Effect of anaesthetic technique on the natural killer cell anti-tumour activity of serum from women undergoing breast cancer surgery: a pilot study

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Editor’s key points
- Anaesthetic technique might influence metastasis after cancer surgery.
- A pilot study compared the effects of serum from breast cancer resection patients receiving different anaesthetics.
- Serum from subjects receiving propofol–paravertebral block resulted in greater NK cell cytotoxicity than serum from subjects receiving sevoflurane/opioid.
- Clinical studies of the impact of anaesthetic technique on immune suppression and metastasis are indicated.

Background. Animal models and retrospective clinical data suggest that certain anaesthetic techniques can attenuate immunosuppression and minimize metastasis after cancer surgery. Natural killer (NK) T cells are a critical component of the anti-tumour immune response. We investigated the effect of serum from women undergoing primary breast cancer surgery, randomized to propofol–paravertebral block (PPA) or sevoflurane–opioid (GA) anaesthetic techniques, on healthy human donor NK cell function and cytotoxicity against oestrogen and progesterone receptor-positive breast cancer cells (HCC1500).

Methods. Ten subjects who donated serum before operation and 24 h after operation in an ongoing randomized prospective trial (NCT 0041857) were randomly selected. Serum from PPA (n=5) and GA (n=5) subjects was co-cultured with HCC1500 and healthy primary NK cells. Serum from PPA subjects did not alter normal NK marker expression or secretion of cytokines. Serum from GA subjects reduced NK cell activating receptor CD16 [from mean (SEM), 82 (2)% to 50 (4)%,

P = 0.001], IL-10 [from 1700 (80) to 1200 (92) pg ml−1, P = 0.001], and IL-1β [from 68 (12) to 19 (4) pg ml−1, P = 0.01]. An increase in NK cell CD107α [from mean (SEM), 23 (2)% to 37 (3)%,

P = 0.007] and apoptosis of HCC1500 [from 21 (2)% to 11 (1)%,

P = 0.0001] was observed with PPA serum, but not GA serum, treated NK cells.

Conclusion. Serum from women with breast cancer undergoing surgical excision who were randomized to receive a PPA anaesthetic technique led to greater human donor NK cell cytotoxicity in vitro compared with serum from women who received GA.

Clinical trial registration. NCT 041857.

Keywords: anaesthesia, general; anaesthesia regional; breast neoplasms; immune cells, natural killer cells; immunity, innate

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The perioperative period might be a critical time in primary cancer surgery because various factors can influence whether inadvertently displaced tumour cells become established metastases or are eliminated by the immune system. Modifiable factors that could influence perioperative immunosuppression are of ongoing research interest. Previous retrospective clinical studies of both breast and prostate cancer surgery have suggested an association between anaesthetic technique and cancer recurrence. However, other retrospective trials on various forms of cancer have shown no such benefit. Definitive evidence of a causal link is awaited from an ongoing prospective trial (NCT 0041857). Animal studies using an inoculation model of breast cancer have suggested a potential protective effect of certain anaesthetic drugs and techniques against metastatic spread in cancer surgery, especially regional anaesthetic techniques and propofol. The mechanism underpinning any benefit of these techniques remains unclear but preservation of innate immune function and a direct anti-inflammatory effect of local anaesthetics or other anaesthetic drugs have been hypothesized.

Natural killer (NK) cells are CD3− CD56+ lymphocytes and play an important role in the innate immune response against...
tumour cells. Their main function is recognition and killing of virus infected cells or tumour cells. Patients with low levels of NK cell activity are predisposed to tumour progression.21

Our group has previously used patient serum as a tool to evaluate the effect of anaesthetic technique on cancer cell biology. Serum was obtained from breast cancer patients randomized in an ongoing prospective trial to receive two distinct anaesthetic techniques, and its effects on breast cancer cell biology in vitro have been evaluated.18 19

The potential effect of serum from patients who received different anaesthetic techniques on healthy human NK cells in vitro has not been investigated. In this pilot study, we examined the effect of serum from subjects randomized in the ongoing clinical trial on anti-tumour activity of healthy human NK cells towards an oestrogen and progesterone receptor-positive breast cancer cell line (HCC1500).

