

Differential effects of serum from patients administered distinct anaesthetic techniques on apoptosis in breast cancer cells *in vitro*: a pilot study

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Editor's key points

- Some data suggest that anaesthetic technique might affect cancer outcome.
- Serum was obtained from a subset of patients enrolled in a larger clinical trial who were randomized to receive propofol-paravertebral or sevoflurane-opioid based anaesthesia.
- Postoperative serum from the sevoflurane anaesthesia group reduced cancer cell apoptosis *in vitro*. These preliminary findings suggest that anaesthetic technique might affect cancer cell metastatic potential during cancer surgery.

Background. *In vitro* and retrospective clinical studies suggest an association between anaesthetic technique during primary breast cancer surgery and cancer outcome. Apoptosis is an important step in the mechanism of breast cancer metastasis, but whether it is influenced by anaesthetic technique is unknown. Using serum from breast cancer surgery patients randomized to receive distinct anaesthetic techniques, we investigated its effect on apoptosis in oestrogen receptor (ER)-negative breast cancer cells *in vitro*.

Methods. Women with biopsy-proven breast cancer were randomized to receive either propofol general anaesthesia with paravertebral analgesia (PPA) or standard sevoflurane general anaesthesia with opioid analgesia (SGA) in an ongoing, prospective clinical trial (NCT 00418457). Serum from a randomly selected subset of these patients (10 PPA and 10 SGA) who had donated 20 ml venous blood immediately before anaesthetic induction and at 1 h after operation was exposed to ER-negative MDA-MB-231 cells. Apoptosis was measured using ApoLive-Glo Multiplex Assay™.

Results. Exposure of MDA-MB-231 cells to postoperative serum of PPA patients resulted in higher luminescence ratio (apoptosis) than SGA patients, median (25–75%), 0.40 (0.35–0.43) compared with 0.22 (0.21–0.30), respectively ($P=0.001$). The luminescence ratio of postoperative serum from SGA was reduced compared with preoperative SGA 0.22 (0.21–0.30) compared with 0.3 (0.25–0.35) ($P=0.045$).

Conclusions. Serum from patients given sevoflurane anaesthesia and opioids for primary breast cancer surgery reduces apoptosis in ER-negative breast cancer cells to a greater extent than serum from patients given propofol-paravertebral anaesthesia. Anaesthetic technique might affect the serum milieu in a manner that impacts cancer cell apoptosis, and thereby tumour metastasis.

Keywords: anaesthesia, general; anaesthesia, paravertebral; anaesthesia, regional; apoptosis; breast cancer

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Breast cancer is one of the main causes of cancer-related death in women.¹ Associated morbidity and mortality relates mainly to metastatic disease and not the primary tumour. The process of metastasis is complex, with tumour cells requiring the ability to resist apoptosis, survive conditions of stress, seed, proliferate, and induce angiogenesis.²

A number of perioperative factors during primary breast cancer surgery, including anaesthetic technique, might influence whether minimal residual micrometastases are eliminated by the immune system or become full-blown metastatic disease.^{3–7} More than 50% of primary neoplasms

have defects in cellular apoptotic mechanisms and the process of apoptosis is a key regulator of cancer cell growth, including metastatic potential. Apoptosis is influenced by multiple factors, including immune cytokine signalling.^{8,9} In the perioperative period, apoptosis in minimal residual cancer (including micrometastatic deposits, shed tumour cells at the time of surgery, and circulating cancer cells) could plausibly be influenced by many factors, including perioperative immune suppression, the stress response to surgery, acute pain, and opioids. All of these factors can potentially be modified by anaesthetic technique.⁷ We have previously studied

serum from breast cancer patients as a marker of the overall effect of anaesthesia and surgery on patients' physiological status, and showed that serum from breast cancer patients differentially affected breast cancer cell functional biology.¹⁰

The expression of oestrogen receptors (ERs) in breast cancer cells is an important predictor of response to therapy and prognosis.¹¹ ER-negative breast cancer tends to be associated with more resistance to apoptosis and with poorer outcomes in the clinical context. Therefore, we investigated the potential effect of serum from breast cancer patients who had received different anaesthetic techniques on ER-negative breast cancer cell apoptosis *in vitro*. The primary endpoint was the effect of postoperative serum from breast cancer surgery patients who had received different anaesthetic techniques on apoptosis compared with the effect of serum taken before operation from the same patients.

