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Combined Spinal and General Anesthesia Attenuates Liver Metastasis by Preserving Th1/Th2 Cytokine Balance

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Background: **Many studies have shown that regional anesthesia improves postoperative outcome and particularly lessens infection by attenuating perioperative immunosuppression related to the stress response to surgery and general anesthesia. However, it remains to be determined whether regional anesthesia improves oncologic outcome after surgery.**

Methods: **C57BL/6 mice were subjected to laparotomy during sevoflurane general anesthesia alone or combined with spinal block achieved with bupivacaine (5 μg) and morphine (1.25 μg). Control groups were anesthetized only or were untreated. Liver was removed 5 h after surgery to assess antitumor killer cell activity and production of interferon γ and interleukin 4 by liver mononuclear cells, or mice were inoculated intravenously with liver-metastatic EL4 cells and hepatic metastases were counted 12 days later.**

Results: **Laparotomy during sevoflurane anesthesia significantly increased the number (± SD) of liver metastases from 15.5 ± 8.7 (control) and 19.4 ± 5.4 (sevoflurane alone) to 33.7 ± 8.9. Sevoflurane anesthesia plus spinal block significantly reduced this increase to 19.8 ± 9. The *in vitro* killer activity of liver mononuclear cells against EL4 cells decreased from 32.7% (control) and 29.4% (sevoflurane alone) to 18.5% after sevoflurane plus laparotomy, and the addition of spinal block increased activity to 26.6%. The interferon-γ/interleukin-4 ratio decreased from 89.3 (control) and 95.7 (anesthesia alone) to 15.7 after sevoflurane plus laparotomy, and the addition of spinal block increased the ratio to 46.5.**

Conclusions: **The addition of spinal block to sevoflurane general anesthesia accompanying surgery attenuates the suppression of tumoricidal function of liver mononuclear cells, presumably by preserving the T helper 1/T helper 2 (Th1/Th2) balance, and thereby reduces the promotion of tumor metastasis.**

SUPPRESSION of the postoperative immune response may affect infection rate and the rate and size of tumor metastases disseminated during surgery.[1] Surgery and the resultant stress response have been shown to lead to depression of natural killer (NK) cell function in the spleen and peripheral blood, which is associated with increased tumor growth and metastasis in animal models.[2–5] NK cells are considered to be major effector cells that are active before the induction of adaptive immunity by T cells in the early stages of tumor growth.[6,7] Although this immunosuppression is a result of many factors,[8,9] including surgical stress[1,10,11] and anesthesia,[12,13] it has been shown to be dependent on the severity of surgery in clinical[14,15] and animal[4,16,17] studies.

Regional anesthesia, including epidural and spinal block, has been shown to reduce the excessive stress response after surgery,[18,19] which is thought to be one of the mediators of postoperative immunosuppression.[20,21] However, there are no clinical studies evaluating the effect of choice of anesthesia and analgesia on outcome after oncologic surgery, mainly because of ethical limitations.[20,22] In 2001, Bar-Yosef *et al.*[23] reported that the addition of spinal blockade to general halothane anesthesia markedly attenuates the promotion of pulmonary metastasis after surgery in Fischer-344 rats. However, peripheral blood NK activity was reduced to a similar degree by general anesthesia alone and by surgery; it was further reduced by the addition of spinal block, despite its favorable effect on metastatic development.[23] A number of factors, including pulmonary NK activity, may account for this discordance.[24] In their model, pulmonary NK activity may be of greater importance than that in peripheral blood because the metastases are formed in the lungs.[23]

Liver mononuclear cells (MNCs) include NK cells and natural killer T (NKT) cells,[25–28] major effectors against hepatic metastasis, and may migrate and inhibit pulmonary metastases.[25,29] Antitumor cytotoxicity of NK cells and NKT cells, accompanied by interferon γ (IFN-γ) produced by these cells, plays major roles against hepatic metastasis.[25,29,30] Surgical stress depresses the cytotoxic activity of liver MNCs and enhances the growth of metastatic liver tumors.[17] Liver MNC function may be more susceptible to the influence of surgical stress than peripheral blood mononuclear cell function.[17] We hypothesized that regional anesthesia maintains the function of liver MNCs by attenuating alterations in the T helper 1 (Th1)/T helper 2 (Th2) balance in response to surgery, thereby reducing the promotion of metastasis after surgery. IFN-γ, a Th1 cytokine, is known to be involved in increasing the cytotoxic activities of T cells and NK cells.[31] In contrast, interleukin 4 (IL-4), a Th2 cytokine, is reported to be involved in increasing humoral immunity and in suppressing Th1 response.[31]

We investigated the involvement of liver MNC function in the protective effect of adding spinal anesthesia to general anesthesia on surgery-induced acceleration of

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hepatic metastasis in mice. We examined (1) the numbers of EL4 tumor cell metastases in the liver, (2) killer activity of liver MNCs against tumors, and (3) IFN-γ and IL-4 production in liver NKT cells stimulated with the synthetic ligand α-galactosylceramide (α-GalCer) as markers of the Th1/Th2 balance.[32–35]

Materials and Methods

This study was conducted in accordance with the guidelines of the Institutional Review Board for the Care of Animal Subjects at the National Defense Medical College, Tokorozawa, Japan.

