Impaired Immune Function in Patients Undergoing Surgery for Bone Cancer

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Abstract. *Background: Natural killer (NK) cells undergo quantitative and functional changes after oncological surgery. Patients and Methods: After Institutional Review Board approval, the count and function of NK cells from patients with malignant bone tumors were assessed only days 1, 3, 5 and during first postoperative visit, and compared with preoperative values. The serum concentrations of interleukins (IL)-2, -4 and -6 were also measured before and after surgery. Results: Complete clinical and laboratory data were analyzed from 17 patients with different bone malignancies. The number of NK cells significantly decreased postoperatively as well as their function. The maximum deterioration in their function occurred 5 days postoperatively. The serum concentrations of IL-2 and IL-4 did not change perioperatively. In contrast, a significant increase in the concentrations of IL-6 was observed on day 1, 3 and 5 postoperatively. Conclusion: A significant inflammatory response and innate immune suppression occurred after surgery for malignant bone tumors.*

Among the wide variety of human cancers, primary bone tumors are relatively uncommon. According to the Cancer Statistics review of the National Cancer Institute, the incidence of bone and joint tumors is approximately one per 100,000 persons per year, being more frequently found in young individuals (1). Among these tumors, osteosarcoma is the most common, accounting for 35% of cases of bone tumors in all ages, followed by chondrosarcoma 25% and Ewing sarcoma 16% (2). The primary objective in the treatment of bone cancer is to achieve long-term disease-free survival by performing adequate resection of the tumor (3). However, during tumor resection, there is a release of malignant cells into the systemic circulation and there are micrometastases (also known as minimal residual disease, MRD) that could, after seeding and growth, develop into future clinical metastases (4, 5). During the perioperative period, the immune system can eliminate the growth of MRD by inducing apoptosis of the malignant cells through the killing activity of natural killer (NK) cells, (6, 7). A reduction in the cytotoxic activity of NK cells, possibly mediated by factors including surgical stress, analgesics and anesthetics, has been described after oncological surgery (8, 9). Several studies have shown that patients undergoing curative resection for breast, lung or colorectal cancer experience a decrease in the function of NK cells that is independent of the type of anesthetic technique and surgical site; however, there exists limited evidence regarding the biology of these cells from primary malignant tumors of bone. The purpose of this study was to investigate the behavior of NK cells, the plasma concentrations of lymphocyte T-helper (Th) cell interleukins (IL2 and IL4) and the inflammatory marker (IL6) before and after surgery for primary bone malignancies. We specifically hypothesized that postoperative innate immune suppression and a strong inflammatory response occur in patients with primary malignant tumors of bone. We believe that investigating changes in the concentrations of inflammatory cytokines and count and function of NK cells is relevant in order to establish correlation with clinical outcomes in future studies.

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Materials and Methods

Patients and perioperative care. After receiving an Institutional Review Board approval (IRB # PA12-0508), we obtained written informed consent from all patients. Patients older than 5 years of

age who had primary resective surgery for malignant bone tumors were eligible in the study. We excluded pregnant patients, children younger than 5 years old, those with an American Society of Anesthesiologists (ASA) physical status 4 or higher, those with history of opioid use at the time of surgery or had received chemotherapy within 2 weeks of surgery patients, those who had planned palliative surgery or hemipelvectomies and those who did not agree to participate in the study. All procedures were performed between January 2012 and January 2015.

All patients received balanced general anesthesia induced intravenously (*i.v.*) with a combination of a hypnotic drug (1-3 mg/kg propofol), a muscle relaxant (0.6-1.1 mg/kg succinylcholine, 0.6 mg/kg rocuronion or 0.2 mg/kg cisatracurium) and fentanyl (1-3 μg/kg). General anesthesia was maintained with 3-6% desflurane, oxygen and air to achieve a bisprectal index (BIS) level from 40-60. Supplemental *i.v.* intermittent doses of sufentanil (1-10 μg) or fentanyl (50-100 μg), or a continuous infusion of sufentanil (0.05-0.2 μg/kg/hour) were given at the discretion of the attending anesthesiologist to provide intraoperative analgesia. At the completion of surgery, muscle relaxation was reversed with *i.v.* neostigmine (0.5-2 mg) and *i.v.* glycopyrrolate 0.2-0.6 mg based on clinical judgment. Patients also received 1 g of acetaminophen *i.v.* and postoperative nausea and vomiting prophylaxis at the discretion of the attending anesthesiologist. Immediate postoperative pain control was achieved according to routine care at our Institution which typically includes the intravenous administration of 12.5-50 μg of fentanyl or 0.01-0.04 mg/kg hydromorphone. None of the patients enrolled in the study received intraoperative ketamine, ketorolac, celecoxib or regional anesthesia blocks at any time of the study. Subsequent postoperative pain was managed patient-controlled analgesia with morphine, fentanyl or hydromorphone *i.v.* and transitioned to oral analgesics.