Methods

Patient selection

After approval from the Ethics Committee of the Mater Misericordiae University Hospital and written informed consent, women undergoing surgery for biopsy-proven primary breast cancer were randomized into an international, multicentre, prospective clinical trial (NCT 00418457). In our centre only, patients were also consented to contribute a sample of venous blood before operation, and at 1 h after operation for this study of the effect of anaesthetic technique on cancer cell biology. After centrifugation at 400g, serum was divided into multiple aliquots and stored at -20°C . Inclusion criteria were women aged 18–85 yr undergoing mastectomy or wide local tumour excision with or without axillary node sampling or excision (i.e. believed to be tumour stages 1–3, nodes 0–2). Exclusion criteria were prior breast cancer surgery (except diagnostic biopsy); presence of inflammatory breast cancer; ASA physical status IV; any contraindication to paravertebral anaesthesia; or any general anaesthetic agent.

Randomization

Patients had been randomly assigned for the long-term follow-up clinical trial (NCT 00418457) from a web-based system that automatically recorded their study number and study group allocation. Patients received either combined propofol general anaesthesia with paravertebral analgesia (PPA) or standard sevoflurane general anaesthesia with opioid analgesia (SGA). From the patients enrolled to each arm of this study at our centre who had contributed serum samples, we randomly selected 10 patients from each arm for inclusion in the present study.

Anaesthetic technique

For patients who received PPA, a catheter was positioned in the ipsilateral paravertebral space at the level of the second thoracic vertebra using a standard technique. A 20 ml bolus of levobupivacaine 0.25% was administered before surgery. Total i.v. general anaesthesia was then commenced using a target-controlled infusion of propofol (DiprifuorTM). Fentanyl

$1-3\ \mu\text{g}\ \text{kg}^{-1}$ was administered at induction. Maintenance of the airway was through a laryngeal mask airway (LMA) with patients breathing spontaneously. Postoperative analgesia was a continuous infusion of levobupivacaine 0.25% at $5-10\ \text{ml}\ \text{h}^{-1}$ via paravertebral catheter. Paravertebral catheters were removed at 24 h. Rescue analgesia if needed was triggered by a visual analogue scale (VAS) pain score ≥ 3 , consisting of morphine $0.1\ \text{mg}\ \text{kg}^{-1}$ i.m. every 3–4 h as required.

In the SGA group, anaesthesia was induced with fentanyl $1-2\ \mu\text{g}\ \text{kg}^{-1}$ and propofol $1.5-2\ \text{mg}\ \text{kg}^{-1}$. Anaesthesia was maintained with sevoflurane (end-tidal concentrations 1–3%) in oxygen/air mixture. Intraoperatively, morphine $0.1-0.15\ \text{mg}\ \text{kg}^{-1}$ was given at the discretion of the anaesthetist. Patients received postoperative patient-controlled analgesia morphine, bolus 1 mg, lockout 6 min, and 4 h dose limit 30 mg. Paracetamol 1 g i.v. was given to all patients during surgery. Venous blood was sampled before and 1 h after surgery. Samples collected before surgery and 1 h after surgery were used for further analysis in this study.

Cell culture

The ER-negative MDA-MB-231 cell line from European Collection of Cell Cultures (ECACC) was used for analysis of cell viability and apoptosis. Cells were cultured in Leibovitz's L-15 medium to which 10% fetal bovine serum, L-glutamine, and 1% penicillin–streptomycin solution was added. Cells were incubated at 37°C in air with 5% CO_2 .