Animals

Male C57BL/6 mice (20–25 g; Charles River Inc., Yokohama, Japan), aged 11–13 weeks, were maintained and fed under specific pathogen-free conditions according to a 12:12-h lighting regimen. All experiments were conducted during the first half of the light phase. Mice were housed four per cage with free access to food and water.

Study Design and Procedure

In a preliminary study, we evaluated the duration and strength of analgesia under each drug administration. We then conducted four experiments as described below. Mice were assigned to one of six groups: control, sevoflurane general anesthesia, sevoflurane plus spinal block, laparotomy conducted with sevoflurane anesthesia, laparotomy conducted with sevoflurane plus spinal block, and laparotomy conducted with sevoflurane plus systemic morphine.

In the first experiment, mice were injected intravenously with EL4 cells 5 h after the end of anesthesia. Twelve days later, livers were removed and the number of metastases was counted.

In the second experiment, after depletion of NKT cells or depletion of both NK cells and NKT cells, mice were injected intravenously with EL4 cells 5 h after the end of anesthesia. Twelve days later, livers were removed and the number of metastases was counted.

In the third experiment, livers were removed 5 h after the end of anesthesia, and MNCs were prepared to assess their activity.

In the fourth experiment, livers were removed 5 h after the end of anesthesia, and MNCs were prepared to measure IFN-γ and IL-4 production.

These experiments were conducted several times. Each set of experiments included mice from all experimental groups or from subgroups.

General Anesthesia, Spinal Anesthesia, and Morphine Administration

General anesthesia was induced with sevoflurane and maintained at 1.5–2.5% sevoflurane in room air *via* a vaporizer. Mice breathed spontaneously throughout the anesthesia, and the sevoflurane concentration was adjusted according to the respiratory pattern and heart rate.

The spinal injection technique was adapted from a previously described method.[36] After mice were gently held, a 30-gauge, 0.5-in stainless-steel needle attached to a 10-μl microsyringe was inserted between the L5 and L6 or the L4 and L5 vertebrae without general anesthesia. Tail or hind leg movement was used to confirm penetration into the spinal canal, and 5 μl bupivacaine,[36] 0.5%, containing 1.25 μg morphine sulfate was injected. This drug regimen was determined on the basis of the results of the preliminary experiment. Sevoflurane general anesthesia was induced 10 min after spinal injection. In experimental groups without spinal injection, mice were gently held for 15 s to simulate spinal puncture.

Morphine sulfate was prepared at a concentration of 1 mg/ml in saline and injected intraperitoneally at a dose of 5 mg/kg with a 30-gauge needle. After injection was performed during sevoflurane anesthesia, mice awoke; 10 min later, sevoflurane general anesthesia was reinduced for lower abdominal incision.

Evaluation of Analgesia

To evaluate the analgesic effect of spinal block and systemic morphine, we conducted a preliminary experiment. Analgesia was evaluated by the tail-flick (TF) test. The light beam from a projector bulb was focused on the middle portion of the tail to produce a spot of intense heat, and the TF latency was measured. The intensity of the thermal stimulus was adjusted so that the baseline TF latency was 1.0–2.5 s. A 10-s cutoff time was imposed to minimize tissue damage. In all experiments, the TF latency was determined three times for each mouse. Measurements were obtained before intervention and at 10, 30, 60, 120, 180, 240, 300, and 360 min after awaking from anesthesia. We tested five regimens of spinal block (25 μg bupivacaine alone, 25 μg bupivacaine plus 0.5 μg morphine, 25 μg bupivacaine plus 1.25 μg morphine, 25 μg bupivacaine plus 2.5 μg morphine, and 25 μg bupivacaine plus 5 μg morphine) and systemic injection of morphine (5 mg/kg intraperitoneally[23]). We also tested two control conditions: no intervention and intrathecal injection of 5 μl saline. Three mice were used for each test. Each mouse received a single injection. To aid in the evaluation of analgesic effect, motor function was assessed on the muscle tone of the lower limbs and abdomen.

