Sevoflurane promotes the expansion of glioma stem cells through activation of hypoxia-inducible factors in vitro

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Editor’s key points

- Glioma stem cells (GSCs) were exposed to sevoflurane to assess the mechanisms involved in possible anaesthetic mediated tumour recurrence.
- Sevoflurane promoted GSC proliferation via hypoxia-inducible factors.
- Sevoflurane may enhance tumour growth by effects on tumour stem cells.

Background. Growing evidences indicate that inhalational anaesthetics can enhance the growth and malignant potential of tumour cells and may affect tumour recurrence after surgery. Tumour stem cells play a vital role in tumour recurrence. This study investigates the effect of sevoflurane on glioma stem cells (GSCs) in vitro and the underlying molecular mechanisms in this process.

Methods. Cultured GSCs were exposed to clinically relevant concentrations and durations of sevoflurane exposure. Cell proliferation and self-renewal capacity were determined. Expression of the stem cell marker CD133, vascular endothelial growth factor (VEGF), hypoxia-inducible factors (HIFs), and phosphorylated Akt, which is a protein kinase involved in multiple cellular processes, were measured using western blotting. Small interfering RNAs and an Akt inhibitor were used to investigate specific pathways.

Results. Compared with controls, cells exposed to 2% sevoflurane for 6 h induced a larger number of proliferated cells (31.2 ± 7.6% vs 19.0 ± 5.8%; P < 0.01). Levels of CD133, VEGF, HIF-1α, HIF-2α, and p-Akt were up-regulated by sevoflurane in a time- and concentration-dependent manner. Small interfering RNA against HIFs decreased the percentage of proliferating GSCs after sevoflurane exposure and pre-treatment of cells with an Akt inhibitor abrogated the expression of HIFs induced by sevoflurane.

Conclusions. Sevoflurane can promote the expansion of human GSCs through HIFs in vitro. Inhaled anaesthetics may enhance tumour growth through tumour stem cells.

Keywords: AC133 antigen; glioma; hypoxia-inducible factor-1α; neoplastic stem cells; sevoflurane

Accepted for publication: 9 September 2014

Surgery remains the main treatment for the majority of solid tumours. Unfortunately, postoperative recurrence and metastasis occur frequently and the mechanisms involved remain obscure. Perioperative events including surgical stress, anaesthetic technique, and the anaesthetic/analgesic agents used may play an important role in cancer recurrence after surgery.1-5

Recent evidence indicates that perioperative anaesthetics have direct effects on tumour cells and might influence tumour recurrence, metastasis, and long-term survival.6-7 Sevoflurane was observed to increase proliferation, migration, and invasion in breast cancer cells.8 Isoflurane was also found to promote the growth of several types of tumour cells in vitro9-11 and the mechanism may involve up-regulation of hypoxia-inducible factors (HIFs).9

Tumour stem cells possess the ability to undergo extensive self-renewal, differentiation into multiple lineages, and recapitulation of the original tumour.12 These cells, such as glioma stem cells (GSCs), play a central role in tumour initiation, metastasis, and recurrence and so have become a target of tumour treatment.13

HIFs are the major regulators of tumour cell adaptation to hypoxic stress.14 In GSCs, HIFs also can be induced by numerous stimuli other than hypoxia, including insulin, insulin-like growth factors, epidermal growth factor (EGF), and heavy metals.15 Stabilizing HIF-1α expression may promote the self-renewal of the CD133-positive GSCs.16 CD133 is a stem cell marker and Akt is a protein kinase involved in many cellular processes including cell survival.

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Therefore, the aim of this study was to determine the effect of sevoflurane on the HIF pathway in GSCs and to define its potential impact on proliferation of tumour stem cell *in vitro*. We hypothesized that sevoflurane would up-regulate the HIF pathway and promote proliferation of GSCs.

**Methods**

**Cell culture**

Human primary GSCs were derived from tumour resection. Tumour tissue was enzymatically dissociated and cultured as floating neurospheres in serum-free medium (Dulbecco's modified eagle medium/F-12) supplemented with 20% bovine serum albumin, insulin and transferrin serum-free supplement (Stemcell Technologies, Canada), EGF, and basic fibroblast growth factor (20 ng ml⁻¹; PeproTech, USA). Cells were cultured at 37°C, 95% relative humidity and 5% CO₂ with 21% oxygen. As demonstrated previously, these GSCs were multipotent, expressed markers of the undifferentiated phenotype, and showed self-renewal.

**RNA interference**

HIF-1α, HIF-2α, and control small interfering RNA (siRNA) oligonucleotides were obtained commercially (Santa Cruz Biotechnology). The day before transfection, 5 × 10⁶ GSCs were dissociated into single cells and transferred to culture flasks. Cells were then transfected with siRNA using a nucleofector according to the manufacturer's protocol (Bio-Rad, USA). Cells were harvested for sevoflurane exposure after 24 h.

**Akt inhibition**

GSCs were pre-treated with 10 μM LY294002, a commonly used Akt inhibitor, in 0.02% dimethyl sulphoxide (Sigma-Aldrich, USA), and incubated for 2 h before sevoflurane exposure. Naïve control (NC) cells were treated with the same volume of solvent.

