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Translational Research

TRANSLATIONAL RESEARCH

Isoflurane enhances the malignant potential of glioblastoma stem cells by promoting their viability, mobility *in vitro* and migratory capacity *in vivo*

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Abstract

Background: Isoflurane is one of the most common general anaesthetics used during surgical procedures, including tumour resection. However, the effects of isoflurane on the viability and migration capacity of cancer cells, specifically in the context of brain cancer cells, remain unclear. Therefore, the aim of this study was to evaluate the influence that isoflurane has on the function of glioblastoma stem cells (GCSs) in regards to cell proliferation, survival and migration.

Method: U251-GSCs were exposed to isoflurane at clinically relevant concentrations and incubation times. The effects on proliferation, survival and migration capacities of the cells were evaluated *in vitro*. The potential risk was assessed in mice by intracranial injection of U251-GSCs pretreated with isoflurane. Furthermore, the average tumour volume and migration distance of U251-GSCs from the tumour centre were calculated.

Results: Exposure of U251-GSCs to 1.2% isoflurane for 6 h resulted in increased proliferation ($P<0.05$) and decreased apoptosis rate ($P<0.05$) when compared with the control group. In addition, isoflurane exposure caused increased migration capacity *in vitro* ($P<0.05$) and the distance migrated was increased *in vivo* ($P<0.05$).

Conclusion: Clinically relevant concentrations and incubation times of isoflurane could promote the viability and mobility of U251-GSCs, suggesting this general anaesthetic may have detrimental effects in glioblastoma by facilitating its growth and migration.

Key words: glioblastoma; isoflurane; proliferation and invasion; stem cells

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Editor's key points

- The choice of anaesthetic in cancer patients may have implications for tumour recurrence or metastasis.
- The effects of isoflurane exposure on glioblastoma stem cells was studied *in vitro* and *in vivo* in mice.
- Isoflurane exposure decreased apoptosis and increased proliferation and migration of cells.
- In mice, isoflurane pre-exposure of stem cells prior to injection increased cell invasion but had no effect on tumour volume.
- Isoflurane appears to facilitate the growth and invasion of glioblastoma.

Glioblastoma is one of the most common primary malignant brain tumours in adults, with an annual incidence of 5.26 per 100 000 population or 17 000 new diagnoses per year.[1 2] Although treatment options have been expanding and improving, the overall prognosis remains poor, with an average overall survival time of 15–17 months.[3] Early surgical resection may be first-line treatment, even in suspected cases of low-grade glioma.[4] There are numerous events within the perioperative and postoperative periods that can influence tumour recurrence, metastasis, and survival. The effect of anaesthetics has been implicated in tumour growth, with a number of retrospective studies suggesting that general anaesthetics are associated with an increased risk of recurrence or metastasis in a variety of tumours, including melanoma[5] and cancers of the breast,[6] prostate,[7] colon and rectum.[8 9]

Isoflurane, one of the most commonly used general anaesthetics in clinical practice, has been shown to have cytotoxic properties in different types of cultured cells.[10–13] Moreover, an increasing number of studies suggest that isoflurane may be cytoprotective *in vitro*[14–17] and *in vivo*.[18–20] These studies suggest that different effects may be observed based on the particular cell type used. It has been reported that isoflurane increases cell proliferation and enhances malignancy in head and neck squamous cell carcinoma cell lines, ovarian cancer cell lines and renal cell carcinoma cell lines.[21–23] However, few studies have evaluated the effects of isoflurane on glioblastoma, specifically the viability and migration capacity of glioblastoma stem cells (GSCs). Thus, the aim of this study was to investigate the effects of clinically relevant concentrations of isoflurane on cell proliferation, apoptosis and migration of human GSCs *in vitro* and *in vivo*.

Methods

GSCs isolation and cell culture

The human glioblastoma cell line U-251 MG (Glioblastoma U251) was obtained from the China Center for Typical Culture Collection (CCTCC, Wuhan, China). The CD133-positive glioblastoma stem cells (GSCs) from U251 were isolated using flow cytometry. The stemness of the cells was verified using Nestin and DAPI staining (Supplementary data, Fig. 1). U251-GSCs were cultured in GSC complete media [DMEM/F12 (GIBCO) with 20 ng ml^{-1} epidermal growth factor (Millipore), 20 ng ml^{-1} fibroblastic growth factor (Millipore), 2% B27 and 1% penicillin/streptomycin (GIBCO)]. U251-GSCs were then seeded on laminin-coated plates (Sigma; 1 mg cm^{-2}) or cultured as GSC spheres with GSC complete media. These cells were maintained in a humidified incubator at 37°C and an atmosphere of 5% CO_2.

Anaesthetic exposure

To evaluate the anaesthetic effects on U251-GSCs, the cells were exposed to different concentrations of isoflurane and for different durations. Isoflurane was delivered from an agent-specific vaporizer carried by humidified air 1 min^{-1}/5% CO_2. The flow rate to the sealed plastic chamber was initially 5 litre min^{-1} for the first 2 min for anaesthesia maintenance. An infrared Ohmeda 5330 agent monitor (Coast to Coast Medical, Fall River, MA, USA) was used to continuously monitor the delivered isoflurane concentration.

Cell proliferation assays

Cell proliferation was evaluated by testing mitochondrial dehydrogenase activity, which reduces 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma). U251-GSCs were exposed to 1.2% isoflurane for 3, 6, 9, 12 or 24 h then cultured in complete media for 3 days (Supplementary data, Fig. 2) prior to assay.

Nuclear Ki-67 expression was used to identify U251-GSCs in the proliferative phase. Cells were exposed to isoflurane for 3 or 6 h at various concentrations (0.6, 1.2 and 2.4%). After 3 days, the cells were fixed in 4% paraformaldehyde and treated with 0.1% Triton X-100 prior to incubation with human anti-Ki67 (RM-9106; Thermo). The secondary antibody (Alexa 594; GIBCO) and DAPI were used for visualization and counterstaining the cell nuclei. The percentage of Ki-67-positive cells was recorded with a fluorescence microscope at 200× magnification from nine random fields and quantified by a single blinded observer.

Cell apoptosis analysis

Annexin V and propidium iodide (PI) were used (Joincare Biosciences, China) to detect apoptosis of U251-GSCs. The assay was performed 2 days after treatment. Cells were stained with annexin V for 15 min and counterstained with PI for 5 min. Annexin V and PI incorporation were quantified via flow cytometry (Becton Dickinson). Three replicate experiments were performed for each sample and data were analysed with FlowJo 7.6.1.

In vitro cell migration assay

Boyden transwell chambers (Corning) were used to analyse the migration capacity of U251-GSCs. Cells were pretreated in different concentrations of isoflurane (0.6, 1.2 and 2.4%) for different durations (3 or 6 h) before being added to the upper chamber. Cells were allowed to migrate into the lower chamber into GSC complete medium at 37°C and then non-migrated cells were removed with a cotton swab. Migrated cells were fixed in 100% methanol for 2 min, stained with haematoxylin-eosin solution and counted at 200× magnification. Nine randomly selected fields per membrane were analysed from independent experiments.

In vivo studies

All animals were treated in accordance with the ethical guidelines set by Huazhong University of Science and Technology (HUS) and relevant sections of the ARRIVE Guidelines were followed.

To investigate the effect of isoflurane on U251-GSCs *in vivo*, 4–6-week-old male BALB/c athymic nude mice were divided into three groups (n=15 per group) and stereotactically injected with U251-GSCs [control (Ctrl) group] or GSCs pretreated for 6 h with either 0.6% isoflurane or 1.2% isoflurane into the right