RESEARCH ARTICLE

Over Expression of BCL2 and Low Expression of Caspase 8 Related to TRAIL Resistance in Brain Cancer Stem Cells

Ling Qi[1], Kuang Ren[2], Fang Fang[3], Dong-Hai Zhao[1], Ning-Jiang Yang[1], Yan Li[3]*

Abstract

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has been investigated as an effective agent to treat various cancers. Cancer stem cells are resistant to TRAIL treatment, but the mechanism of TRAIL resistance remains unknown. In this study, brain cancer stem cells were isolated by CD133 magnetic sorting, and the number of CD133 positive cells detected by flow cytometry. The self-renewing capacity of brain cancer stem cells was examined by a neurosphere formation assay, and the percentage of cell death after TRAIL treatment was examined by an MTS assay. Expression of DR5, FADD, caspase 8 and BCL2 proteins was detected by western blot. The amount of CD133 positive cells was enriched to 71% after CD133 magnetic sorting. Brain cancer stem cell neurosphere formation was significantly increased after TRAIL treatment. TRAIL treatment also reduced the amount of viable cells and this decrease was inhibited by a caspase 8 inhibitor or by the pan-caspase inhibitor z-VAD ($P<0.05$). Brain cancer stem cells expressed lower levels caspase 8 protein and higher levels of BCL2 protein when compared with CD133 negative cells ($P<0.05$). Our data suggest that TRAIL resistance is related to overexpression of BCL2 and low expression of caspase 8 which limit activation of caspase 8 in brain cancer stem cells.

Keywords: Brain cancer stem cells - TRAIL - caspase 8 - BCL2

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Introduction

Glioblastoma is the most common and aggressive brain tumor in humans, and until now no curative treatments were available (Qi et al., 2011a). Recent studies suggest that cancer stem cells are tumor-initiating cells, and that these cells are responsible for resistance to radiation and chemotherapy (Wang et al., 2010; Dirks et al., 2011). Neurospheres are cancer stem cells isolated from glioblastomas using CD133 magnetic sorting (Singh et al., 2004). When cultured in serum-free medium containing growth factors these cells retain the genomic properties of the original glioblastomas (Li et al., 2006). CD133 was recently reported to be a cancer stem cell marker (Zheng et al., 2014). Further studies have shown that targeting hypoxia genes of stem-like cancer cells in glioblastoma inhibits cancer growth and progression (Li et al., 2009).

The aim of cancer therapies is either to initiate cell death or inhibit cancer cell proliferation. Apoptosis, necrosis, autophagy and mitotic catastrophe have been identified as cell death pathways and recent studies suggest that tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) can initiate apoptosis in glioblastoma cells (Ding et al., 2011). TRAIL induces apoptosis through both the receptor-mediated extrinsic pathway and the mitochondrial intrinsic pathway. In brief, TRAIL binds to death receptors 4 (DR4) and 5 (DR5) on the cell surface, it then recruits Fas-associated death domain (FADD) and caspase 8 to assemble the death-inducing signaling complex (DISC) (Bellail et al., 2010). The extrinsic apoptotic pathway impacts on the intrinsic mitochondrial pathway through caspase 8 cleavage, and BCL2 contributes to drug resistance in tumor cells (Zhao et al., 2014). These reports impelled us to study how CD133 positive cells derived from glioblastomas respond to TRAIL treatment. In the present study, we have isolated CD133 positive cells that we called brain cancer stem cells (BCSC) from glioblastoma cells using CD133 magnetic sorting. We explored TRAIL response in BCSC, and tried to ascertain the possible mechanisms of TRAIL resistance, thus providing further data for the use of TRAIL as a clinical anti-cancer drug.

Materials and Methods

Cell culture

Human glioblastoma primary cells SC326 (Qi et al., 2011a) were cultured according to the protocol reported previously (Yuan et al., 2004). In brief, SC326 cells were plated in plastic dishes at a density of $l\times10^4$ cells/cm2 in stem cell neurobasal medium (SCM, neurobasal medium supplemented with 0.5×B27, 50 ng/ml each of epidermal growth factor and basic fibroblast growth factor (Invitrogen, USA). The culture was fed every 5 to 7 days

*[1]Department of Pathology, [2]Department of Pharmacology, [3]Department of Immunology, Jilin Medical College, Jilin, China *For correspondence: liyan2310@126.com*

by changing half of the medium. Once the neurospheres reached approximately 200-300 cells in size, they were dissociated by repeatedly triturating.