Cell viability and apoptosis assay

ApoLive-GloTM Multiplex Assay from Promega (Southampton, UK) was used to evaluate cell viability and apoptosis. Cells were cultured in L-15 Medium (Leibovitz) supplemented with 10% FBS, 1% penicillin–streptomycin solution, and L-glutamine at 37°C , with 5% CO_2 for 48 h. They were then harvested by trypsinization, resuspended in medium, and added to opaque, clear bottom 96-well plates at a density of 5000 cells per well. Culture plates were subsequently incubated in full medium for 24 h at 37°C to allow cell attachment. Thawed serum were diluted in medium to produce 10% serum concentrations as previously described.¹⁰ Serum was added in triplicate and culture plates incubated for a further 24 h. Viability Reagent (20 μl) was then added to the wells, and mixed by orbital shaking. Plates were incubated for 30 min at 37°C and fluorescence measured at $400_{\text{Ex}}/505_{\text{Em}}\ \text{nm}$ (viability). Caspase-Glo 3/7 reagent (100 μl) was then added and mixed briefly by orbital shaking (300–500 rpm for $\sim 30\ \text{s}$). Plates were then left for 30 min at room temperature. Luminescence was analysed using GloMax[®]-Multi Microplate Multimode Reader (Glomax, Promega, Southampton UK).

Statistical analysis

GraphPad Prism v 6 (GraphPad, San Diego, CA, USA) was used for analysis. For parametric continuous data, the unpaired *t*-test was used for comparisons between the groups regarding patient characteristic data. Differences in categorical data were tested using the Fisher exact test. Luminescence data were normalized to control luminescence without serum, and

expressed as a ratio of control luminescence. After confirming that luminescence ratio data were not normally distributed using the D'Agustino–Pearson omnibus normality test, these ratios were expressed as median (inter-quartile range) and compared using the Wilcoxon rank paired test for before–after differences between the groups. Being a pilot study, we did not prospectively evaluate a sample size to detect a specific change in apoptosis.

Results

All 20 patients (10 per group) randomly selected for this study completed it according to the protocol. The same anaesthetist and surgeon performed all procedures. Each paravertebral block was successful. None of the PPA patients received morphine in the postoperative period. The mean age and weight of specimen excised was slightly higher in the SGA group, but not statistically or clinically significant. Otherwise, patients in the PPA and SGA treatment groups were well-balanced regarding age, weight, surgical procedure, and cancer pathology (Table 1).

There was no significant difference in ER-negative breast cancer cell viability ratio between groups in both preoperative and postoperative serum samples (Fig. 1). There was, however, a significant difference in apoptosis between the two groups. Exposure to postoperative serum from SGA patients resulted in lower luminescence ratio (less apoptosis) than from PPA patients, median (25–75%), 0.22 (0.21–0.30) compared with 0.40 (0.35–0.43), respectively ($P=0.001$). The luminescence ratio for postoperative SGA serum was also reduced compared with preoperative SGA serum, 0.22 (0.21–0.30) compared with 0.3 (0.25–0.35) ($P=0.045$) (Fig. 2).

Discussion

We used serum from patients enrolled in a single-centre long-term clinical follow-up study to assess the effect of two different anaesthetic techniques on apoptosis in ER-negative breast

Table 1 Patient and breast cancer characteristics. Data are presented as mean (range), mean (sd), or number

	Sevoflurane/ opioid (n=10)	Propofol/ paravertebral (n=10)
Age (yr)	62.9 (43–79)	56.0 (33–73)
Weight specimen excised (g)	248 (90–690)	169 (70–460)
Mastectomy and axillary node clearance (n)	3	2
Wide local excision and sentinel node biopsy (n)	7	8
Oestrogen receptor positive (n)	10	10
Progesterone receptor positive (n)	10	10
HER2 positive (n)	0	0
Node positive (n)	2	6