Methods
Clinical protocol
Primary breast cancer surgery patients aged 18–85 yr were randomized in an ongoing trial (NCT 00418457) to either propofol and paravertebral block (PPA) or sevoflurane and opioid (GA) anaesthetic techniques, using a secure web-based randomization system according to a table of random numbers. Additional consent was obtained retrospectively by telephone contact from subjects randomly selected to participate in this add on serum study. Exclusion criteria were inflammatory breast cancer, prior breast cancer surgery, other cancer, ASA status ≥IV, contra indications to paravertebral block or midazolam, propofol, sevoflurane, fentanyl, or morphine. Written informed consent and Research Ethics Committee approval were obtained. In the PPA group, a paravertebral catheter was placed using a landmark technique and 20 ml of 0.25% levobupivacaine was administered. Induction and maintenance of anaesthesia was with propofol target-controlled infusion to 4 μg ml−1 and oxygen/air. Paravertebral catheter infusion of 8–10 ml h−1 0.25% levobupivacaine was continued for 24 h after operation. Rescue analgesia, if indicated, was given for verbal pain response score >3 and consisted of a paravertebral bolus 0.25% levobupivacaine 10 ml. If this did not alleviate pain in <15 min, morphine 0.1 mg kg−1 i.v. was administered.

The GA group received 1–3 μg kg−1 fentanyl, 2–4 mg kg−1 propofol, and maintenance with sevoflurane and oxygen/air to maintain arterial pressure and heart rate within 20% of preoperative baseline values. I.V. morphine was titrated to a respiratory rate of 12–14 bpm. Postoperative analgesia was provided as patient-controlled analgesia morphine or bolus i.m. morphine 0.1 mg kg−1 for 24 h. Blood samples were obtained from all patients before operation and 24 h after operation, centrifuged, and stored at −80 °C. Serum from PPA (n=5) and GA subjects (n=5) was randomly selected from among >100 patients already randomized to the long-term clinical trial.

Buffy coat isolation
Healthy donor blood was supplied by the Irish Blood Transfusion Service, St James Hospital, Dublin, Ireland. This was treated with 5% ethylenediaminetetraacetic acid and diluted (1:2) with hank’s balanced salt solution (HBSS) (Bio-Sciences, Oxford, UK) supplemented with 1% fetal calf serum (FCS) and 10 mM hydroxyethyl piperazineethanesulfonic acid (HEPES). Treated blood was layered over the density gradient medium Lymphoprep (STEMCELL Technologies, Grenoble, France) and centrifuged at 400 g (Hettich Universal 320R, DJB Labcare Ltd, Buckinghamshire, UK) for 25 min. The interface cells were collected and washed once in HBSS and suspended in RPMI 1640 medium (Bio-Sciences) supplemented with 10% FCS, 10 mM HEPES, 2 mM l-glutamate, 100 U penicillin ml−1, and 100 μg streptomycin ml−1.

NK cell isolation
CD56+ cells were purified from peripheral blood mononuclear cells, using magnetic-activated cell sorting Anti-CD56 conjugated microbeads (Miltenyi Biotec, Surrey, UK) according to the manufacturer’s protocol. Flow cytometry routinely determined a purity of >95% for NK cells.

Tumour cell culture
The human oestrogen, and progesterone receptor-positive primary breast cancer cell line HCC1500, was cultured in ATCC-formulated RPMI-1640 Medium supplemented with 10% FCS. Media were renewed every 3–5 days and subcultivation was performed using the trypsin method.

Cell co-culture in serum samples
RPMI-1640 medium was supplemented with 10% of sample serums. Ten per cent of serum concentration was chosen as this was shown to be the optimum serum concentration for in vitro cell culture.18 NK and human cancer cells (HCCs) were resuspended in this supplemented medium before coculture at a ratio of 1:10 for 24 h.

Enzyme-linked immunosorbent assay
Supernatants were retrieved after 24 h co-incubation with healthy CD 56 NK cells and HCC1500 cell line. ELISA kits (R&D Systems, Abingdon, UK) were used to quantify levels of IL-8, IL-10, IL-2, IFN, and IL-1β according to the manufacturer’s protocol.