Magnetic cell sorting and flow cytometry

When the number of SC326 cells reached 2×10^7 in total, they were labeled with 1 μl CD133/1 microbeads (Miltenyi Biotec, Germany) per one million cells and sorted with CD133 magnetic sorting as previously described (Singh et al., 2004). CD133 positive and negative cells were cultured for further experiments. Aliquots of CD133 positive and negative cells were evaluated for purity by flow cytometry with a FACSCalibur machine (BD Biosciences), using CD133/2 (293C3)-PE antibody (Miltenyi Biotec, Germany).

Neurosphere formation assay

Suspensions of CD133 positive and negative cells were plated at 100 cells per well in 96-well plates and grown in SCM with or without 300 ng/ml rhTRAIL (PeproTech, USA) for 7 days. The number of neurospheres in each well were then counted. The experiment was repeated in triplicate. Rate of neurosphere formation was calculated based on the formula, Rate of neurosphere formation % = (number of neuroshpere/number of plated cells)×100%.

Cell proliferation assay

Neurospheres were dissociated and plated in 96-well plates at 5×10^3 cells per well. Cells were untreated or treated with 300 ng/ml TRAIL, and in the presence or absence of a caspase 8 inhibitor (z-IETD, R&D, USA) or a pan-caspase inhibitor (z-VAD, ICN Pharmaceuticals, USA) for 24 h. Then cell proliferation was quantified based on the ATP present using CellTiter-Glo Luminescent Cell Viability Assay kit (Promega, USA) according to the manufacturer's protocol. The experiment was performed in triplicate. Cell inhibition was calculated based on the formula, cell inhibition % = [1 - (luminescent density of cells treated/luminescent density of cells untreated)]×100%.

Western blot assay

Neurospheres were lysed in a cell lysis buffer (50 mM Tris, 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 1% protease inhibitor mixture and 1 mM PMSF). Fifty micrograms of total protein from each lysate was separated using SDS PAGE and transferred to nitrocellulose membranes. The membranes were incubated with primary antibodies against DR5 (ProSci. Inc, USA), FADD (BD Biosciences, USA), caspase 8 (Cell Signaling, USA), Bcl-2 and β-actin (Santa Cruz, USA). The experiment was repeated three times. Results were analyzed using Image-Pro Plus image analysis and management systems (USA).

Statistical analysis

Data were expressed as mean±standard deviation ($\bar{x}\pm$sd). Statistical comparisons between different groups were done using one-way ANOVA (SPSS17.0 software, USA). Significance was determined at $P<0.05$.

Results

Percentage of CD133 positive cells before and after CD133 magnetic sorting

Glioblastoma primary cells were cultured and sorted by CD133 magnetic sorting to obtain CD133 positive cells. The percentage of CD133 positive cells was analyzed by flow cytometry. CD133 positive cells were only 3% of the total population before CD133 magnetic sorting but after sorting CD133 positive cells reached 71% (Figure 1A and B). The CD133 positive cell population isolated from glioblastoma primary culture was referred to as BCSC and used in further experiments.

Neuroshpere formation in BCSC

BCSC and CD133 negative cells were cultured and the neuroshpere formation was performed, and the morphology of cells was observed under an inverted microscope. We found that CD133 negative cells cultured in SCM displayed poor ability of neuroshpere formation and very little growth at 24 h, and only a small number of neurospheres was observed after 3 days (Figure 2A). In contrast, BCSC suspended in SCM, started to form small neurospheres after about 6 h, with larger neurospheres after approximately 24 h, and huge neurospheres were observed after 3 days (Figure 2B). The rate of neurosphere formation was 2.44% in CD133 negative cells, and 28.67% in BCSC, the capacity of neurosphere formation in BCSC is significantly increased when compared with CD133 negative cells ($P<0.01$; Figure 3A).

Effect of TRAIL on neurosphere formation in BCSC

A neurosphere formation assay was performed after 7 days of treatment with TRAIL (300 ng/ml) in BCSC and CD133 negative cells. After TRAIL treatment the rate of neurospheres formation was 0.64% in CD133 negative cells and 12.33% in BCSC, demonstrating a greater capacity for neurosphere formation in BCSC compared with CD133 negative cells ($P<0.01$; Figure 3B).

Effect of TRAIL on proliferation in BCSC

An MTS assay was used to measure cell proliferation on BCSC and CD133 negative cells after treatment with 300 ng/ml TRAIL and a caspase 8 inhibitor or a pan-caspase inhibitor for 24 h. After incubation with TRAIL, cell proliferation was inhibited by 44.06% in BCSC. Co-incubation of TRAIL with the caspase 8 inhibitor z-IEDT reduced cell proliferation to 7.00% in BCSC, which was a significant decrease when compared with cells treated with TRAIL alone ($P<0.05$, Figure 4). Co-incubation of the pan-caspase inhibitor z-VAD with TRAIL reduces cell proliferation to 9.35% in BCSC, and likewise was a significant decrease when compared with TRAIL treated cells ($P<0.05$, Figure 4).