We recorded demographic variables including age, gender, height, weight, ASA physical status, tumor variables, intraoperative depth of anesthesia, opioid consumption and postoperative pain scores (verbal rating scale from 0 "no pain" to "10" worse pain ever) in postoperative acute care unit (PACU) and on the first, second and third postoperative day. Fentanyl equivalents were calculated as 1 μg fentanyl:sufentanil 0.1 μg: hydromorphone 10 μg and morphine 100 μg (10).

Laboratory studies. Blood specimens were obtained before the administration of any sedatives, anesthetics, or analgesics, typically at the time of intravenous catheter placement in the preoperative area and on days 1, 3 and 5 after surgery and during the first follow-up visit (typically 1-2 weeks after surgery). Tubes containing heparin-sulfate and ethylene diamine tetra-acetic acid were used to collect blood to obtain peripheral blood mononuclear cells and plasma, respectively. Peripheral blood mononuclear cells were isolated using Ficoll-Paque (GE Healthcare, Uppsala, Sweden) density centrifugation and stored in liquid nitrogen in a solution of 10% dimethyl sulfoxide until used (Sigma–Aldrich, St. Louis, MO, USA) and heat-inactivated human AB serum (Sigma–Aldrich, St. Louis, MO, USA), while plasma was obtained by centrifugation and also stored at –80˚C until used.

Cytotoxicity assays. Natural killer cells were isolated by positive selection using CD56 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and used as effector cells in cytotoxicity assays. K562 cells American Type Culture Collection, (Manassas, VA, USA) were used as target cells, after being cultured in RPMI medium enriched with 10% fetal bovine serum and 1% penicillin/streptomycin. A lactate dehydrogenase (LDH) release assay was used to calculate the percentage of NK cell cytotoxicity (NKCC) at an effector:target ratio of 10:1 in all patients (8, 11). We also expressed the cytotoxicity activity of the NK cells against K562 as the absolute LDH optic density (OD) value measured by our plate reader (Molecular Devices, Inc., Sunnyvale, CA, USA).

Cytokine measurements. The plasma concentrations of IL2, -4 and -6 were measured using an enzyme-linked immunosorbent assay (ELISA) from R&D Systems, Inc, Minneapolis, CA, USA. The assays were read in duplicates using an automated micro plate reader (Molecular Devices, Inc.) and results are reported in picograms per milliliter.

Statistical analysis. Demographic, intraoperative, and postoperative data were summarized using median (with interquartile range, IQR) or mean (with standard deviation). Based on a previous study, we considered patients who experienced a reduction of more than 50% of their preoperative NK cell activity as relevant (8). With 20 patients enrolled in the study, we would be able to detect an absolute difference in means between preoperative and postoperative percentage of NK cell function of 16 units with a α error of 0.05 and power of 80%. Patients with missing perioperative clinical data were excluded from the statistical analysis. ANOVA followed by Dunn's multiple comparisons test were used to test for significant differences in the postoperative count and function of NK cells, and in the concentration of cytokines in comparison to their preoperative values. A *p*-value of less than 0.05 was considered statistically significant. Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA) was used for all statistical analyses.

Results

Demographics and intraoperative data. Twenty patients initially consented to participate in the study; three patients were excluded because they were taking opioids at the time of surgery. The demographic data and tumor-related variables are summarized in Table I. The median age of the patient population was 24 (IQR=16-64) years and there were more male (n=13) than female (n=4) patients. Osteosarcoma was the most common histology (n=9), six patients had previous history of cancer, eight had received neoadjuvant chemotherapy and two preoperative radiation. None of the patients was receiving cyclo-oxygenase inhibitors before surgery.

