Impact of isoflurane on malignant capability of ovarian cancer in vitro

X. Luo1,3†, H. Zhao3†, L. Hennah3, J. Ning3, J. Liu1, H. Tu2 and D. Ma3*

1 Department of Anaesthesiology and 2 Department of Neurosurgery, Taihe Hospital, Hubei University of Medicine, Hubei, China
3 Section of Anaesthetics, Pain Medicine and Intensive Care, Department of Surgery and Cancer, Faculty of Medicine, Imperial College London, Chelsea and Westminster Hospital, London, UK
* Corresponding author. E-mail: d.ma@imperial.ac.uk

Editor’s Key Points

- Metastatic recurrence of ovarian cancer after surgery is common.
- The effect of isoflurane on ovarian cancer cell behaviour was measured.
- Isoflurane exposure increased cancer cell proliferation and expression of markers related to metastatic potential.
- Isoflurane may increase metastatic potential of cancer cells.

Background. Metastatic recurrence of ovarian cancer is the foremost cause of postoperative mortality. With recent research indicating that inhalation of anaesthetics may influence cancer cell behaviour, this study investigated the effects of isoflurane on the expression of tumorigenic markers and proliferative capacity in ovarian cancer cells.

Methods. Ovarian cancer (SK-OV3) cells were cultured and then exposed to 2% isoflurane for 2 h. The expression of markers involved in cell proliferation, angiogenesis, and migration were assessed up to 24 h after treatment using immunofluorescence staining, western blotting, and flow cytometry. The effects of isoflurane on in vitro angiogenesis and migration were also determined.

Results. Isoflurane exposure significantly increased insulin-like growth factor (IGF)-1 and IGF-1R expression, cell cycle progression, and cell proliferation in SK-OV3 cells. Increased expression of the angiogenic markers vascular endothelial growth factor (VEGF) by 56% (P<0.05) and angiopoietin-1 by 62% (P<0.05) was also observed 24 h after isoflurane exposure together with an enhanced in vitro angiogenesis. Cell migration was significantly increased after exposure to isoflurane together with increased production of both matrix metalloproteinases 2 and 9 (both P<0.05) by almost five-fold relative to control. These effects were abolished when IGF-1R signalling was blocked either by neutralizing antibody or by small interfering RNA.

Conclusions. Our data indicate that isoflurane increases the malignant potential of ovarian cancer cells through the up-regulation of markers associated with the cell cycle, proliferation, and angiogenesis. This study warrants further investigations.

Keywords: anaesthetics; angiogenesis; insulin-like growth factor; isoflurane; migration; ovarian cancer; proliferation

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Ovarian cancer is one of the commonest cancers in women. The best treatment option is surgery and chemotherapy. Despite medical and surgical advances, progression-free survival remains as low as 20–30%. Metastatic recurrence after surgery is the major cause of mortality, with current research suggesting that perioperative factors could potentially affect tumour cell signalling and promote metastatic disease. During this crucial period, three principal factors influence the process by which the cancer can establish itself as metastatic disease. First, the surgical technique can influence recurrence rates because the handling and disruption of the neoplasm during surgery is thought to release tumour cells into the circulation. Secondly, the immune system is suppressed during the perioperative period, with reduced circulating natural killer (NK) and cytotoxic T cells. Whilst surgical stress alone can induce this immune suppression, anaesthetic drugs such as isoflurane have also been suggested to independently activate pathways that suppress immune function. Recently, several studies have indicated the importance of anaesthetic technique on postoperative clinical outcome. Retrospective analyses have indicated that regional (RA) rather than general anaesthesia (GA) may improve survival rates of cancer patients. Whilst these studies highlight the important role of anaesthetic techniques, there may be other effects on the immune system.

The direct cellular effects of anaesthetics on cancer cell biology remain elusive and more research is required to...
improve our understanding of the mechanisms by which anaesthetic drugs can influence cancer cells. Isoflurane is one of the most commonly used volatile anaesthetics and is used extensively in gynaecological surgery. In this study, we explored the underlying mechanisms of how isoflurane may influence ovarian cancer cell biology.