**Sevoflurane exposure**

Cells were placed in air-tight, humidified chambers. Briefly, an in-line anaesthetic vaporizer (Drager, Germany) fed by a supply of a gas mixture containing 21% oxygen and 5% carbon dioxide balanced with nitrogen was used to deliver sevoflurane at a rate of 2 litre min⁻¹ for at least 5 min until the desired sevoflurane concentration (1–6%) was achieved. Concentrations of sevoflurane and oxygen were monitored every hour using an anaesthetic analyser (Drager Vamous, Germany) in the outlet. Cells in control conditions defined as NC were placed in an identical gas chamber under 21% oxygen and 5% carbon dioxide balanced with nitrogen. Once sealed, the chambers were placed in a 37°C incubator.

**Proliferation**

Proliferation of GSCs was measured using a modified sphere-forming assay as described earlier and an 5-ethynyl-2-deoxyuridine (EdU) incorporation kit (RiboBio, China) according to the manufacturer's instructions. Briefly, GSCs were exposed to 2% sevoflurane for 6 h and incubated with 20 nM of EdU for an additional 12 h at 37°C. Cells were fixed and treated with 0.5% Triton X-100 for 20 min. After washing, cells were incubated with 1 × Apollo reaction cocktail for 30 min. DNA was stained with Hoechst33342. The nuclear intensity threshold for an EdU-positive cell was defined by the intensity of EdU positive staining in matched control cells. Images were captured using a Nikon Eclipse 80i microscope (Nikon, Japan) and merged using the ImageJ 2 software (National Institutes of Health). The percentages of EdU-positive cells were measured.

**Western blot**

GSCs were washed and collected in radio immunoprecipitation assay lysis buffer (Sigma-Aldrich). After brief sonication, lysates were clarified by centrifugation and the protein content was measured. Protein samples were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis gels and transferred to polyvinylidene fluoride membranes (Millipore). Membranes were blocked for 1 h at room temperature and incubated with primary antibodies overnight at 4°C. The following antibodies were used: CD133 which is a stem cell marker (Abcam), HIF-1α, HIF-2α (Novus Biologicals), β-actin, vascular endothelial growth factor (VEGF), and phospho-Akt (Cell Signaling Technology). After washing, membranes were further incubated with secondary antibody for 1 h at room temperature. Bound antibody was detected with an enhanced chemiluminescence reagent (Pierce), and images were analysed with the Quantity One Software. Each assay was performed in triplicate and all tests were repeated three times.

**Statistical analysis**

All data were normally distributed and expressed as mean and standard deviation (SD). Difference in the proliferation between the NC and sevoflurane group and the results of RNA interfering were evaluated by Student’s t-test. The remaining data were analysed using one-way analysis of variance. Statistical analysis was based on two-tailed hypothesis testing, and analysis was performed using the GraphPad prism (version 5.0, USA). P-values <0.05 were considered significant.

**Results**

The number of actively proliferating cells was significantly increased in cells exposed to sevoflurane compared with NC (P <0.01, Fig. 1a). The sphere-forming abilities of the dissociated single cells were also increased (P <0.01, Fig. 1a). Immunoblots showed increasing expression of CD133 total protein level in a time- and concentration-dependent manner under sevoflurane exposure (Fig. 2a and b).

Significant increases in HIF-1α protein levels were seen in samples exposed to 2% sevoflurane at 0, 2, 4, and 6 h (P <0.05; Fig. 2a). Sevoflurane also induced HIF-1α production in a concentration-dependent manner (Fig. 2a). Similar changes were seen for HIF-2α with significant increases observed at 0, 2, 4, and 6 h (P <0.05; Fig. 2a). The level of HIF-2α increased after exposure to 2, 4 and 6% sevoflurane.
Fig 1 GSCs exposed to either 2% sevoflurane or NC. (a) Representative image of EdU staining (red) with Hoechst 33342 counterstain (blue). (b) Representative image of sphere-forming ability and number of spheres (mean and SD, **P<0.01).

Fig 2 Representative images of western blots of CD133, VEGF, HIFs, p-Akt, and β-actin after exposure of GSCs to 2% sevoflurane for up to 6 h. (a) Exposure to 0–6% sevoflurane for 4 h. HIF, hypoxia induced factor; NC, naïve control; Sevo, sevoflurane.
(P<0.05; Fig. 2a). Similarly, VEGF expression also increased significantly after sevoflurane exposure (P<0.05; Fig. 2a and 2b).

Transfection of GSCs in the presence of 2% sevoflurane with HIF-1α siRNA, abrogated the expression of HIF-1α and decreased the EdU-positive GSCs (P<0.01; Fig. 3A). The HIF-1α depletion experiment performed without sevoflurane had no effect on sphere-forming capacity (data not shown). Depletion of HIF-2α showed a similar effect on proliferation (P<0.05; Fig. 3a).