Laparotomy

After induction of general anesthesia by sevoflurane, hair was trimmed, the incision area was cleaned with 70% alcohol, and a 12-mm lower midabdominal incision was made. The abdomen was left open for 5 min and

was closed in a single layer with 3-0 polyglactin sutures. Mice awoke 3–5 min after suturing.

EL4 Tumor Cell Line and Injection of Tumor Cells

EL4 T-cell lymphoma cells, which are of B6 origin and are highly metastatic to the liver, were used for metastasis experiments. EL4 T-cell lymphoma cells were propagated in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, and 25 mM $NaHCO_3$, in a 95%-humidified atmosphere containing 5% CO_2 at 37°C. Cell cultures were washed three times with phosphate-buffered saline and were suspended at 1×10^5 cells/0.2 ml phosphate-buffered saline. Aliquots (0.2 ml) of tumor cell suspension were injected into mice through the tail vein 5 h after anesthesia.

Counting of EL4 Cell Metastases in the Liver

Mice were killed 12 days after EL4 tumor cell injection. Livers were removed and fixed in Bouin solution to facilitate visualization of tumor colonies for quantification.[23,37] After livers were washed in ethanol, visible tumor colonies on the surface of livers were counted.

In Vivo *Cell Depletion*

Anti-NK1.1 antibody treatment depletes both NK cells and $NK1^+$ intermediate $\alpha\beta$ T cell receptor cells (NKT cells), and anti-asialo GM1 (anti-AGM1) antibody treatment depletes NK cells alone.[38,39] Mice were injected through the tail vein with 200 μg anti-NK1.1 antibody or 50 μg anti-AGM1 antibody. This antibody treatment depletes the respective cell populations for at least 5 days. For tumor metastasis experiments, mice were injected with antibodies 3 days before tumor inoculation and after tumor inoculation on day 0, day 3, and day 9.[37]

Preparation of Liver MNCs

During sevoflurane anesthesia, mice were bled from the subclavian artery and vein. Livers were then removed. Hepatic lymphocytes were prepared as described previously.[39] Briefly, the liver was passed through a stainless-steel mesh and suspended in RPMI 1640 medium containing 5% FBS. After one washing, cells were resuspended in osmolarity- and pH-adjusted 33% Percoll containing 100 U/ml heparin and were centrifuged at 2,200 rpm for 20 min at room temperature. Cell pellets were resuspended in RBC lysis solution (0.17 mM NH_4Cl, 0.01 mM EDTA, 0.1 M Tris, pH 7.3) and then washed twice in RPMI 1640 plus 10% FBS. Cells were counted, and viability was determined by trypan blue exclusion. Prepared liver MNCs were used in cytotoxicity assays and cytokine measurements.

Liver MNC Cytotoxicity Assays

Cytotoxicity of liver MNCs against EL4 lymphoma cells was measured. Each target cell group (2×10^6 cells) was labeled with 200 μCi $Na_2(^{51}Cr)O_4$ for 60 min at 37°C in RPMI 1640 plus 10% FBS, washed three times with medium, and subjected to cytotoxicity assay. Labeled target cells (10^4/well) were incubated in a total volume of 200 μl with effector cells in RPMI 1640 plus 10% FBS in 96-well, round-bottomed microtiter plates. Effector-to-target (E:T) cell ratios were 80:1, 40:1, and 20:1. The plates were centrifuged after incubation for 8 h, and the supernatant was harvested and counted with a gamma counter. Cytotoxicity was calculated as the percentage of releasable counts after the subtraction of spontaneous release, according to the following formula: cytotoxicity (%) = experimental release − spontaneous release/maximum release − spontaneous release × 100. Spontaneous release was less than 15% of maximum release.

Assays for IFN-γ and IL-4 Production

Before the production of IFN-γ and IL-4 was measured, liver MNCs (5×10^5 cells) were suspended in a total volume of 200 μl in RPMI 1640 plus 10% FBS and stimulated with 100 ng/ml α-GalCer, a synthetic ligand specific for NKT cells and known to induce NKT cells to produce IFN-γ and IL-4.[32-35] After liver MNCs were cultured in 5% CO_2 at 37°C in 96-well, round-bottomed plates for 24 h, supernatants were harvested and stored at −80°C for enzyme-linked immunosorbent assay. Cytokines (IFN-γ and IL-4) were measured in duplicate by enzyme-linked immunosorbent assay according to the manufacturer's instructions (Endogen, Woburn, MA).

Statistical Analysis

Analysis of variance was used to analyze metastases and cytokine production. Cytotoxicity was analyzed by repeated-measures analysis of variance (different E:T ratios as the repeated measures). *Post hoc* Bonferroni procedures were conducted as appropriate, correcting for multiple comparisons. Probability values of $P < 0.05$ were considered significant. The results are expressed as mean ± SD unless stated otherwise. We used Graphpad Prism 4.00® software (Graphpad Software, Inc., San Diego, CA) for analysis.