**Methods**

**Cell culture**

The human ovarian epithelial carcinoma cell line SK-OV3 (European Cell Culture Collection) and human umbilical vein endothelial cells (HUVECs) (Lanza Biosciences, UK) were used for this study. Cell cultures were kept at 37 °C in a humidified atmosphere containing 5% CO₂. SK-OV3 cells were cultured in McCoy’s 5A medium (Sigma-Aldrich, Dorset, UK), containing 10% fetal bovine serum (Thermo Scientific, Epsom, UK), 2 mM L-glutamine and 1% penicillin (Sigma-Aldrich). HUVECs were cultured in EGM-2 medium supplemented with growth supplements (EGM-2 MV bullet kit) (Lonza, Walkersville, MD, USA) under standard culture conditions.⁹

**Gas exposure**

The SK-OV3 cells were cultured in 60 mm Petri dishes at a density of 1 × 10⁶. Cell media was replaced with serum-free media before the dishes were placed in a 1.5 litre airtight gas chamber. The gas chamber contained inlet and outlet valves and a fan to ensure equal distribution of gas within the chamber. A sensor (Datex-Ohmeda, Bradford, UK) was used to monitor the concentration of gas before cell exposure and also to sample the gas within the chamber. The chamber was connected to calibrated flow meters and an in-line vaporizer used to deliver the desired composition (Datex gas monitor, Finland) of isoflurane (1.0, 1.5 or 2.0%) (Abbott Laboratories Ltd, Maidenhead, UK) in 21% oxygen and 5% carbon dioxide, balanced with nitrogen (BOC, UK). Initially, the concentration-related response of isoflurane on cell proliferation was determined and the further effects of 2% isoflurane on cell malignancy were then focused on. Under our experimental conditions, 1% isoflurane in gas phase was ~0.21 mM in the aqueous phase when fully equilibrated.¹⁰ The chamber was pre-flushed with the gas mixture to ensure a stable gas composition and a closed system was established to prevent leakage. Cells were exposed to isoflurane concentration for 2 h at 37 °C. The cells were then removed and the serum-free medium was replaced with full culture medium and the Petri dishes were returned to a standard incubator containing humidified air and 5% carbon dioxide at 37 °C for further analysis. Cells used as the treatment control group (N₂ group) were placed in an identical gas chamber containing 21% oxygen and 5% carbon dioxide balanced with nitrogen at 37 °C. Cells were analysed at different time points ranging from 0 to 24 h post gas exposure.

**Cell treatments**

Small interfering (si) RNA transfections were carried out using lipofectamine (Invitrogen, Paisley, UK). siRNA targeting human insulin-like growth factor (IGF-1R, SI00017521, Qiagen, Crawley, West Sussex, UK) was administered at 20 nM to SK-OV3 cells with scrambled siRNA as a negative control. Cells were incubated with siRNA for 6 h at 37 °C, and then medium was removed and replaced with experimental medium followed by isoflurane gas treatment. Some cells were also treated with IGF-1R neutralizing antibodies α1R3 (Millipore Ltd, UK) after or at the same time as isoflurane exposure.

**Immunofluorescence**

SK-OV3 cells were fixed in paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), after being incubated in 10% normal donkey serum. Samples were then washed three times with 0.1 M PBS and incubated at 4 °C overnight with the following primary antibodies: rabbit anti-cyclin D (1:200), rabbit anti-cyclin E (1:200), mouse anti-VEGF (1:200), rabbit anti-angiopoietin (1:200, Abcam, Cambridge, UK) or mouse anti-Ki-67 (1:200, DAKO, St Thomas Place, Ely, UK) followed by fluorochrome-conjugated secondary antibodies for 1 h. The slides were counterstained with the nuclear dye DAPI and mounted with VECTASHIELD Mounting Medium (Vector Lab, USA) and immunofluorescence was quantified using ImageJ (National Institutes of Health, MD, USA). Fluorescence was assessed by a technician blinded to the treatment groups. Values were then expressed as percentages of the mean value for naive controls and expressed as % fluorescence intensity (FI).

**Western blot analysis**

Cells were mixed with tissue lysis buffer (pre-treated with protease and phosphatase inhibitor) for 30 min. After centrifugation the supernatant was collected and removed for protein quantification by Bradford Protein Assay (Bio-Rad, Hemel Hempstead, UK). For protein extracts (40 μg sample⁻¹) were heated for 10 min in a 95 °C water bath and denatured in sodium dodecyl sulphate (SDS) sample buffer (Invitrogen Ltd, Paisley, UK). The samples were loaded on a NuPAGE 4 to 12% Bis–Tris gel (Invitrogen) for electrophoresis and then transferred to a PVDF membrane. The membrane was blocked using non-fat milk (Cell Signalling, Hitchin, UK) dissolved in Tris–buffered saline with Tween (TBS-T; 10 mmol litre⁻¹ Tris–HCl, 150 mmol litre⁻¹ NaCl, 0.1% Tween; pH 8.0) for 2 h, then the membrane probed with rabbit anti-human IGF-1 (1:1000, Abcam) overnight, followed by an HRP-conjugated secondary antibody for 1 h. The loading control was α-tubulin (1:10 000, Sigma–Aldrich). Blots were visualized with the enhanced chemiluminescence (ECL) system (Santa Cruz, USA), and the protein bands were captured with using GeneSnap (Syngene, Cambridge, UK) and analysed using GeneTools software (Syngene, Cambridge, UK) protein bands were normalized to α-tubulin expression and reported as ratio of control.

