated with BV at its IC_{50} concentration for 48 h.

MMT assay: Effect of BV on A172 and L929 cell viability was determined using MTT assay as described previously (Mortazavian et al., 2012; Ghorbani et al., 2015). Briefly, 10 μl of MTT reagent (5 mg/ml) was added to each well, and the plates were incubated further for 4 h in 37 °C. At the end of incubation time, media was removed and formazan crystals were dissolved by adding 100 μl dimethyl sulfoxide. Finally, absorbance was read at 545 nm using ELISA plate reader (Stat fax-2100). The assay was carried out in triplicate.

Apoptosis analysis: After treatment with BV, the floating and adherent cells were harvested and incubated with a hypotonic buffer containing propidium iodide for 30 min (Mortazavian et al., 2013; Sadeghnia et al., 2014). The samples were then subjected to the flow cytometry for determination of apoptotic cells.

RNA extraction and cDNA synthesis: The cells were cultured in T25-flasks and treated with different concentrations of BV (0–10 μg/ml). Then total RNA was extracted using Parstous RNA extraction kit (Iran) according to the manufacturer's instruction. Quality of extracted RNA was checked by running on 1% agarose gel in the presence of cyber safe or green viewer (Parstous). Synthesis of cDNA was performed using Parstous kit according to the manufacturer's instruction.

Real time-PCR: MMP-2 primers were designed as follows: forward; 5′-AACTACGATGACGACAGCAAGT-3′ and reverse; 5′-AGGTGTAAATGGGTCCCATCA-3′. Quantitative RT-PCR was carried out on Stratagene 3000 instrument. Net volume of PCR reaction was 20 μl containing 1 μl cDNA, 1 μl mixed primer, 10 μl Sybergreen dye, 0.4 μl Rox dye and 7.6 μl distilled water. Temperature profile was designed as an initial denaturation phase at 95 °C (5 min). Following this, reaction continued in 35 cycles with temperature profile as denaturation (94 °C, 30 s), annealing (57 °C, 30 s) and extension (72 °C, 45 s). A final period of 72 °C for 5 min was considered to ensure maximum production of PCR products. The expression of MMP-2 gene was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as housekeeping gene.

Gelatin zymography: To assess enzymatic activity of MMP-2, gelatin zymography was performed to identify 72 kDa (pro-MMP-2) and 62 kDa (active-MMP-2) isoforms of the protein. The cells were cultured in 96 well plates (1×10^4 cells/well) for 12 h. Then they were washed with PBS, and subjected to treatment with different BV concentrations (0–10 μg/ml) in serum free medium for 24 h. To evaluate the direct effect of BV on MMP-2, different BV concentrations were added to the A172 cellular supernatant and incubated for 24 h. Then, the media were resolved on 8% SDS-PAGE containing 1% gelatin as enzyme substrate. The SDS was removed and the enzyme activity regenerated by washing the gels in 2.5% triton-X100 for three times. Subsequently, gels were incubated in developing buffer (Tris 50 mM pH 7.4, $CaCl_2$ 10 mM, NaN_3 0.02% and sterile dH_2O) at 37 °C for 42 h. The area of digested gelatin was visualized by counterstaining using Coomassie brilliant blue R-250, and quantified as relative numerical values with arbitrary units using NIH ImageJ 1.42q software.

Statistical analysis

All results are presented as mean ± standard error of the mean (SEM). The values were compared using the one-way analysis of variance followed by Tukey's post hoc test for multiple comparisons. The *p*-values less than 0.05 were considered to be statistically significant.

Results

Effect of BV on cell viability

As shown in Fig. 1, BV decreased viability of glioblastoma cells in a concentration-dependent manner with the IC_{50} value of 28.52 and 28.3 μg/ml for 24 and 48 h, respectively. After 24 h of incubation, viability of cells treated with 5, 10, 20, 40, 80 and 160 μg/ml of BV was 76 ± 3.5, 66 ± 2.5, 61 ± 0.5 ($p < 0.05$), 41 ± 0.5 ($p < 0.001$), 27 ± 0.5 ($p < 0.001$) and 25 ± 5% ($p < 0.001$) of control (100 ± 4%), respectively. At concentrations of 40–160 μg/ml, the antiproliferative effect of BV was more than that of 70 μg/ml cisplatin. This effect of BV was also more than cisplatin when the cells incubated for 48 h. Compared to untreated cells, cell viability was 70 ± 4.5, 63 ± 2.5, 60 ± 3 ($p < 0.05$), 41 ± 1 ($p < 0.001$), 25 ± 0.5 ($p < 0.001$) and 25 ± 1% ($p < 0.001$), respectively.

The effect of BV on viability of L929 fibroblast cells is presented in Fig. 2. At the end of 48 h incubation, BV did not show any cytotoxic effect on normal fibroblast cells (Fig. 2).

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