NK cell receptor and CD107a expression
Activating receptor expression was determined by flow cytometry. After 24-h culture, NK cells were stained with fluorescence conjugated antibodies for the detection of CD56, Nkp30, Nkp44, Nkp46, 2b4, CD16, or NKG2D expression. NK cells expressing each particular receptor were analysed by FACSCalibur™ Flow Cytometer and CellQuest software (BD Bio-sciences, Oxford, UK). CD107a expression was for NK cells cultured alone or co-cultured with HCC cells in subject serum-supplemented medium.
Apoptosis assay
HCC cells were cultured either alone or with NK cells in medium supplemented with subject serum. After 24 h, cells were stained with FITC-Annexin V and propidium iodide using FITC-Annexin V Apoptosis Detection Kit 1 (BD Pharmingen, Oxford, UK) according to the manufacturer’s protocol with analysis by flow cytometry.

Statistical analysis
Normally distributed data were analysed using parametric tests and non-parametric tests were applied to data not normally distributed. There were 10 serum samples (n=5 each anaesthetic technique), and three healthy donor NK sets, with each experiment carried out in triplicate. Statistics were performed using GraphPad Prism™ V5 for windows (GraphPad Software, San Diego, CA, USA). Data were compared using unpaired t tests for parametric data and the Mann-Whitney U-test for non-parametric data. Data are expressed as mean (SEM).

Results
Ten subjects were randomly selected as a subgroup of an ongoing prospective randomized trial (NCT 00418457). Both groups were balanced regarding weight, height, oestrogen and progesterone receptor status, and ASA physical status (Table 1). Two subjects in GA and three in PPA had node positivity. All subjects underwent wide local excision and axillary clearance apart from one subject who had mastectomy PPA. One subject in PPA received 5 mg morphine as rescue analgesia clear ance after operation in IL-10 [1700 (80) to 1200 (92) pg ml⁻¹, P=0.001] expression in NK cells treated with GA serum. No significant reduction was seen in IL-8 [260 (8) to 240 (9) pg ml⁻¹, P=0.1 PPA; 260 (10) to 250 (8) pg ml⁻¹, P=0.6 GA], IFN-γ [1910 (88) to 2040 (130) pg ml⁻¹, P=0.2 PPA; 2010 (133) to 2080 (102) pg ml⁻¹, P=0.5 GA], and IL-2 [560 (40) to 580 (54) pg ml⁻¹, P=0.7 PPA; 590 (86) to 550 (56) pg ml⁻¹, P=0.7 GA] expression with either anaesthetic technique (Supplementary Fig. S1).

PPA serum maintained normal NK cell CD16 activating receptor expression [mean (SEM) preoperative to postoperative; 87 (1)% to 85 (2)%]. However, there was a significant reduction after operation in CD16 expression [82 (2)% to 50 (4)%]. P=0.001] in GA serum-treated NK cells (Fig. 1a and a). No reduction was seen in NKP30 [35 (1)% to 33 (2)%], P=0.6 PPA; 40 (2)% to 40 (2)%), P=0.8 GA], NKP44 [35 (2)% to 36 (2)%), P=0.7 PPA; 37 (1)% to 32 (2)%), P=0.06 GA], NKP46 [47 (4)% to 48 (4)%), P=0.5 PPA; 51 (5)% to 49 (2)%), P=0.4 GA], 2b4 [31 (1)% to 29 (1)%), P=0.4 PPA; 30 (1)% to 34 (1)%), P=0.01 GA], or NKG2D marker [47 (4)% to 45 (4)%), P=0.4 PPA; 43 (4)% to 41 (3)%), P=0.4 GA] expression following either anaesthetic technique (Supplementary Fig. S2).

Serum from subjects receiving PPA caused an increase in NK cell expression of CD107a when co-incubated with HCC tumour cells [23 (2)% to 37 (3)%], P=0.007]. GA serum did not increase the percentage of NK cells expressing CD107a when exposed to HCC cells [15 (1)% to 17 (2)%], P=0.3] (Fig. 2a and a). This effect was reproduced against three different healthy NK donors.