**MMT assay**

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Merck KGaA, Darmstadt, Germany).
Enzyme-linked immunosorbent assay
MMP-2 and MMP-9 were measured in medium from SK-OV3 cells using enzyme immune assay (R&D Systems, Oxon, UK), following the manufacturer’s instructions.

Determination of cell cycle through flow cytometry
Flow cytometry was used to analyse the cell cycle. Briefly, cells were washed with PBS and detached using cell dissociation buffer (Sigma-Aldrich), then fixed with ethanol and stained with propidium iodide (PI, 0.8 μg ml⁻¹, Sigma-Aldrich) in PBS. A minimum of 20 000 cells per sample were analysed with flow cytometry (FACS Calibur, Becton Dickinson, Sunnyvale, CA, USA). Data were analysed by FlowJo software (TreeStar), which showed basic statistics such as the fraction of cells in G1, S, and G2, the positions of the G1 and G2 peaks and their widths. The percentage of cells in different phases of cell cycle was therefore determined.

Determination of protein expression by flow cytometry
SK-OV3 cells were incubated with fluorochrome-conjugated anti-IGF-1R antibody (e-bioscience, UK) or its isotype control for 30 min at 4°C then washed with FACS buffer.

Immunofluorescence intensity was analysed using flow cytometry (FACSCalibur; Becton Dickinson, Sunnyvale, CA, USA).

In vitro angiogenesis assay
Angiogenesis was analysed using the angiogenesis assay (Endothelial Tube Formation Assay, Invitrogen, UK), following the manufacturer’s instructions. Plates (24-well) were coated with matrigel and previously untreated HUVEC and HUVEC treated with conditioned medium (cell culture medium collected from SK-OV3 cells exposed to gas, mixed with HUVEC medium in 1:1 ratio) were seeded at 3 × 10⁴ cells well⁻¹. Tube formation was examined and monitored by digital camera images taken using a phase-contrast microscope (Olympus CK30, Tokyo, Japan) and the number of vascular tubes in 4 fields was then counted.

Scratch assay
The scratch assay (wound healing assay) was performed for assessing tumour cell migration. Briefly, SK-OV3 cells were cultured in 24-well plates. After confluence, one artificial gap per well was scratched with a sterile plastic 1000 μl micropipette tip to generate a uniform gap that was devoid of adherent
cells. Gap closure was monitored by digital camera images taken in a phase-contrast microscope (Olympus CK30, Tokyo, Japan) across the gap. The gap closure (healing) was quantified by calculating the mean percentage of the remaining cell-free area 24 h after treatment compared with the area of the initial wound.14

Statistical analysis
Data are expressed as mean (SD) and analysed using one-way ANOVA followed by the post hoc Student–Newman–Keuls test (GraphPad Prism 5.0 Software). A $P$-value of $<0.05$ was considered to be statistically significant.

Results
Increased production of IGF-1 was observed 24 h after exposure of SK-OV3 cells to isoflurane ($P<0.01$, Fig. 1A and B). Expression of IGF-1R also increased 24 h after exposure to isoflurane (Fig. 1c). Exposure to isoflurane also resulted in a significantly greater number of viable cells than the nitrogen control groups ($P<0.05$) (Fig. 1a). Using Ki-67 as a marker of proliferation it was found that isoflurane increased the number of replicating cells at 24 h when compared with the control group ($P<0.05$, Fig. 1e and f). The cyclins D and E, which control cell cycle entry into the S phase, were up-regulated in the isoflurane group when compared with control (Fig. 2A–D). This was confirmed by cell cycle analysis using flow cytometry, which showed an increased percentage of cells at S phase when the isoflurane dose was gradually increased (Fig. 2E).