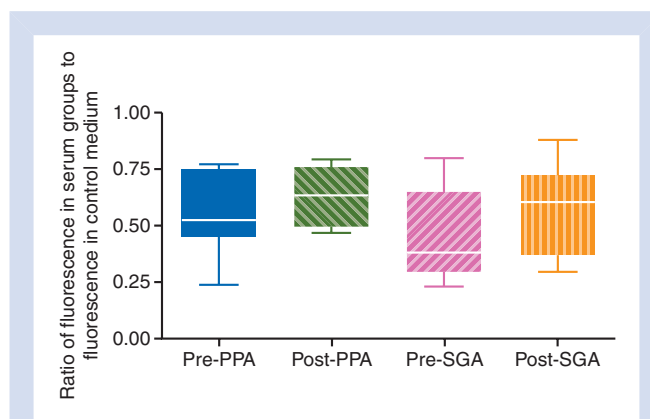


Fig 1 Effects of serum on breast cancer cell viability. No significant difference in the effect of serum from patients receiving the two anaesthetic techniques on cell viability ratio. Cell viability measured as fluorescence emitted relative to control. PPA, propofol/paravertebral anaesthesia group; SGA, sevoflurane general anaesthesia group.

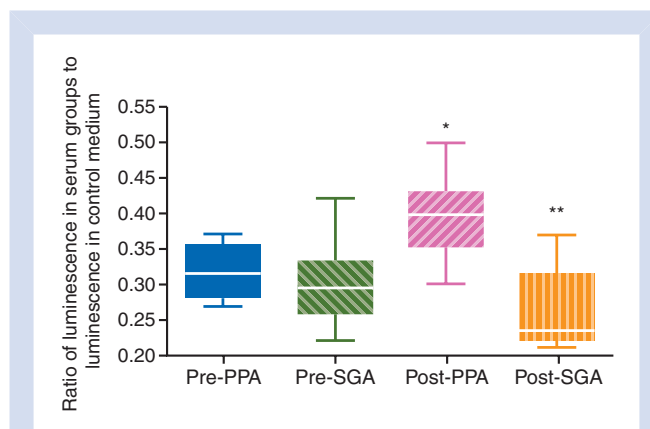


Fig 2 Effects of serum on breast cancer cell apoptosis. Decreased apoptosis in postoperative serum from the SGA group compared with the postoperative PPA group ($P=0.001$) compared with the preoperative SGA group ($P=0.04$). Apoptosis measured as luminescence relative to control.

cancer cells *in vitro*. Since all patients were enrolled and randomized as women with primary breast cancer, the observed differences can be attributed to differences in anaesthetic technique. Our main finding is that apoptosis of the human breast carcinoma cell line MDA-MB-231 was significantly reduced when cells were treated with postoperative serum from the SGA group compared with the PPA group. Breast cancer cell viability was similar in both groups.

Apoptosis is programmed cell death distinct from necrotic cell death. Apoptosis can be aberrant and therefore contribute to the metastatic process. Proteases known as caspases are central to apoptotic mechanisms. Whether the effects we observed are mediated by a caspase-3 mechanism is unknown. We chose the ER-negative MDA-MB-231 cell line for this study because it is an aggressive tumour cell, associated clinically with poorer prognosis compared with ER-positive breast

cancer cells. Previous work from our group suggests that the ER-positive breast cancer cell line MCF-7 does not express caspase-3, in contrast to ER-negative MDA MB-231 cells, which do express caspase-3 (data not shown). The Glomax assay we used for this study used caspase activation, in particular caspase-3 activation, as a marker for apoptosis.

Defective apoptosis is a known causative factor for development and progression of cancer.¹² Volatile anaesthetics apparently affect apoptotic mechanisms in different cell types. In normal myocardial cells, for example, volatile anaesthetics appear to protect against apoptotic cell death.¹³ In human colon cancer cells, isoflurane reduces the apoptotic effect of tumour necrosis factor via a mechanism involving caveolin-1.¹⁴ While volatile anaesthetics and opioids have been shown to impair immune function,¹⁵⁻¹⁸ they also impair apoptosis differently in other cell types, including T-cells and neonatal neuronal tissue.^{19, 20} However, evidence is contradictory regarding the effect of anaesthetic agents on the neuroblastoma cell line SH SY5Y, with some data showing increased apoptosis after exposure to volatile anaesthetic agents and others not.^{21, 22} Evidence is also conflicting regarding the effect of morphine on apoptosis in cultured human peripheral lymphocytes.^{23, 24} Propofol does not suppress natural killer (NK) immune cell activity, but it reduces apoptosis in normal hepatocytes²⁵ and renal cells.²⁶ Lidocaine, but not ropivacaine, has been shown to induce apoptosis in T-lymphocytes.^{27, 28}

