Surgical Stress Impairs Natural Killer Cell Programming of Tumor for Lysis in Patients with Sarcomas and Other Solid Tumors

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Background. Natural killer (NK) cells may provide a first line of defense against the metastatic implantation of circulating tumor emboli. Because tumor emboli are discharged systemically in patients undergoing solid tumor resection, it is important to determine the nature of surgical-stress impairment of perioperative NK cell cytotoxic function.

Methods. The authors studied 85 patients undergoing surgical resection of solid tumors, most of whom had an abrupt and marked decrease in NK cell cytotoxicity that was detectable within 18 hours of surgical resection.

Results. This impairment was not caused by rapidly emerging suppressor cells (measured in autologous effector cell mixing studies) or decreased NK cell frequency in the peripheral blood (assessed phenotypically and morphologically). Instead, surgical stress exerted a direct "toxic" effect on NK cells that could be localized to a specific phase of the NK cell tumor lysis cycle. Tumor binding and the first round of tumor lysis were intact postoperatively (measured in single-cell assays). However, postbinding programming for lysis was decreased sharply after surgery (assessed by calcium pulse assays). In addition, the kinetics of lysis and the rate of lytic programming were slower after surgery (assessed in target saturation kinetic chromium-51 release tests).

Conclusions. These latter defects probably were related to the programming for lysis deficiency because postprogramming NK cell maximal recycling capacity was not affected by surgical stress. Cancer 1992; 70: 2192–2202.

Key words: sarcoma, surgical stress, natural killer cells.

The natural killer (NK) cell is an important immune effector cell that can kill tumor cells rapidly without prior sensitization or major histocompatibility complex restrictions. In animals, and possibly humans, the NK cell may provide a first line of defense against the metastatic implantation of circulating tumor emboli. Morphologically and phenotypically, the NK cell can be distinguished from T-cells, B-cells, and monocyte-macrophages; NK cells are also the major cellular constituent of the lymphokine-activated killer cell program. A multistep sequence of NK cell lysis of tumor has been identified. This consists of tumor target binding, NK cell-dependent programming of bound tumor for lysis (delivery of lethal hit), and NK cell-independent lysis with concomitant NK cell detachment from dying tumors and recycling to new tumor targets.

Surgical resection of solid tumor provides a major opportunity for cure in many patients; however, surgical procedures may cause perioperative tumor embolization from mechanical manipulation of the primary tumor. Successful implantation of such emboli may be correlated with the early local and distant tumor recurrence observed clinically. In addition, surgical stress impairs NK cell cytotoxicity; however, the nature of this impairment in humans is obscure. In small animal models, we elucidated a highly complex process underlying surgical-stress impairment of NK cell cytotoxicity. Small animal models also suggest a possible connection between surgical stress and the incidence, rate, and size of subsequent metastases.

These considerations may be relevant particularly to patients with sarcomas because of the aggressive and almost exclusively intravascular (i.e., nonlymphatic) pattern of metastasis, especially to the lungs, and the particularly radical surgical resection techniques usually required for sarcoma extirpation. In light of these factors, it may be important to determine the nature of surgical-stress impairment of human NK cell
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Cytotoxicity in patients with sarcoma and other solid tumors to improve their outcome.

Materials and Methods

Preparation of Fresh Effector Cells

We collected 30–60-ml aliquots of peripheral blood in heparinized syringes (typically 3–4 × 10^7 cells). Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation on a Ficoll-Hypaque gradient (Pharmacia, Piscataway, NJ) and adjusted to 2 × 10^7 PBMC/ml in supplemented RPMI-1640 tissue culture solution (sRPMI) as described elsewhere.

Preparation of Cryopreserved Normal Donor Lymphocytes

Normal donor lymphocytes were obtained by leukopheresis, and the buffy coats were collected. The buffy coat PBMC then were assayed against K562 targets in a 3-hour chromium-51 release assay before freezing. We used 1-ml aliquots of normal donor PBMC (3 × 10^7 PBMC in 1 ml of RPMI-1640 with 20% fetal calf serum and 10% dimethyl sulfoxide at 4°C). These were stepwise frozen down to −70°C (4°C for 30 minutes, −20°C for 2 hours, −70°C for up to 6 months storage before use). For thawing, frozen vials of cryopreserved PBMC were placed in a 37°C water bath for 2–4 minutes until thawed. The resultant cell suspension was mixed with 1 ml of sRPMI warmed to 37°C, washed twice at 200 × g for 10 minutes at room temperature, and then resuspended in sRPMI.