Results

Preliminary Experiment: Evaluation of Analgesia

Systemic injection of 100 μg (5 mg/kg) morphine showed analgesia beginning 10 min after injection and lasting for 180–240 min (fig. 1). Spinal injection of 25 μg bupivacaine plus 1.25 μg morphine showed analgesia beginning 10 min after injection and lasting for 180–240 min. Spinal injection of bupivacaine alone or with a lower dose of morphine showed shorter duration of analgesia. Spinal injection of bupivacaine with higher doses of morphine showed longer duration of analgesia (fig. 1). However, it also showed slight respiratory failure

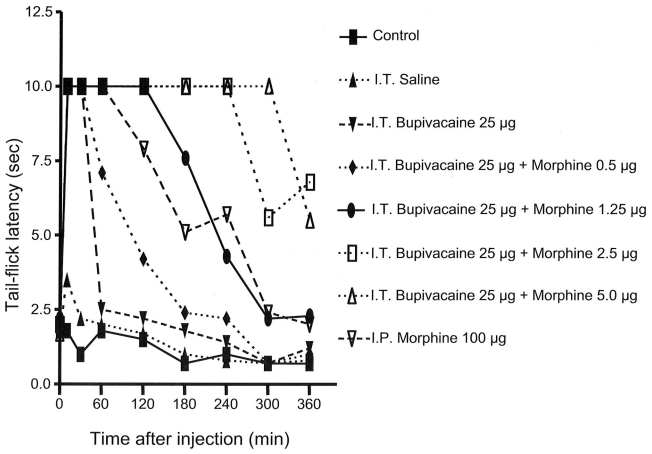

Fig. 1. Duration of analgesia measured by tail-flick latency after injection of either morphine intraperitoneally (I.P.) or bupivacaine with or without morphine intrathecally (I.T.). Control mice did not receive any injection. *Symbols* denote mean value for three animals.

(decreased respiratory rate) and marked reduction of motor activity. Flaccidity of the lower limbs started 1 min and disappeared within 30 min after intrathecal bupivacaine alone, but did not disappear 30 min after intrathecal bupivacaine with morphine. Signs of motor block, including relaxation of the abdominal musculature, started 2–3 min, continued at least 30 min, and disappeared 60 min after intrathecal bupivacaine with morphine; motor disturbances were not observed up to 2 weeks after lumbar puncture in all regimens.

Effect of Lower Abdominal Surgery and Anesthesia on Liver Metastasis of EL4 Tumor Cells

Liver surface metastases 12 days after EL4 injection were shown (fig. 2). Laparotomy conducted with sevoflurane general anesthesia increased the number of EL4 liver metastases from 15.5 ± 8.7 in the control group (19.4 ± 5.4 in the sevoflurane group and 19.1 ± 8.6 in the sevoflurane plus spinal block group) to 33.7 ± 8.9 ($P < 0.05$ for all; fig. 3). The addition of spinal block to sevoflurane general anesthesia significantly reduced the number of metastases after surgery from 33.7 ± 8.9

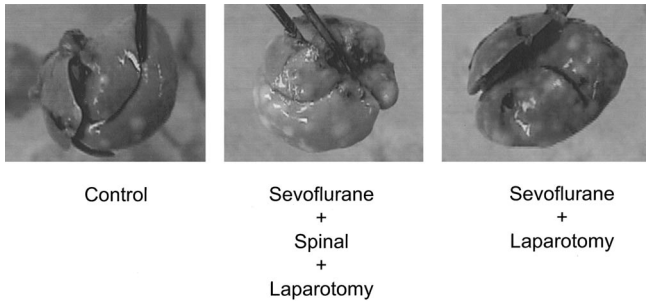

Fig. 2. Liver metastases 12 days after EL4 cell injection. Three of 48 livers in the control, sevoflurane, sevoflurane + spinal, sevoflurane + laparotomy, sevoflurane + spinal + laparotomy, and sevoflurane + systemic morphine + laparotomy groups are shown. *White spots* on the surface of livers are metastases.