Significant increases in expression of angiogenic markers were observed in the isoflurane-treated cells, with a significant increase in angiopoietin intensity between the isoflurane and nitrogen groups at 4 and 24 h after exposure ($P<0.05$, Fig. 3A and B). VEGF expression was also higher in cells exposed to isoflurane compared with control ($P<0.05$, Fig. 3C and D). This was supported by enhanced in vitro HUVEC angiogenesis and increased

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**Fig 2** Representative images of (a) cyclin D and (c) cyclin E immunofluorescent staining in SK-OV3 cells at 4 and 24 h after 2% isoflurane exposure (nuclei counterstained with DAPI, scale bar: 50 μm). Fluorescence intensity of (b) cyclin D and (d) cyclin E ($n=8$). (e) Cell cycle analysis (percentage of cells in S phase) 24 h after exposure to 1–2% isoflurane ($n=4$). Data are expressed as mean (SD). *$P<0.05$ and ***$P<0.001$. N2, nitrogen; NC, naı¨ve control; Iso, isoflurane.
vascular tube formation in HUVEC exposed to medium from SK-OV3 cells previously exposed to isoflurane (Fig. 3E and F).

Using the scratch assay, enhanced cell migration was seen up to 48 h after isoflurane exposure ($P<0.001$) (Fig. 4A and B). Both MMP and MMP-9 concentrations were also higher in medium after isoflurane exposure compared with control (Fig. 4C and D).

Treatment of cells with an antibody to αIR3 abolished the increased cell cycle progression induced by isoflurane exposure (Fig. 5C). Up-regulation of Ki-67, angiopoietin, and VEGF was reversed by αIR3 treatment (Fig. 5A, B and C–G). In addition IGF-1R siRNA treatment of SK-OV3 before isoflurane exposure significantly decreased the expression of proliferative and angiogenic markers (Fig. 6).

**Discussion**

There are limited laboratory studies of the direct effects of specific anaesthetic agents on cancer cell biology, although clinical evidence suggests anaesthetics/techniques could potentially affect cancer progression after surgery. For example, Deegan and colleagues$^{15}$ showed that serum from breast cancer surgery patients who received propofol–paravertebral anaesthesia inhibited proliferation of one type of breast cancer cells in vitro compared with serum from patients receiving sevoflurane/opioid anaesthesia. In this study, the effects of a clinically relevant exposure of cancer cells to isoflurane were determined by monitoring the expression of tumorigenic markers of ovarian cancer. This study demonstrates, for the first time, that direct exposure to isoflurane influences the behaviour of ovarian cancer cells through up-regulation of markers associated with cell proliferation, angiogenesis, and metastasis.

Cancer is often defined by uncontrolled growth and the ability to continue replicating compared with normal cells where regulation is achieved by proteins which control the cell cycle, ensuring replicative accuracy.$^{16}$ The ability of...
cancer cells to down-regulate growth-inhibitory signals and sustain growth-promoting signals enables this increased replicative potential. The cyclins D and E promote the transition of cells from the G1 to the S phase of the cell cycle and over-expression accelerates the cell cycle, driving cell proliferation. Cyclin D expression has been shown to be predictive of poor survival.

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**Fig 4** (A) Representative image of SK-OV3 cell migration at 24 h and 48 h after isoflurane exposure using scratch assay. (B) Percentage healing (gap closure) after isoflurane exposure (n=8). Concentration of MMP-2 (C) and MMP-9 (D) in culture medium from SK-OV3 cells at 24 or 48 h after isoflurane exposure (n=4). Data are expressed as mean (SD). *P<0.05, **P<0.01 and ***P<0.001. N2, nitrogen; NC, naive control; Iso, isoflurane.

**Fig 5** (A) Representative image of immunostaining of Ki-67 (nuclei counterstained with DAPI) and (B) percentage of Ki-67+ SK-OV3 cells 24 h after isoflurane exposure. (C) Percentage of cells in S phase, 24 h after isoflurane exposure. Representative images of immunostaining of (D) angiopoietin and (E) VEGF (nuclei counterstained with DAPI) and fluorescence intensity of (F) angiopoietin and (G) VEGF in SK-OV3 cells 24 h after αR3-isoflurane treatment. Data are expressed as mean (SD) (n=4). *P<0.05, **P<0.01 and ***P<0.001. NC, naive control; Iso, isoflurane. Scale bars: 50 μm.
clinical outcome in ovarian cancer and cyclin E is up-regulated in ovarian cancer cells.\textsuperscript{18}

Ki67 is strictly associated with cell proliferation and present only in active cells and absent in resting cells at G0 of the cell cycle. It has a prognostic use in many cancer types including breast and prostate cancer\textsuperscript{19} and increased Ki67 expression is seen in higher grade ovarian tumours.\textsuperscript{20,21}