There was an increase in the levels of p-Akt in GSCs at 0, 2, 4, and 6 h (P<0.05; Fig. 2A). Dose–response tests on p-Akt revealed increases after 2, 4, and 6% sevoflurane exposure (P<0.05; Fig. 2a). A significant decrease in p-Akt, HIF-1α, HIF2α, and CD133 in the group of cells treated with both the inhibitor and 2% sevoflurane was seen compared with cells treated with 2% sevoflurane alone (P<0.05; Fig. 4A). There was significantly lower cell proliferation in the cells treated with LY294002 plus sevoflurane than with sevoflurane alone (P<0.05; Fig. 4a and c).

**Discussion**

Determining the effect and mechanisms of the inhaled anaesthetics on tumour stem cells will enhance our understanding of tumour recurrence after surgery. In this study, we investigated the effect of sevoflurane on GSC maintenance and growth and the potential mechanisms involved. Our results showed that sevoflurane promoted the growth of GSCs through activation of HIFs. Blockade of the PI3 K/Akt pathways abrogated the growth-promoting effect.

GSCs are responsible for recurrence of malignant glioma, and possess stem cell-like characteristics, including the capacity for self-renewal and long-term proliferation, and the ability to differentiate into multiple nervous system lineages. According to our results, exposure to sevoflurane at clinically relevant concentration and durations did not affect cell viability but promoted the proliferation of GSCs.

Other studies have indicated that sevoflurane had different effects in tumour cells and stem cells. Tumour cells from human carcinoma of the colon or larynx, pancreas, and lung were reported to be inhibited and undergo apoptosis after exposure to sevoflurane. However, the proliferation, migration, and invasion of breast tumour cells increased after sevoflurane exposure. In neural stem cells (NSCs), clinically relevant concentrations of sevoflurane could increase cell proliferation, while prolonged sevoflurane exposure might decrease the self-renewal capacity of hippocampal NSCs.

These conflicting results may be attributable to the heterogeneity of responses to the sevoflurane depending on cell type, concentration, duration of exposure, or all.

Our results showed that exposing the GSCs to 2% sevoflurane for 6 h increased cell proliferation by ~12% along with increased expression of CD133. Previous research demonstrated that injection of as few as 100 CD133+ cells could produce a tumour that could be serially transplanted. Most importantly, glioblastoma multiforme is characterized by a diffuse tissue-distribution pattern, with extensive dissemination of the tumour cells within the brain that hinders complete surgical resection. Thus, exposure of these cells to sevoflurane may play a vital role in glioma recurrence after surgery.

To elucidate the underlying mechanism, we examined the effects of sevoflurane on HIFs, as they have been reported to be involved in the growth, self-renewal, and differentiation processes of GSCs. Time- and concentration-dependent expression of HIFs was found in GSCs after sevoflurane exposure. This result combined with the expression level of the stem cell marker CD133 and abrogation of the growth effect by HIF siRNA, strongly indicated the crucial role of HIFs in sevoflurane-induced GSC growth.

Akt, also known as protein kinase B, is the main downstream effector of PI3 K. PI3 K/Akt is one of the main signal pathways controlling the expression of HIFs. Previous research has demonstrated that this can be activated by inhaled anaesthetics in other several cancer cells. As we expected,

**Fig 3** Representative western blots of HIFs and β-actin expression and percentage of EdU-positive cells after exposure of GSCs to 2% sevoflurane for 6 h (mean and SD, **P<0.01, # P<0.05) after treatment with (a) HIF-1α siRNA and (b) HIF-2α. Cont, control siRNA.
Sevoflurane increased the level of p-Akt, and pre-treatment with the Akt inhibitor LY294002 abolished the up-regulation of p-Akt and HIFs after sevoflurane exposure. These results indicate that this pathway plays an important role in sevoflurane-induced expression of HIFs. The mechanism of activation of PI3K/Akt by sevoflurane and other signal pathways, such as extracellular signal-regulated kinase/mitogen-activated protein kinase, needs to be further investigated.

This work has limitations in some respects. First, in vitro systems do not faithfully replicate in vivo conditions. GSCs are located in specialized microenvironments (niches) within tumours in vivo. For example, hypoxia exists in GSC niches. This suggests that inhaled sevoflurane may not reach all stem cell niches and not all GSCs may be affected by sevoflurane. As such, extrapolation of in vitro data to the clinical situation must be done with caution. Secondly, we examined...
only one type of tumour stem cell and only one anaesthetic agent, and as such the generalizability of the results is restricted. Other general anaesthetics such as isoflurane and propofol and other tumour stem cells should be studied.

In conclusion, our study showed that sevoflurane can promote the self-renewal and proliferation of GSCs through HIFs. This suggests that inhaled anaesthetics may enhance tumour growth through tumour stem cells and perioperative usage of general anaesthetics may play a crucial role in cancer recurrence after surgery. Our results, combined with previous research, indicate that anaesthetics may be an important factor impacting the prognosis of cancer patients.

Authors’ contributions

Acknowledgements
The authors thank H. J. Xu, J. T. Yang, and L. H. Tang (MSc, Department of Anaesthesiology, Second Affiliated Hospital, Zhejiang University School of Medicine), for their technical assistance in cell culture, western blot and image analysis.

Declaration of interest
None declared.

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Handling editor: H. F. Galley