NK cells conditioned with serum from PPA subjects exhibited strong cytotoxicity by inducing apoptosis in HCC cells [HCC; 11 (1)% vs 21 (2)%], P=0.0001]. However, NK cells cultured in GA serum displayed a marked impairment in their ability to induce apoptosis in HCC cells [12 (2)% to 12 (2)%], P=0.9] (Fig. 3A and a). This effect was reproduced across three different healthy NK cell donors.

Table 1 Subject characteristics and breast cancer staging. Data presented as median (interquartile range) or number (%)

<table>
<thead>
<tr>
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<th>GA (sevoflurane–opioid)</th>
<th>PPA (propofol–paravertebral block)</th>
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<tbody>
<tr>
<td>Height (cm)</td>
<td>155 (152–162)</td>
<td>165 (159–174)</td>
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<tr>
<td>Weight (kg)</td>
<td>69 (52–73.6)</td>
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<tr>
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<tr>
<td>ASA II</td>
<td>2 (40%)</td>
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<tr>
<td>Node positive</td>
<td>2 (40%)</td>
<td>3 (60%)</td>
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<tr>
<td>Oestrogen receptor positive</td>
<td>5 (100%)</td>
<td>5 (100%)</td>
</tr>
<tr>
<td>Progesterone receptor positive</td>
<td>5 (100%)</td>
<td>5 (100%)</td>
</tr>
<tr>
<td>HER positive</td>
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<tr>
<td>Morphine (mg)</td>
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<td>0 (0–2.5)</td>
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Discussion
Serum from women with breast cancer who had been randomized to receive propofol–paravertebral anaesthesia for their primary breast cancer surgery maintained healthy donor NK anti-tumour cell activity compared with serum from women receiving a sevoflurane–opioid anaesthetic technique.

In the perioperative period, a brief profound inflammatory stress response occurs that is conducive to tumour progression through suppression of cell-mediated immunity.20 21 The stress response is characterized by sympathetic nervous system mediated β-adrenergic stimulation with release of catecholamines and prostaglandins and activation of the hypothalamic–pituitary axis with glucocorticoid production. A reduction in number of NK cells occurs and the ratio of TH1/TH2 cells is shifted towards pro-inflammatory TH2 development with an increase in plasma concentrations of TH2 cytokines.20

Laparotomy increases animal corticosterone levels with a simultaneous decrease in NK cytotoxicity, whereas dexamethasone reduces NK cell cytotoxicity and expression of cytolytic enzyme granzyme A.21 22 Cytokines IL-1β, IL-6, IL-8,
and IL-12 are down-regulated by glucocorticoids with up-regulation of IL-10 and the TH2 response. NK cells express β adrenergic receptors. Upon activation, an increase in cAMP and protein kinase A results in decreased NK cytotoxicity. This effect is prevented by β adrenergic receptor block. Suppression of NK cell cytotoxicity was demonstrated in rats undergoing laparotomy through splenic nerve sympathetic activation via brain opioid receptor activation. This effect was antagonized by naloxone. Opioids have conflicting immunomodulatory effects. Fentanyl depressed NK cell function in a rat laparotomy model, while tramadol prevented metastasis in animal surgery and increased postoperative NK activity in cancer patients. Pain also suppresses cell-mediated immunity. This effect is attenuated by opioid analgesia in animal pain models. The increased cytotoxic response of PPA-treated NK cells exposed to tumour cells in our study, which was not seen in the GA group, could result from an immunomodulatory effect of opioids or from reduced pain with the use of regional anaesthesia.

Anaesthetic agents including ketamine, thiopental, and volatile agents reduce NK cell activity and increase breast cancer metastasis in animal models, while propofol did not suppress NK cell cytotoxicity. Propofol suppresses tumour growth in a mouse model with preservation of in vitro cytotoxic T lymphocyte activity. Propofol also reduces PGE2 production in vitro, which is a known mediator of pain and inflammation that inhibits NK cell cytotoxicity through EP2 and EP4 receptors. This attribute of propofol compared with volatile agents is consistent with the increased cytotoxicity of PPA serum-treated NK cells noted in our pilot study.