As shown in Table II, the intraoperative data analysis showed that the median duration of anesthesia was 367 (IQR=230.5-496.5) minutes. The median consumption of fentanyl equivalents and desflurane was 500 (IQR=250-900) μg and 4.97% (IQR=4.25-5.37%), respectively. The median BIS of our patient population was 41.95 (IQR=38.72-47.42), indicating moderate levels of depth of anesthesia. The postoperative fentanyl equivalent consumption and pain intensity in PACU were 50 μg (IQR=37-175) and 3.4 (IQR=2-4.7), respectively. Table II also shows the postoperative pain scores and opioid use after surgery. Briefly,

Table I. *Patients' demographic characteristics.*

Variable	Patients (n=17)
Age years, median (IQR)	24 (16-64)
Gender, F/M, n	4/13
Height cm, median (IQR)	174 (167-183)
Weight kg, median (IQR)	91.5 (72.25-107.3)
ASA physical status, n (%)	
1/2/3	0 (0)/2 (12%)/15 (88%)
Cancer histology, n (%)	
Osteosarcoma (yes/no)	9 (53%)/8 (47%)
Tumor size (mm)	70 (32.5-100)
Tumor location, n (%)	
Bone/soft tissue	15 (88%)/2 (12%)
Neoadjuvant chemotherapy, n (%)	
Yes/no	8 (47%)/9 (53%)
Neoadjuvant radiation, n (%)	
Yes/no	2 (12%)/15 (88%)
Use of COX inhibitors, n (%)	
Yes/no	0 (0%)/17 (100%)

IQR: Interquartile range; ASA: American Society of Anesthesiologists physical status, COX: cyclo-oxygenase.

Table II. *Intraoperative and postoperative data.*

Variable	Median (IQR) (n=17)
Intraoperative data	
Anesthesia duration (min)	381 (230.5-474)
Intraoperative fentanyl equivalents (μg)	500 (250-900)
Desflurane consumption (ET%)	4.97 (4.45-5.37)
BIS score	41.95 (38.72-47.42)
Total fluids (ml)	2976 (1700-4046)
Postoperative data	
Pain scores postoperative day 1	3 (1.75-4.24)
Postoperative day 2	2.75 (1.4-4.86)
Postoperative day 3	2 (0.62-4.46)
Opioid consumption in fentanyl equivalents (μg)	
Postoperative day 1	306 (106-677)
Postoperative day 2	144 (94-307)
Postoperative day 3	128 (94-234)

ET: End tidal; IQR: interquartile rage; BIS: bispectral index.

Table III. *Serum concentrations of interleukin 2 (IL-2) and 4 (IL-4). Data are the median and interquartile range.*

Time of blood collection	IL-2 pg/dl	IL-4 pg/dl
Preoperative	49 (1.75-125.5)	21.18 (14.02-195.8)
Post-op day 1	50 (0.62-147.3)	29 (15.52-169.4)
Post-op day 3	48 (0.8-172.6)	30 (14.87-166.4)
Post-op day 5	50 (0.13-204.7)	24 (14.34-153)
First follow-up	53 (0.13-204.7)	24 (14.47-134.5)
p-Value overall effect	0.709	0.235

Friedman test with Dunn's test for multiple comparisons.

pain scores indicated mild-to-moderate postoperative pain that peaked in day 1 after surgery (median=3, IQR=1.75-4.24) and that was accompanied by a median consumption of 306 μg (IQR=106-677) of fentanyl equivalents.

Laboratory results. The count and function of NK cells of patients who did not and did receive neoadjuvant treatment was compared to determine any potential effect of preoperative chemotherapy. Although, the analysis demonstrated that the median preoperative function of NK cells was not significantly different between patients who received chemotherapy (29.21%, IQR=14.38-54.23) and those who did not (29.12%, IQR=12.06-39.05; p=0.74); the count of NK cells was significantly lower in those who receive neoadjuvant therapy (4.8×10^5, IQR=$2.68\text{-}8.8\times10^5$) than those who did not (8.8×10^5, IQR=$5.27\text{-}10.4\times10^5$; p=0.012). The data analysis indicated that the percentage of NK cells in the PBMCs population remained unchanged postoperatively compared to preoperative values (p=0.516); however, the absolute number of NK cells significantly decreased over time compared to preoperative values (p=0.019). The nadir in the absolute count of NK cells was reached on day 1 after surgery (p=0.044) and remained significantly low through postoperative day 5 (p=0.03). At the time of the first postoperative follow-up, the absolute count of NK cells was still low compared to preoperative counts but it was not statistically significance (p=0.162). We also observed a statistically significant decrease in the postoperative function of NK cells, measured as both the percentage of NKCC (p=0.001) and LDH release O.D. values (p=0.002). The maximum decrease in NK cell function was found on postoperative day 5 when there was reduction in the activity of 82.53% (rank sum difference=28, p=0.003) compared to preoperative values (Figure 1). On the first postoperative outpatient follow-up visit, the function of NK cells was still lower than preoperative values but the difference did not reach statistical significance (p=0.092).