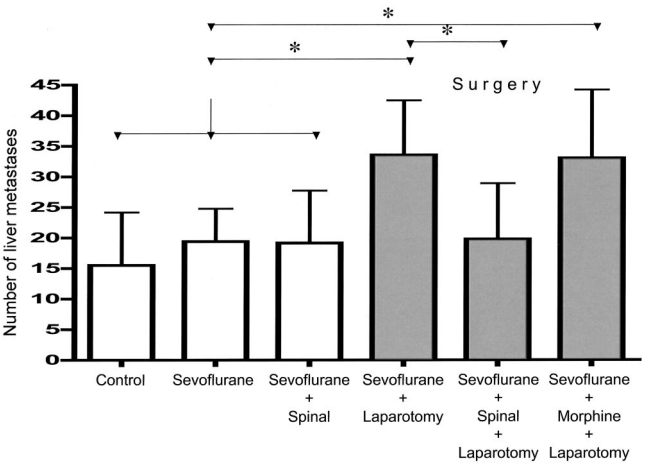

Fig. 3. Effect of surgery and anesthesia on the number of liver metastases 12 days after EL4 cell injection. Compared with control, sevoflurane, and sevoflurane + spinal groups, sevoflurane + laparotomy significantly increased liver metastases (*). Spinal anesthesia attenuated this metastasis-promoting effect of surgery (*), whereas systemic morphine did not (*). *Bars* denote mean value of eight animals; *error bars* = SD. * $P < 0.05$.

to 19.8 ± 9.1 ($P < 0.05$), whereas the addition of systemic morphine had no significant effect (33.7 ± 8.9 *vs.* 33.0 ± 11.2; $P > 0.05$), and liver metastases remained significantly elevated compared with the control group ($P < 0.05$; fig. 3). No significant difference was found between the group that underwent surgery plus sevoflurane and morphine and the group that underwent surgery plus sevoflurane and spinal block.

Liver Metastasis of EL4 Tumor Cells in Response to In Vivo *Cell Depletion*

The numbers of EL4 cell metastases in the liver were greatly increased by cell depletion. The addition of spinal block to sevoflurane general anesthesia significantly increased the number of metastases 5.0-fold compared with control after anti-AGM1 treatment and 7.7-fold after anti-NK1.1 treatment ($P < 0.01$ for both). The difference between these two treatment groups was statistically significant ($P < 0.01$; fig. 4).

Effect of Lower Abdominal Surgery and Anesthesia on Activity of Liver MNCs

The cytotoxic activity of liver MNCs against EL4 tumor cells was not significantly suppressed by anesthesia (29.4 ± 2.4% in the sevoflurane group and 30.4 ± 2.3% in the sevoflurane plus spinal block group), compared with control (32.7 ± 2.3%) (at an 80:1 E:T ratio). However, cytotoxic activity was greatly decreased to 18.5 ± 4.5% in response to sevoflurane plus surgery ($P < 0.01$ for all; fig. 5). Interestingly, the addition of spinal block to general anesthesia in mice undergoing surgery preserved liver MNC activity (26.6 ± 3.6%). There was a significant difference between surgery plus sevoflurane general anesthesia and surgery plus sevoflurane and spinal block at an 80:1 E:T ratio (18.5 ± 4.5 and 26.6 ± 3.6%; $P < 0.01$;

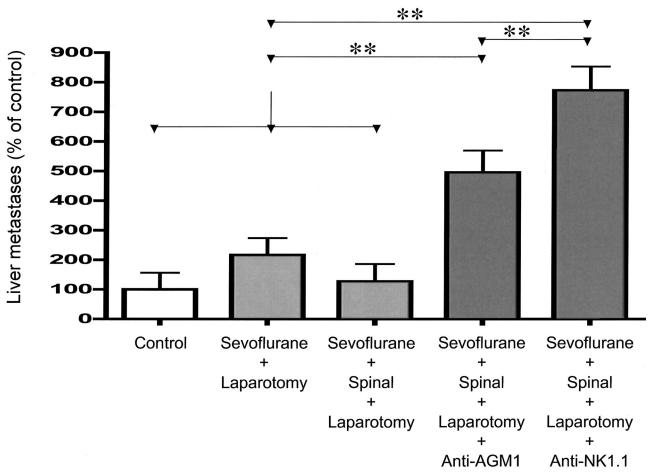

Fig. 4. Effect of depletion of natural killer cells or depletion of natural killer cells and natural killer T cells on the number of liver metastases 12 days after EL4 cell injection. Values are expressed as percentage of the control value (± SD). Both anti-AGM1 and anti-NK1.1 treatment significantly increased the number of metastases compared with the control, sevoflurane + laparotomy, and sevoflurane + spinal + laparotomy groups (**). Between the two drug treatment groups, the difference was statistically significant (**). *Bars* denote the mean value of four animals in the drug treatment groups and eight animals in the other groups; *error bars* = SD. ** $P < 0.01$.

fig. 5). No significant difference was observed between groups at 40:1 or 20:1 E:T ratios, with the exception of control and surgery plus sevoflurane general anesthesia at a 40:1 E:T ratio (20.8 ± 1.5 *vs.* 13.9 ± 3.3%; $P < 0.05$; fig. 5).