Several factors in the perioperative period might promote metastasis of residual cancer after surgical resection of the primary tumour. Although surgery successfully de-bulks the primary tumour, this can inadvertently release tumour cells into the circulation and also reduce anti-angiogenic factors, which has tumour-promoting effects.³ Additionally, a large fraction of patients already harbour micrometastases at the time of surgery. These micrometastases often lie dormant for months to years and are known to have a three-fold higher incidence of apoptosis than the primary tumour cells.⁴ In addition, surgery *per se* induces a stress response,⁷ causing suppression of NK cell activity, which are important in host immune resistance to tumour development.²⁹

Anaesthesia *per se* might also promote metastasis.³⁰ Anaesthetics impair a number of immune functions, including neutrophil, macrophage, T-cell, and NK cell function. Opioids are known to inhibit human cellular and humoral immune function,¹⁸ and animal studies have demonstrated a dose-response effect with increasing immunosuppression resulting from greater doses of morphine.³¹ The literature is divided regarding the effect of opioids on tumour cell function. High-dose opioids (usually outside of the therapeutic range) appear to be tumour suppressive while lower doses, typical of those used perioperatively, appear to be tumour promoting.^{32, 33} In addition, morphine has been shown to be both pro-angiogenic³¹ and anti-angiogenic.³³ Postoperative pain suppresses cell-mediated immunity and enhances the tumour-promoting effects of surgery,³⁴ suggesting that optimum perioperative analgesia might enhance metastasis resistance in cancer patients having surgery.

Greater cancer cell apoptosis produced by postoperative serum from the PPA group compared with the SGA group could be attributable to an alteration in the molecular profile of the serum as a result of anaesthetic technique. We have previously shown that PPA compared with SGA for primary breast cancer surgery decreases serum concentrations of pro-tumorigenic cytokines and matrix metalloproteinases (IL-1 β , MMP-3, and MMP-9) and increases serum concentration of the anti-tumorigenic cytokine IL-10.³⁵ Separately, it has been shown that the PPA technique is associated with reduced angiogenesis-inducing factor VEGF compared with SGA.³⁶ It is unlikely that sevoflurane or opioids have a persistent effect at 1 h that is directly causing the difference in apoptosis we observed. Rather, it seems more plausible that these agents or those used in the PPA group induce changes in serum that indirectly affect cancer cells or immune cells. Clinical data from the ongoing prospective clinical trials will be the definitive evidence whether anaesthetic technique can influence cancer outcome.

In summary, serum from patients receiving a standard sevoflurane and opioid anaesthetic technique reduced apoptosis in MDA-MB-231 ER-negative breast adenocarcinoma cells compared with serum from patients receiving a propofol and paravertebral anaesthetic technique. These preliminary results suggest that anaesthetic technique might affect the serum milieu in breast cancer patients to influence metastasis potential.

Authors' contributions

A.I.J. contributed to design of the project, all experimental laboratory work, statistical analysis, and drafted and critically reviewed the manuscript. G.F. contributed to design of the project, assisted with experimental laboratory work, and critically reviewed the manuscript. H.C.G. contributed to design of the project, technical conduct of experimental laboratory work, and critically reviewed the manuscript. D.J.B. instigated the clinical trial from which patients' serum was derived, conceived the study design, provided funding, analysed the data, and substantially drafted the manuscript.

Declaration of interest

D.J.B. is a member of the editorial board of *British Journal of Anaesthesia*.

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