The kinetics in plasma concentrations of IL2, IL4 and IL6 are shown in Table III and Figure 2. The analysis showed no statistically significant change in the postoperative concentrations of IL2 and IL4 compared to their preoperative values (p=0.709 and p=0.235). On the other hand, as shown in Figure 2, the concentrations of the pro-inflammatory cytokine IL6 showed a statistically significant increase (overall effect, p=0.011) that peaked on postoperative day 1 (rank sum difference=–26.5; p=0.016) after surgery and returned to preoperative concentrations on the first outpatient follow-up day (p=0.7).

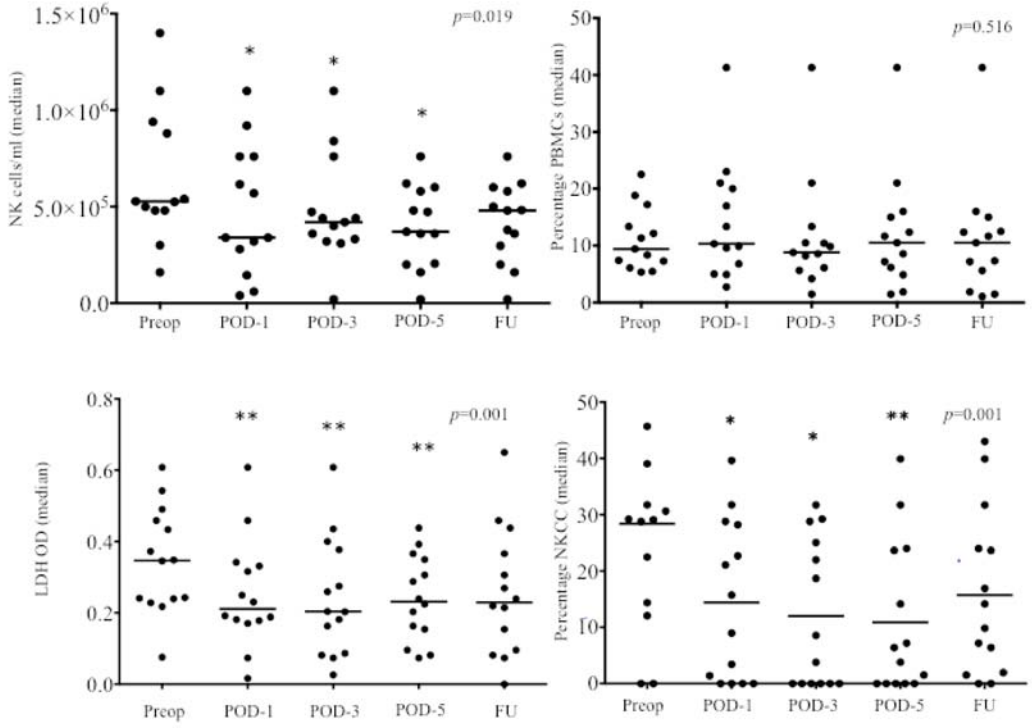

Figure 1. *Natural killer cell count and function. The figure illustrates the postoperative count [absolute and as percentage of peripheral blood mononuclear cells (PBMCs)] and function [absolute release of lactate dehydrogenase (LDH) and percentage of natural killer cytotoxicity (NKCC)]. FU: Follow-up; POD: postoperative day. Friedman test with Dunn's test for multiple comparisons. *p<0.05, **p<0.01, compared to preoperative values.*

Discussion

Our study demonstrates that the count and function of NK cells of patients undergoing surgery for primary bone cancer is lower postoperatively. The findings of this study are new in this population of patients and similar to those that we previously published in patients undergoing lung, breast and colorectal cancer surgery, but they also demonstrate that this period of immune suppression can last up to 2-3 weeks after surgery (8, 12). Our findings are in line with experimental animal models suggesting that the function of the NK cells decreases following a surgical insult up to 5 days postoperatively and in humans up to 28 days (13, 14). Although as far as we are aware of, there is no literature regarding the perioperative function of NK cells and development of metastasis in humans with primary bone tumors, several studies indicate that the activity of these cells is an important prognostic factor for survival in patients with other types of solid tumors (15-17). For instance, it has been suggested that patients with pancreatic and colorectal cancer with poor NKCC might have unfavorable prognosis (16). Tartter *et al*. demonstrated that the preoperative NKCC is a prognostic factor for cancer recurrence after colorectal cancer surgery (15). Another observational study showed that NKCC 4 weeks after non-small cell lung cancer surgery was strongly associated with recurrence-free survival. Briefly, those patients who had a NKCC higher than 20% showed the best survival compared to those with functions that were between 10%-20% or less than 10% (18). We also found a significant decrease in the count of NK cells that interestingly did not follow the same timeframe as the decrease in function of these cells. This finding is clinically relevant because in patients with solid tumors, such as pancreatic and colorectal cancer, recent evidence indicates that a high number of NK cells positively correlates with improved survival (19). Furthermore, a favorable response to immune therapy, as indicated by an increased number of circulating NK cells was associated with good prognosis in patients with squamous cell carcinoma of the head and neck, and ovarian cancer (20, 21).