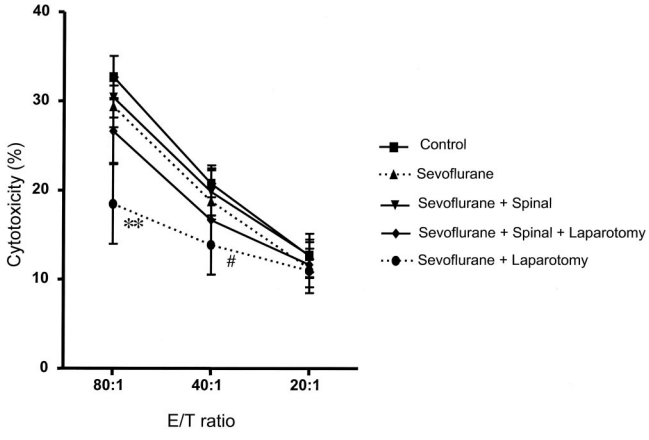

Fig. 5. Effect of anesthesia and surgery on cytotoxic activity of liver mononuclear cells against EL4 cells. Five hours after the end of anesthesia, liver mononuclear cells from each group were obtained and incubated with ^{52}Cr-labeled EL4 cells at the indicated effector:target (E/T) ratios. Laparotomy with sevoflurane significantly reduced cytotoxicity compared with the control, sevoflurane, sevoflurane + spinal, and sevoflurane + spinal + laparotomy groups at an 80:1 E/T ratio (**), and compared with the control group at a 40:1 E/T ratio (#). *Symbols* denote mean value for five or six animals; *error bars* = SD. ** $P < 0.01$ *versus* control, sevoflurane, sevoflurane + spinal, and sevoflurane + spinal + laparotomy groups. # $P < 0.05$ *versus* the control group.

Effect of Lower Abdominal Surgery and Anesthesia on IFN-γ and IL-4 Production and on IFN-γ/IL-4 Ratio in Liver MNCs after α-GalCer Stimulation

IFN-γ Production. The production of IFN-γ after α-GalCer stimulation significantly increased in response to surgery, compared with control ($P < 0.01$ for sevoflurane plus spinal plus laparotomy, $P < 0.05$ for sevoflurane plus laparotomy; fig. 6A). IFN-γ level increased in response to surgery, compared with anesthesia alone (sevoflurane or sevoflurane plus spinal) ($P < 0.01$ for sevoflurane plus spinal plus laparotomy, not significant for sevoflurane plus laparotomy; fig. 6A). In the surgical groups, IFN-γ was produced significantly more in response to surgery plus sevoflurane and spinal block, compared with surgery plus sevoflurane ($P < 0.01$; fig. 6A).

IL-4 Production. Interleukin-4 level also increased significantly in response to surgery, compared with control or anesthesia alone ($P < 0.05$ for sevoflurane plus spinal plus laparotomy, $P < 0.01$ for sevoflurane plus laparotomy) and was increased more in response to sevoflurane plus laparotomy compared with sevoflurane plus spinal plus laparotomy, but not significantly (fig. 6B).

Ratio of IFN-γ to IL-4. Surgery conducted with sevoflurane general anesthesia plus spinal block significantly reduced the IFN-γ/IL-4 ratio from 89.3 ± 16.8 in the control group or from 95.7 ± 25.5 in the anesthesia group to 46.5 ± 16.8 ($P < 0.01$ for both). Surgery with sevoflurane general anesthesia significantly reduced the IFN-γ/IL-4 ratio to 15.7 ± 6.7 compared with control, anesthesia, and sevoflurane plus spinal plus laparotomy ($P < 0.01$ for control and anesthesia, $P < 0.05$ for sevoflurane plus spinal plus laparotomy; fig. 6C).

Discussion

The results of this study indicate that innate tumor immunity is impaired by inhibition of the cytotoxic Th1 response of liver MNCs after lower abdominal surgery and that spinal block attenuates this impairment, thereby inhibiting the promotion of liver metastasis after surgery.