Regional anaesthesia through afferent sensory and efferent sympathetic block attenuates the stress response to surgery. Spinal anaesthesia preserves NK cell function and TH1/TH2 balance and might also reduce opioid and intraoperative volatile requirements. In animal studies, spinal anaesthesia is associated with preserved NK cell function and reduced lung metastasis after laparotomy. However, a meta-analysis found no association between regional anaesthesia and NK cell function, but was limited by number and heterogeneity of existing studies. Our findings build upon the scarce existing literature evaluating potential mechanisms for effects of anaesthetic technique on metastasis in primary cancer surgery.

We observed decreased IL-10 expression in GA serum-treated NK cells. IL-10 is produced by Type 2 helper T cells and is involved in inhibition of pro-inflammatory cytokines and down-regulation of cell-mediated immunity. Previous in vitro studies of breast and ovarian cancer found increases in serum IL-10 with use of regional anaesthetic techniques. IL-10 inhibits tumour metastasis through an NK-dependent mechanism. Breast cancer surgery patients receiving sevoflurane and opioid displayed significantly lower serum IL10/IFN-γ ratio than those receiving propofol and paravertebral. This is in keeping with our findings; however, our study was unique in that we measured cytokines from supernatants retrieved after co-incubation of NK cells and HCC1500 tumour...
cells in serum rather than directly from patient serum in order to look at the effect of NK cell-related cytokine production.

We found a decrease in the IL-1β expression by GA serum-treated NK cells. IL-1β is a mediator of the acute phase response resulting in elevated prostaglandin levels, tachycardia, and accelerated catabolism. It has been shown to facilitate nociception during neuropathic and inflammatory pain. IL-1β stimulates T and B cells, prostaglandin production, myelopoiesis, and adrenocorticotrophic hormone (ACTH) release. Laparoscopy compared with open laparotomy recto-sigmoid carcinoma excision resulted in significantly lower elevation in IL-1β. A study of breast cancer patients’ serum found reduced serum IL-1β after operation with PPA anaesthesia compared with GA. Another clinical study of ovarian cancer showed attenuated increase in serum IL-1β with a regional anaesthetic technique. However, sedation with propofol-ketamine did not modify plasma concentrations of IL-1β, IL-2, IL-6, IL-10, IL-8, IL-12p70, or IFN-γ or the T helper cell 1/2 ratio in children with leukaemia undergoing bone marrow aspiration. Isoflurane suppressed glial cell IL-1β up-regulation and plasma ACTH but not plasma IL-1β in intraperitoneal lipopolysaccharide inoculated mice. Our findings of reduced IL-1β expression by the GA group differs from those of previous studies; however, results might vary between studies depending on stimulation methods and cell type.

NK cells express activating receptors that are involved in tumour lysis and control of metastatic spread, including Nkp30, Nkp44, Nkp46, 2b4, CD16, and NKG2D. Nkp30, Nkp44, and Nkp46 are natural cytotoxicity receptors expressed mainly on NK cells. CD16 is an important NK activating receptor involved in direct tumour cell lysis, antibody-dependent cellular cytotoxicity, cytokine production, and post-activation NK cell apoptotic death. When NK cells become cytotoxic, they degranulate and express the surface protein CD 107a. The expression of this protein correlates with target tumour cell death. The reduction in CD16 and failure to increase CD107a NK receptor expression in response to HCC in GA serum correlated with functional changes in NK cell cytotoxicity seen through the effects of the GA serum on HCC1500 apoptosis.

Caution should be exercised in interpreting these data and extrapolating implications to the clinical scenario. Healthy NK cells from donors could exhibit different immunological behaviour compared with NK cells of women with breast cancer. The scope of cytokine function and interactions is large with alterations occurring in inflammation, pain, surgery, and trauma.
which might behave differently in vitro. NK cells play an important role in the cancer immune response. The findings of our study are in keeping with the hypothesis that regional anaesthesia and avoidance of volatile agents and opioids could contribute to reduced perioperative residual disease. Further studies to examine the influence of anaesthetic technique on NK cells from cancer patients are warranted.

Authors’ contributions

Supplementary material
Supplementary material is available at British Journal of Anaesthesia online.

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Declaration of interest
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