Influence of General Anesthesia with Isoflurane Following Propofol-Induction on Natural Killer Cell Cytotoxic Activities of Peripheral Blood Lymphocytes in Dogs

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ABSTRACT To investigate influence of general anesthesia on immunological anti-tumor activity, the natural killer (NK) cytotoxic activity of peripheral lymphocytes (PBLs) was measured in 7 dogs anesthetized for 3 hr with isoflurane following propofol-induction (anesthesia group) and 6 dogs without anesthesia (control group). Blood samples were collected before (baseline) and 24, 120 and 192 hr after the anesthesia. The PBLs were isolated via centrifugation with Ficoll-Hypaque solution (density, 1.073), and adherent cells were removed. The NK cytotoxic activity of the isolated PBLs against canine thyroid cancer cells was detected by the colorimetric rose Bengal assay. Significant decrease in the NK cytotoxic activity was observed at 24 hr after the anesthesia, compared with the baseline values and the control group. The NK cytotoxic activities were recovered to the baseline values until 120 hr after the anesthesia. The general anesthesia with isoflurane following propofol-induction decreased the NK cytotoxic activities of PBLs in dogs. This finding has a clinical relevance to the risk of tumor recurrence or metastasis induced by the suppression of immunological anti-tumor activity after general anesthesia in dogs. The results further emphasized the importance of the need to evaluate immune suppression following general anesthesia in animals.

KEYWORDS anesthesia, canine, NK cytotoxic activity.


Both adaptive and innate immunities play important roles in immune responses to tumors. Although adaptive immunity is a specific system controlled by T and B cells, innate immunity is a nonspecific system consisting of monocytes, macrophages, polymorphonuclear neutrophils, and lymphocytes, and (The same applies to the following.) Natural killer (NK) cells [6, 11]. The NK cytotoxic activity is a measure of the cytotoxic targeting of tumor cells and is mediated by NK cells, NK T cells, killer T-lymphocytes, CD8+ T cells and possibly other cells [3, 6, 15]. The NK cytotoxic activities are considered to have important functions in the defense against oncoprogenicity and cancer metastasis, as they strengthen immunity against tumors [5, 15].

In human medicine, most anesthetics, sedatives and opioid drugs directly inhibit immune cell activities [11]. For example, isoflurane decreases cytokine production in lymphocytes and NK cell activity [7] and can also induce apoptosis of lymphocytes [8]. To our knowledge, effects of anesthetics on anti-tumor immunity have not been reported for veterinary medicine.

The purpose of the study reported here was to assess the influence of general anesthesia with isoflurane following propofol-induction on the NK cytotoxic activity toward tumor cells in dogs. We hypothesized that the NK cytotoxic activity in dogs decreased by the general anesthesia induced with propofol and maintained with isoflurane. Effects were evaluated via a cytotoxicity test protocol that involves the use of Canine thyroid adenocarcinoma cell line (CTAC) target cells [3].

MATERIALS AND METHODS

Animals: Thirteen healthy adult Beagles (4 males and 9 females) were included in the study. Mean age was 1.8 years (range, 1.5 to 2.1 years). Food was withheld from dogs for 12 hr and water was withheld for 6 hr prior to the experiment. The study was approved by the Ethics Committee of Nippon Veterinary and Life Science University.

Experimental procedures: Dogs were randomly allocated to 2 groups (7 dogs in the anesthetized group and 6 dogs in the control group). A catheter was placed into their cephalic vein. In each dog of the anesthesia group, anesthesia was induced with an intravenous injection of propofol (7 mg/kg) (Rapinovet®, Schering-Plough Animal Health, Tokyo, Japan) and maintained for 3 hr by administration of isoflurane (2.0% of end-tidal concentration) (Escaín®, Mylan pharmaceutical, Tokyo, Japan). The dogs were orotracheally intubated following the induction of anesthesia and administered isoflurane in oxygen (flow rate: 2 l/min) using an anesthetic circle system (FO-20A, Acoma Medical Industry Co., Ltd., Tokyo, Japan) with the isoflurane vaporizer (MKIII ai, Acoma Medical Industry Co., Ltd.). During anesthesia, the dogs were administered lactated Ringer’s solution (Solulact®, Terumo Corporation, Tokyo, Japan) at an infusion rate of 10 ml/kg/hr via the catheter placed into the cephalic vein. End-tidal concentration of isoflurane and

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heart rate, noninvasive mean arterial blood pressure, percutaneous arterial oxygen saturation (SpO₂), end-tidal partial pressure of carbon dioxide (EtCO₂) and rectal temperature were monitored using medical monitor (Bioscope AM120, Fukuda ME Kogyo Co., Ltd., Tokyo, Japan). EtCO₂ was maintained within the reference range (35 to 45 mmHg) [13] by controlled mandatory ventilation using a ventilator (mini Vent-3 animal, Kimura Medical Instrument Co., Ltd., Tokyo, Japan). Rectal temperature was maintained at > 36°C using a heat mat (minimatt, Watanabe Industry Co., Ltd., Osaka, Japan). Each dog of the control group was administered lactated Ringer’s solution at an infusion rate of 10 ml/kg/hr via the catheter placed into the cephalic vein for 3 hr without anesthesia.