Factors Affecting NK Cell Function after Surgery

Surgery and the resultant stress response have been shown to lead to depression of NK cell function in the spleen and peripheral blood,[10,21,40] which is associated with increased tumor growth and metastasis.[3,4] It has been reported that oncologic surgery outcome may be affected by many factors, including blood transfusion,[8,41] hypothermia,[9,42] chemotherapy,[43] psychological stress,[44] and anesthetic agents.[12,13] However, surgical trauma is generally considered to have a greater role than anesthesia in altering the immune response.[1,11,45] Anesthetic treatment alone does not induce suppression

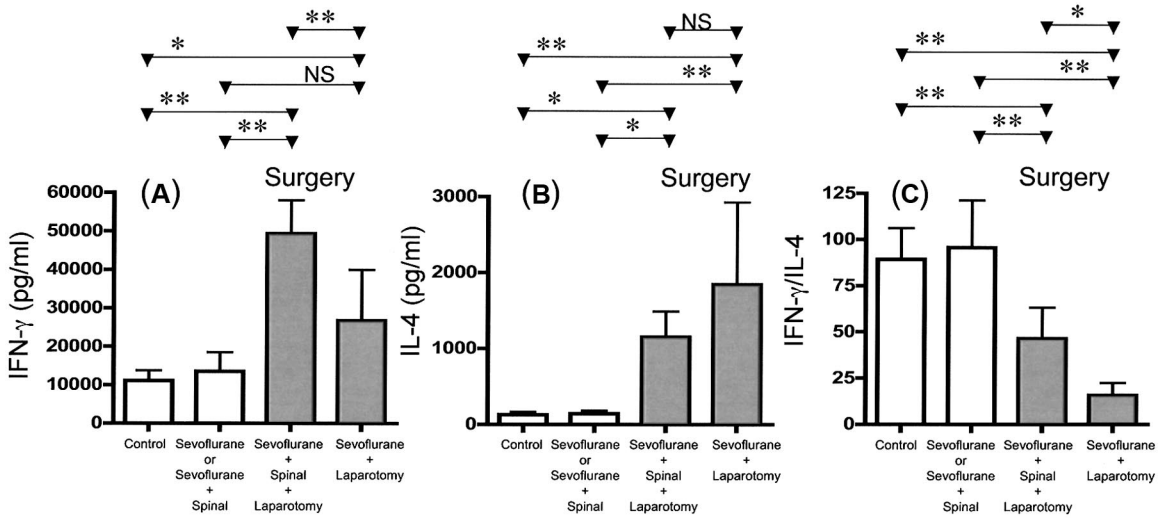

Fig. 6. Interferon (IFN)-γ (*A*) and interleukin (IL)-4 (*B*) production of liver mononuclear cells and the IFN-γ/IL-4 ratio (*C*) after α-GalCer stimulation (24-h incubation) assessed 5 h after the end of anesthesia. (*A*) Laparotomy significantly increased IFN-γ production compared with control (or *). IFN-γ production increased in response to laparotomy, compared with sevoflurane or sevoflurane + spinal group (** for sevoflurane + spinal + laparotomy, not significant [NS] for sevoflurane + laparotomy). (*B*) Laparotomy significantly increased IL-4 production compared with control, sevoflurane or sevoflurane + spinal group (** or *). (*C*) Laparotomy significantly reduced the IFN-γ/IL-4 ratio compared with control, sevoflurane or sevoflurane + spinal group (**). In the sevoflurane + laparotomy group, the IFN-γ/IL-4 ratio was significantly more reduced than in the sevoflurane + spinal + laparotomy group (*). *Bars* denote mean value for six animals, in the sevoflurane or sevoflurane + spinal group, three animals in sevoflurane and three in sevoflurane + spinal; *error bars* = SD. ** $P < 0.01$. * $P < 0.05$.**

of NK cell cytotoxicity in mice, whereas hind limb amputation does.[10] The current results are in agreement with these reports.

Regional Anesthesia, Stress Response, and Oncologic Prognosis

Regional anesthesia in humans attenuates surgery-induced stress responses, including increases in levels of corticosteroid hormone and catecholamine.[40] These stress hormones are thought to play important roles in depressing immune function.[20] Bar-Yosef *et al.*[23] reported that the addition of spinal block to halothane general anesthesia markedly attenuated the promotion of pulmonary metastasis induced by surgery in rats. However, peripheral blood NK activity was reduced to a similar degree by general anesthesia alone or by general anesthesia plus surgery and was further reduced by the addition of spinal block, despite its favorable effect on metastatic development.[23] In the current study, we showed for the first time that addition of spinal block to sevoflurane general anesthesia attenuates the promotion of liver metastasis induced by surgery through preservation of tumoricidal function of liver MNCs and the Th1/Th2 cytokine balance.

To assess the involvement of liver NK and NKT cells in the effect of regional anesthesia on oncologic immunity, anti-NK1.1 antibody or anti-AGM1 antibody was used. Many more liver metastases were found after administration of either of these antibodies. Therefore, both NK and NKT cells are believed to be involved in protection against metastasis in the liver. In addition, the suppression of liver MNCs by lower abdominal incision seems to be less strong than depletion of NK cells or of both NK and NKT cells.