Expression of DR5, FADD, caspase 8 and BCL2 proteins in BCSC

The expression of DR5, FADD, caspase 8 and BCL2 proteins was performed by western blot. The expression of DR5 and FADD was similar in BCSC and CD133

negative cells, with no significant statistical difference in expression in BCSC when compared with CD133 negative cells ($P>0.05$, Figure 5 and 6). The expression of caspase 8 decreased markedly, while the expression of BCL2 increased markedly in BCSC. These differences were statistically significant ($P<0.05$, Figure 5 and 6).

Discussion

Apoptosis is an important mechanism of death in cancer treatments. Two pathways control the initiation of apoptosis; one is the death receptor-mediated extrinsic pathway, and the other is the mitochondrial-involved intrinsic pathway. TRAIL initiates apoptosis in sensitive cells by binding to DR5 on the cell surface, which is then followed by recruitment of FADD and caspase 8 to form the DISC, which leads to caspase 8 activation, autoproteolytic cleavage of caspase 8 and the initiation of apoptosis (Qi et al., 2011c; Bellail et al., 2012). BCL2 is an anti-apoptotic gene overexpressed in gliomas and correlates with poor tumor prognosis, however, few reports have focused on the expression of caspase 8 and BCL2 in glioma stem cells. Glioma stem cells expressing higher levels of BCL2 may be the reason for the drug resistance of glioma to apoptotic agents (Qiu et al., 2012; Qi et al., 2014).

The mechanism of TRAIL resistance is unknown in BCSC. A recent study reported that stem-cell-like glioma cells are resistant to TRAIL, and exhibit down-regulation of caspase 8 by promoter methylation (Capper et al., 2009). A separate report has shown that cancer stem cells isolated from glioblastomas are responsible for TRAIL resistance (Qi et al., 2011a). Co-operation of TRAIL and cisplatin can partly restore the sensitivity to TRAIL in glioblastoma cells (Ding et al., 2011). The DISC plays a key role in the TRAIL apoptotic pathway, and BCL2 is an important factor in the mitochondrial pathway, with activation of caspase 8 at the intersection of the two pathways. These findings stimulated us to investigate the factors affecting the activity of caspase 8, and its mechanism of activation.

In this study, we isolated BCSC by CD133 immuno-magnetic sorting based on our previous experiments (Qi et al., 2011b). We obtained that CD133 positive cells, which we called BCSC after CD133 immuno-magnetic sorting (Figure 1). The morphology of BCSC and CD133 negative cells were observed under an inverted microscope. A larger number of neurospheres were formed in cultured BCSC compared with cultured CD133 negative cells (Figure 2A and B). These results showed that neurospheres formed easily in BCSC compared with the CD133 negative population.

Self-renewal capacity was checked by a neurosphere formation assay in BCSC and CD133 negative cells. The capacity of neurosphere formation in BCSC was significantly increased when compared with CD133 negative cells in the presence or absence of TRAIL treatment (Figure 3). The results showed that neurosphere formation capacity was better in BCSC compared with the CD133 negative population. Cell proliferation assays were performed to find the reason for the decrease in neurosphere formation in BCSC. After TRAIL treatments, we found that cell proliferation was significantly decreased in BCSC after co-treatment with either a caspase 8 inhibitor or a pan-caspase inhibitor (Figure 4). These experiments showed that caspases, and in particular caspase 8, play a key role in the resistance of BCSC to TRAIL. We also found no evidence to support the involvement of DR5 and FADD protein in the resistance to TRAIL in BCSC. However, low expression of caspase 8 and over expression of BCL2 was shown in BCSC (Figures 5 and 6), both of which may lead to inactivity of caspase 8, eventually culminating in TRAIL resistance.

In short, the BCSC population appears to be the key to TRAIL resistance in glioma. Low expression of caspase 8 and high expression of BCL2 may limit activation of caspase 8 after TRAIL treatment which may subsequently lead to the occurrence of TRAIL-resistance in BCSC. Tumorigenesis is link with multiple factors (Gao CM et al., 2013), drug resistance may occur in the course of cancer treatment. But further exploring the mechanisms of TRAIL resistance will provide new paradigms for the use of TRAIL in the clinic for cancer treatment.

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