Collection of blood samples and preparation of canine PBLs: Venous blood samples were collected from the anesthetized dogs before (baseline) and 24, 120 and 192 hr after anesthesia. Venous blood samples were collected from the control dogs at the same times. Samples (7 ml/sample) were collected; 5 ml of each sample was placed into tubes containing heparin, and 2 ml of each sample was placed into tubes containing ethylenediaminetetraacetic acid (EDTA).

The peripheral blood mononuclear cells (PBMCs) were purified from the sterile heparinized whole blood samples as described previously [3]. Briefly, 5 ml of heparinized venous blood was mixed with 5 ml of RPMI 1640 (Sigma-Aldrich Japan, Tokyo, Japan) culture medium containing a combination of 10,000 U of penicillin/ml (Sigma-Aldrich Japan) and 10,000 µl of streptomycin/ml (Sigma-Aldrich Japan). The mixture was carefully layered onto 2 ml of Ficoll-Hypaque (GE Healthcare UK LTD., Buckinghamshire, UK) (density, 1.073) in a 15-ml conical tube, and tubes were centrifuged at 400 × g at 20°C for 25 min. After centrifugation, PBMCs were removed from the gradient interface and washed in 5 ml of RPMI 1640 by centrifugation at 400 × g at 20°C for 10 min. To prepare PBLs, PBMCs were allowed to adhere to a 60-mm plastic culture dish for 1 hr at 37°C; nonadherent cells were subsequently collected via gentle pipetting.

Functional evaluation for NK cytotoxic activity of PBLs: The NK cytotoxic activity was measured with a method reported in another study [3]. The target cell line was a CTAC (European Collection of Cell Cultures, Health Protection Agency Culture Collections, Salisbury, UK). Vero cells (Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan) were used as negative control cells. To assess NK cytotoxic activity toward tumor cells before and after anesthesia, PBLs were used as effector cells. The colorimetric rose bengal assay was used for the measurement of NK cytotoxic activities. The rose bengal assay is able to measure both apoptotic and necrotic cells [10]. An aliquot (100 µl) of rose bengal dye (0.25% solution) was added to each well; wells then were incubated for 5 min at room temperature. Excess dye was removed by washing the wells twice with phosphate buffered saline (PBS) solution. After wells were washed, rose bengal dye was released into the supernatant by the addition of 200 µl of ethanol-PBS solution (50:50)/well, and the Optical density (OD) was measured with an enzyme-linked immunosorbent assay (ELISA) reader at an excitation wavelength 548 nm. The OD of each well was measured in triplicate, and a mean value was calculated. The NK cytotoxic activity was calculated with the mean values by use of the following equation: NK cytotoxic activity (%) = 100 – (OD test/OD control × 100).

Where OD test was the OD value for the samples obtained from the dogs and OD control was the OD value for the CTAC or Vero cells without effector cells.

Ratio of large granular lymphocytes (LGLs) in isolated PBLs: The PBLs were centrifuged onto microscope slides. Cells were stained with May-Grünwald-Giemsa stain in accordance with a standard procedure [3]. The LGLs, which are large lymphocytes that contain a distinct, often indented nucleus and azurophilic granules in the cytoplasm, were identified morphologically and were counted. The LGLs are recognized by their morphological traits as NK cells, can manufacture and release a variety of cytokines, and are cytotoxic to certain tumors and virally infected cells [2, 15].