Effect of Surgery and Anesthesia on IFN-γ and IL-4 Production

We had expected that IFN-γ production by liver MNCs stimulated with α-GalCer, a synthetic ligand for NKT cells,[30,46] would be suppressed more in surgery groups than in control groups. The production of IFN-γ by NKT cells stimulated with IL-12 is important in the rejection of liver and pulmonary metastases[29]; in the case of α-GalCer injection, activation of NK cells by NKT-produced IFN-γ inhibits hepatic metastasis.[30,46] However, our results differed from what we had expected: IFN-γ production increased after surgery (fig. 6A). Therefore, liver MNCs may be primed to produce IFN-γ by abdominal incision.

The IFN-γ/IL-4 ratio was significantly reduced in surgery groups compared with control groups. Furthermore, this ratio in surgery groups was reduced more in the surgery plus general anesthesia group than in the surgery plus general and spinal anesthesia group. Although α-GalCer is known to increase production of both IFN-γ and IL-4 by NKT cells,[30,46] its precise properties have not been elucidated. Our results suggest an important function of NKT cells: stressors, including surgical stress, may alter their IFN-γ/IL-4 balance.

Th1/Th2 Cytokine Balance and Oncologic Prognosis

IFN-γ and IL-12, Th1 cytokines, are known to be involved in increasing the cytotoxic activities of T cells and

NK cells[31]; IL-4 and IL-10, Th2 cytokines, are reported to be involved in increasing humoral immunity and in suppressing the Th1 response.[31] The Th2-dominant status, which was observed in the current study, is thought to have a negative role in oncologic immunity. Indeed, a previous study showed that a Th2-dominant status of intracellular cytokines was present in patients with advanced cancer.[47] Another study reported that a Th2-dominant status of intracellular cytokines was present after prostatectomy.[31] Taken together with our current results of tumoricidal activity and cytokine production, it is possible that Th2-dominant status is a major factor in depression of cytotoxic activity.

An *ex vivo* study in humans indicated that some stressors may cause selective suppression of Th1 function and a shift toward a Th2 cytokine pattern rather than generalized Th suppression *via* elevation of glucocorticoid and norepinephrine levels[48]; this suggests that a Th1-to-Th2 shift may be responsible for the stress-induced susceptibility of patients to certain infections. Another study showed that the administration of glucocorticoids can lead to excessive production of IL-4 and suppressed production of IFN-γ in humans.[49] These findings suggest, albeit indirectly, that spinal anesthesia may attenuate the postoperative elevation of stress hormones.

General and Spinal Anesthesia

In the current study, we assume that the general anesthesia was enough, because there were no differences in respiratory rate and heart rate in these mice during and after surgery. A deeper level of sevoflurane general anesthesia could not prevent the stress response which might affect tumor cell spread, because sevoflurane general anesthesia could not block the reflection that originated from lower spinal level, but spinal anesthesia could block or blunt it. Other types of inhalational anesthetics may have a different effect. They have not been examined in the current study.

NK and NKT Cell Activity after Surgery

EL4 cells were administered to mice 5 h after surgery because we postulated that immune function within 24 h, and particularly within 6 h, after surgery would affect oncologic prognosis. Many clinical studies regarding peripheral NK activity and cell count after surgery have shown suppression immediate after surgery, and that it continues for 3–10 days.[15,18,43,50,51] Therefore, suppression of liver MNC activity 5 h after surgery would be assumed to continue for many hours, even as long as several days.

Effect of Opioid on Oncologic Immunity

We used morphine, which has been reported to lead to opposing effects on immune function.[52–55] Systemic administration of morphine showed a duration of analgesia similar to that of intrathecal morphine administration in the TF test. However, only intrathecal administration showed a protective effect on liver metastasis. Therefore, there may be different mechanisms, extents of analgesia, or abilities to block stress response between these modes of administration. A previous study reported that postoperative metastases decreased after surgery with systemic administration of morphine and volatile anesthetics[54]; thus, higher doses or continuous postoperative administration of morphine may attenuate tumor metastasis. However, it has also been reported that systemic morphine suppresses NK activity,[52] as does a large dose of systemic opioid,[56] indicating that spinal or epidural administration of morphine with regional anesthetics attenuates immune suppression, because a smaller dose of morphine might have blunted the stress response when administered intrathecally in the current study.

Summary

The current results indicate that combined spinal and general anesthesia may attenuate the suppression of innate tumor immunity, including liver NK and NKT cell activity, and preservation of Th1/Th2 cytokine balance, after surgery. Further investigations regarding methods of anesthesia that blunt excessive stress response must be performed.

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