Although a mechanism of postoperative immune suppression has not been yet elucidated, several factors including inflammation, catecholamines, and the use of opioids or anesthetic agents, have been shown to reduce the function of NK cells and be associated with the tumor growth and promotion of metastasis formation (22-24). Our

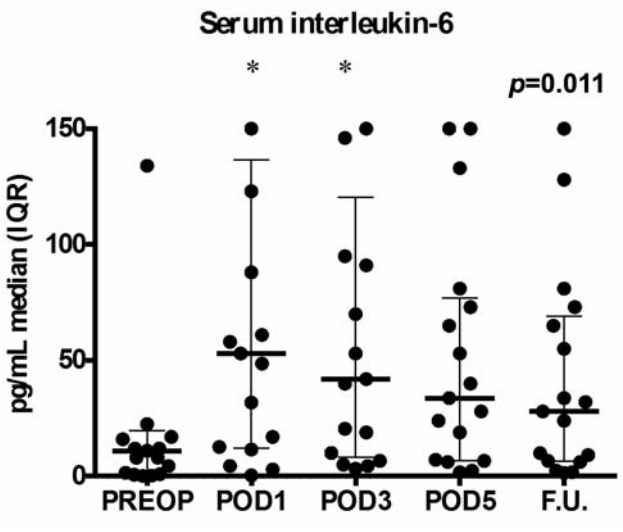

Figure 2. *Serum concentrations of interleukin 6 (IL-6). The figure depicts the change in concentrations of IL-6 during the perioperative period. FU: Follow-up; POD: postoperative day. Friedman test with Dunn's test for multiple comparisons. *p<0.05 compared to preoperative values.*

results demonstrate no changes in the postoperative concentrations of IL2 and IL4; however, we observed a significant increase in that of IL6. IL6 is secreted by a variety of cells, including macrophages, endothelial cells and adipocytes, and is considered as the principal mediator of the inflammatory response, proportional to the magnitude of trauma (22, 25-27). This cytokine can significantly depress the NKCC function and several authors have suggested a link between inflammation and immune suppression after surgery (28, 29). Thus, it is possible to speculate that our findings are consistent with these previous reports suggesting that an acute increase in plasmatic levels of IL6 immediately after surgery can be followed by a significant decrease in NKCC. IL6 signaling involves the activation of Janus kinases, which then trigger tyrosine phosphorylation and activation of signal transducer and activators of transcription (STATs) and other receptor-interacting proteins, such as SH2 (30, 31). Remarkably, an increased phosphorylation of STAT3 would be associated with a decrease in the killing activity of these cells (32).

Our study has several limitations, including a mixed population of pediatric and adult patients, a relatively small sample size, administration of adjuvant therapy, limited period of observation and inclusion of a variety of histological bone cancer types. Although the count of NK cells was lower in patients who received preoperative chemotherapy, we hypothesized that a further decrease in count and function is part of the stress response associated with surgery. Unfortunately, we did not measure the concentrations of other markers of stress response, such as cortisol and catecholamines, therefore we cannot comment on any potential association between the perioperative concentrations of those markers and our findings. Since non-steroidal analgesic drugs and corticosteroids were not used in our patient population, we cannot comment whether the use of these drugs would have modulated either the concentrations of IL6 or the count or function of NK cells. Unfortunately, clinical outcomes of cancer progression or recurrence were not measured in our patient population therefore, whether a transient decrease in count or a sustained impairment in the function of NK cells correlates with tumor progression remains unknown. Lastly, we observed a large spread in the concentrations of IL2 (nearly 100-fold) and IL6 (approximately 10-fold) that can be explained by several factors, including type of surgery, administration of blood products, preoperative adjuvant therapy and bulk of tumor disease.

In conclusion, a significant inflammatory response and innate immune suppression occurred after surgery for malignant bone tumors. We observed this as an increase in the postoperative levels of IL6, which could be related to the decrease in count and activity of NK cells. Nevertheless, further studies are required to confirm our results and investigate the potential link between IL6 and NK cell function. Understanding the response of the immune system to acute surgical stress can lead to development of strategies to avoid or ameliorate perioperative immune suppression.

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