Evaluation of the lymphocyte count and ratio of CD3⁺, CD4⁺, CD8⁺, and anti-B cells: Blood samples collected into tubes that contained EDTA at each time point were processed. The lymphocyte count; number of cells with surface markers CD3⁺, CD4⁺ and CD8⁺; and number of anti-B cells were investigated. Blood smear was prepared and stained with a commercial Romanowski stain (Diff-Quik, Merck KGaA, Darmstadt, Germany). Lymphocyte counts were determined via microscopic examination. Immunophenotyping of PBLs was performed by evaluating cell-surface expression of T-cell markers (CD3, CD4 and CD8) and B-cell marker (anti-B cell) via flow cytometry (Canine-lab Corp, Tokyo, Japan). Blood samples were collected as described previously and treated with lysis buffer (BD Pharm LyseTM, BD Biosciences Pharmingen, Tokyo, Japan) to lyse erythrocytes. The remaining nucleated cells were washed with PBS solution and then labeled with fluorescein-isothiocyanate–conjugated mouse monoclonal antibodies for canine CD3 (clone No. CA17.2A12) and phycoerythrin-conjugated mouse monoclonal anti-B cell antibodies (clone No. CA2.1D6) for the detection of T cells and B cells, respectively. Fluorescein-isothiocyanate–conjugated anti-CD4 antibodies (clone No. YKIX302.9) and phycoerythrin-conjugated anti-CD8 antibodies (clone No. YCATE55.9) were used for the detection of helper and killer T cells, respectively. Cells were analyzed via flow cytometry by use of a forward-scatter versus side-scatter plot for the identification of lymphocytes. Data were analyzed (WinMDI 2.9, The Scripps Research Institute, La Jolla, CA, U.S.A.), and the ratios of CD3⁺ cells to anti-B cells and CD4⁺ cells to CD8⁺ cells were calculated.
rate, noninvasive mean arterial blood pressure, and SpO\textsubscript{2} were maintained within the target ranges. Heart anti-B cells, CD4\textsuperscript{+} cells to CD8\textsuperscript{+}

**RESULTS**

Between control and anesthetized groups were determined mmHg) [13]. SpO\textsubscript{2} was maintained within the reference range (mean 60 to 120 bpm) [13]. Noninvasive mean arterial blood pressure analyses. Values of \( P < 0.05 \) were considered significant. Statistical analysis: All results were expressed as mean \( \pm \) SD, medians and ranges. Differences in NK cytotoxic activity, number of lymphocytes, and the ratios of CD3\textsuperscript{+} cells to anti-B cells, CD4\textsuperscript{+} cells to CD8\textsuperscript{+} cells and LGLs to PBLs between control and anesthetized groups were determined via the Mann-Whitney \( U \) test. The Friedman test was used to determine significant differences between results for samples obtained before and 24, 120 and 192 hr after anesthesia. Commercially available software (SPSS, version 16 for Windows, IBM, Tokyo, Japan.) was used for statistical analyses. Values of \( P < 0.05 \) were considered significant.

**RESULTS**

Heart rate was maintained within the reference range (60 to 160 bpm) [13]. Noninvasive mean arterial blood pressure was maintained within the reference range (mean 60 to 120 mmHg) [13]. SpO\textsubscript{2} was maintained within the reference range (>95%) [13]. During anesthesia, the EtCO\textsubscript{2} and rectal temperature were maintained within the target ranges. Heart rate, noninvasive mean arterial blood pressure, and SpO\textsubscript{2} were able to maintained without needing special management, respectively. Recovery from anesthesia was good and uneventful in all dogs.

The NK cytotoxic activity decreased significantly at 24 hr after anesthesia (2.6 \( \pm \) 3.7%), compared with the baseline NK cytotoxic activity for the anesthetized dogs (20.6 \( \pm \) 5.9\%; \( P = 0.01 \)) and with NK cytotoxic activity for the control group at 24 hr (19.9 \( \pm \) 5.6%, \( P = 0.001 \)). The NK cytotoxic activity in anesthetized dogs returned to baseline values at 120 (15.2 \( \pm \) 4.6%) and 192 hr (21.9 \( \pm \) 8.4%) after anesthesia (Table 1). No NK cytotoxic activity was detected for the negative control Vero cells.

The percentage of LGLs in the PBLs was assessed. Before anesthesia, LGLs comprised 14.5% of PBLs. This percentage did not change significantly in the anesthetized dogs (15.2%, 17.3% and 15.1% at 24, 120 and 192 hr after anesthesia, respectively).

Lymphocyte counts and ratios of CD3\textsuperscript{+} cells to anti-B cells and CD4\textsuperscript{+} cells to CD8\textsuperscript{+} cells were determined. The lymphocyte count at 24 hr after anesthesia was significantly decreased (248 \( \pm \) 99 lymphocytes/\( \mu \)l), compared with the value for the control group (1,242 \( \pm \) 618 lymphocytes/\( \mu \)l). However, at 120 hr after anesthesia (1,409 \( \pm \) 310 lymphocytes/\( \mu \)l), the lymphocyte count had returned to a value similar to that before anesthesia (1,288 \( \pm \) 205 lymphocytes/\( \mu \)l). Measurement of the ratios of CD3\textsuperscript{+} cells to anti-B cells and CD4\textsuperscript{+} cells to CD8\textsuperscript{+} cells revealed no significant differences before and after anesthesia with the combination of propofol and isoflurane.