To sustain uncontrolled replication, blood vessel growth, or angiogenesis, is induced by tumour signalling.\textsuperscript{22} VEGF and angiopoietin-1 play a role in neovascularization of tumours, acting synergistically to meet the increased demands of the cancer cells.\textsuperscript{23} Both angiopoietin-1 and VEGF are up-regulated in ovarian cancer.\textsuperscript{24} VEGF also promotes ascites formation in ovarian cancer by increasing vascular permeability.\textsuperscript{25} MMP-2 and MMP-9 play a key role in invasion and migration by mediating degradation of the extracellular matrix and allowing tumour cells to spread into the surrounding areas. In this study, VEGF, angiopoietin-1 and MMPs were up-regulated after isoflurane exposure.

Our data also showed enhanced IGF signalling after isoflurane exposure. Expression of IGF-1 in ovarian cancer cells was strongly associated with the progression of ovarian cancer.\textsuperscript{26} We also found that inhibiting IGF using a neutralizing antibody or siRNA abolished the enhanced proliferation and angiogenesis seen after isoflurane exposure. In addition, it has been reported that isoflurane can up-regulate hypoxia-inducible factors (HIFs) in cancer and normal tissues.\textsuperscript{27,28} Interestingly, HIF-1 is the downstream transducer of IGF-1 and can interact with IGF-1 to increase downstream targets of transcription.\textsuperscript{29}

including VEGF, angiopoietin-1, transforming growth factor-alpha (TGF-\(\alpha\)), plus CXCR4 and E-cadherin, which promote adhesion and invasion.\textsuperscript{30} Indeed, it has been well documented that HIF-1\(\alpha\) is up-regulated in more aggressive forms of ovarian cancer.\textsuperscript{31,32} Taken together, it may conclude that isoflurane promotes ovarian cancer metastatic potential through the IGF1/HIF signalling pathway.

Our studies are clearly not without limitations. Cancer cell migration was assessed only using the scratch assay. More advanced assessments including in vitro live cell imaging of cancer cell invasion, which is relevant in the context of the changes in MMPs, a marker of cancer cell invasion potential, after isoflurane exposure are needed in future studies. For angiogenesis, VEGF is indeed a validated marker but others [e.g. the pro-angiogenesis factor RANTES (regulated on activation normal T cell expressed and secreted)]\textsuperscript{33} could be considered as further supporting evidence. Importantly, an in vivo study should be considered for future investigation to verify the in vitro findings. Nevertheless, the work reported here provides further understanding of the effect of anaesthetic specific type rather than technique on cancer cell biology.

Recent studies have indicated that the choice of anaesthetics could potentially influence patients’ prognosis after cancer surgery. Furthermore, Looney and colleagues\textsuperscript{34} showed that VEGF was increased in women undergoing surgery for primary breast cancer after general anaesthesia but not after propofol-paravertebral regional anaesthetic technique. Our data show that a commonly used inhalation

\textbf{Fig 6}  \(\text{(A) Representative image of immunostaining of Ki-67 (nuclei counterstained with DAPI) and (a) percentage of Ki-67 + SK-OV3 cells 24 h after isoflurane exposure. \(\text{(c) Percentage of cells in S phase, at 24 h after gas exposure. Representative images of immunostaining of (c) angiopoietin and (d) VEGF (nuclei counterstained with DAPI) and fluorescence intensity of (f) angiopoietin and (g) VEGF in SK-OV3 cells 24 h after siRNA-isoflurane treatment. Data are expressed as mean (SD) (n=4). *P<0.05 and **P<0.01. NC, naive control; Iso, isoflurane; SS, scrambled siRNA; IS, IGF-1R siRNA. Scale bars: 50 \mu \text{m.}\)\)
agent, isoflurane, is able to influence cancer cell behaviour and cellular signalling. The study of the potential impact of anaesthetics on cancer biology is just beginning and it is too early to draw the conclusion that volatile anaesthetics may affect cancer outcomes in patients. However, we have shown a possible link between volatile anaesthetics and cancer recurrence in vitro, which clearly warrants further pre-clinical and clinical studies.

**Authors’ contributions**

X.L., H.Z., L.H., and J.N. conducted the experiment and data analysis; J.L. and H.T. participated in the project; D.M. and H.Z. designed the experiment. All authors contributed to the preparation of the manuscript.

**Declaration of interest**

D.M. is a member of the Board of Management of the BJA.

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