**DISCUSSION**

In the study reported here, the effects of anesthesia on NK cytotoxic activity of PBLs toward tumor cells in dogs were evaluated. The investigation was conducted with a combination of propofol and isoflurane for the induction and maintenance of anesthesia, respectively. Analysis of the data revealed that NK cytotoxic activity in dogs was significantly lower 24 hr after anesthesia. These findings agree with the results of a human study [14]. However, in that report [14], surgical procedures had been performed in addition to the anesthesia. As our finding is based on the use of anesthesia without surgery, the combination of anesthesia and operation...
may more strongly decrease NK cytotoxic activity than anesthesia alone. However, obvious inflammatory lesions have no impact on NK cytotoxic activity [4]. The reason for the postoperative decrease in NK cytotoxic activity is unclear [4], but may involve the effects of invasive surgery. Therefore, future investigations are needed to determine whether invasive surgery decreases NK cytotoxic activity beyond the effect of anesthesia alone.

The mechanism for the reduction in cytotoxic activity is unknown. However, several mechanisms can be considered. First, cortisol concentrations are thought to increase in response to stress caused by anesthesia, which may in turn inhibit NK cytotoxic activity [9]. Second, an experiment in mice revealed that interferon enhancement of NK activity was not detectable after mice were exposed to isoflurane [7]. In addition, it has been reported that NK activity decreases and that the reactivity between NK cells and interferon-γ is suppressed after anesthesia in humans [5]. Third, there may be a direct action of isoflurane on lymphocytes, because a decrease in NK cytotoxic activity has been reported in an ex vivo experiment in which human lymphocytes were exposed to isoflurane [8]. However, it has also been reported that the site of the cytotoxic action of isoflurane on lymphocytes is not the membrane. Therefore, the mechanism is still unclear.

In the present study, NK cytotoxic activity returned to baseline values by approximately 120 hr after anesthesia. In contrast, NK cytotoxic activity then returned to preoperative values by 5 days after anesthesia for surgery in humans [14]. However, surgical stress and anesthesia protocol do not carry out coincidence of the present study, and it might suggest that the effect of anesthesia on NK cytotoxic activity return to baseline values is not related to the existence of surgery.

The percentage of LGLs remained constant in dogs throughout the present study, which suggested that an abundance of LGLs does not contribute to the observed decrease in NK cytotoxic activity. This finding is supported by results of a previous investigation [5] in which investigators found no change in the lymphocyte ratio. Furthermore, the number of NK cells was constant in the present study, although the overall number of lymphocytes decreased in response to anesthesia. However, according to another report [1], the number of circulating NK cells in humans decreases 20 min after anesthesia induction and quickly returns to within the reference range by 50 min after skin incision. Therefore, we cannot exclude the possibility that there was a transient reduction in the percentage of LGLs.

In the present study, the number of lymphocytes decreased 24 hr after anesthesia, but returned to baseline values by 120 hr after anesthesia. Although anesthesia is reported to cause apoptosis of PBLs in dogs [12, 16] as well as in humans [8], the influence of PBL apoptosis on anti-tumor immunity is uncertain. The results of the present study do not clearly show whether any PBLs underwent apoptosis. The reduction in the number of PBLs was not investigated in direct relation to the NK cytotoxic activity in this study, as the latter was assayed using the same number of PBLs. However, in vivo, the decreased number of PBLs may result in an even more marked decrease in NK cytotoxic activity than observed in vitro. In addition, stress hormones can cause changes in the distribution of lymphocytes [16]. These factors might explain, at least in part, the decrease in lymphocyte numbers in the dogs of the study reported here. On the other hand, the ratio of CD3+ cells to anti-B cells and CD4+ cells to CD8+ cells did not change over time; therefore, we concluded that anesthesia does not affect the ratio of B-cells to T cells in PBLs or the ratio of helper T cells to killer T cells in PBLs.

The study reported here emphasized the importance of evaluating immune suppression after anesthesia in a veterinary setting. Analysis of the results of the study suggests a possible role for anesthetic drugs in the general anesthesia decrease in the anti-tumor immune response. It is thought that the anesthetic protocol in the present study was designed to be similar to those used for diagnostic imaging and radiation therapy. Both of these procedures are important in veterinary oncology. So, the anti-tumor immunity after anesthesia becomes important. This finding has a clinical relevance to the risk of tumor recurrence or metastasis induced by the suppression of immunological anti-tumor activity after general anesthesia in dogs. The results further emphasized the need to evaluate immune suppression following general anesthesia in animals. We suggest that the identification of anesthetic protocols that do not compromise tumor-targeting immune activities may be an important area for future investigations.

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REFERENCES