Nylon Wool Column Separation

Up to 5 × 10^7 PBMC were placed onto columns consisting of 0.8 g of presoaked nylon wool (Fenwal, Deerfield, IL) packed into the barrel of a 10-ml syringe. After incubation for 45 minutes at 37°C in a 5% CO₂ humidified atmosphere, nonadherent cells were collected by elution with 20 ml of warm sRPMI. Adherent cells were obtained by vigorously washing the column with cold Hanks balanced salt solution.

Plastic Adherence

Up to 1 × 10^8 cells (1 × 10^7 cells/ml sRPMI) were placed onto a 15 × 100-mm plastic tissue culture-grade Petri dish and incubated at 37°C in a 5% CO₂ atmosphere for 1 hour. Nonadherent cells were collected by decanting the media and washing the plate three times with 10 ml of warm (37°C) sRPMI. Adherent cells were collected by adding cold Dulbecco phosphate-buffered saline without Ca²⁺ or Mg²⁺ to the plate, followed by displacement of the cells using a rubber policeman.

Sheep Erythrocyte Rosetting

Sheep erythrocytes were obtained from the University of Texas Science Park (Smithville, TX). The sheep cells in 20% fetal calf serum in RPMI (2 × 10^9 sheep cells/ml) were mixed at a 100:1 ratio with PBMC in 5% fetal calf serum in RPMI (2 × 10^7 PBMC/ml). We placed 5-ml aliquots of this mixture on a Ficoll-Hypaque gradient (4 ml of 1.0779/ml Ficoll-Hypaque), and the solution was centrifuged at 800 × g for 30 minutes. After centrifugation, the interface cells were collected and washed in Hanks balanced salt solution before use in the assays.

Preparation of Target Cells

The NK-sensitive human erythroleukemia cell line K562 was used as the target in the NK assay and maintained in continuous culture in sRPMI as previously described. For use in the chromium-51 release assay, 2.5 × 10^6 K562 cells were incubated with 100 µCi of sodium chromate for 30 minutes at 37°C in a 5% CO₂ humidified atmosphere. The cells then were washed and adjusted to 1 × 10^6 cells/ml in sRPMI.

Chromium-51 Release Assay

NK cell cytotoxic activity was determined by the release of radioactivity from chromium-51-labeled K562 target cells as previously described.

Briefly, 50 µl of target cells (1 × 10^6 cells/ml) and 100 µl of effector cells (2 × 10^6 cells/ml) were plated in quadruplicate in 96-well microtiter plates. The effector-to-target ratios studied ranged from 200:1–3:1, depending on the experiment. The plates were incubated for 3 hours at 37°C in a 5% CO₂ humidified atmosphere. Released radioactivity was measured using a gamma scintillation counter. Spontaneous release was determined by incubation of labeled targets in medium alone and was consistently 6% or less of the total incorporated label. Percent NK cell cytotoxicity was calculated as [(experimental counts per minute − spontaneous counts per minute)/total incorporated label counts per minute] × 100.

Suppressor Cell Assay

Possible suppressor cell activity was determined by mixing a fixed number of preoperative refrigerated PBMC with increasing doses of fresh postoperative
PBMC (ranging from 1:1–1:3 mixtures). These mixtures were assayed in quadruplicate against radiolabeled K562 targets. To control for cell density, unmixed preoperative and postoperative PBMC effectors were tested against K562 targets at concentrations equal to effector-to-"suppressor" cell mixtures (e.g., 50:1, 100:1, 150:1, and 200:1 corresponded to the 1:0, 1:1, 1:2, and 1:3 effector-to-"suppressor" ratios, respectively).

**Depletion of NK Cells Using Monoclonal Antibody Treatment**

Depletion of NK cells was done using anti-Leu11b monoclonal antibody (Becton Dickinson, Mountain View, CA) and Low-Tox-MA rabbit complement (Accurate Chemical, Westbury, NY). Briefly, 0.5 μg anti-Leu11b was added to 5 × 10⁶ PBMC/ml in sRPMI and incubated for 45 minutes at room temperature. Rabbit complement (diluted 1:2 in sRPMI) then was added, and the resultant solution was incubated for 60 minutes at 37°C in a 5% CO₂ humidified atmosphere. After 60 minutes, PBMC were recovered and washed in sRPMI before use in the assay.

**Phenotypic Analysis**

Analyses were done using a Becton Dickinson FACScan equipped with FACScan research software. Anti-Leu11a conjugated to fluorescein isothiocyanate and anti-Leu19 conjugated to phycoerythrin fluorescent-labeled monoclonal antibodies were obtained from Becton Dickinson. PBMC (1 × 10⁶ PBMC/50 μl in Dulbecco phosphate-buffered saline and 1% sodium azide) were labeled with 20 μl of anti-Leu11a–fluorescein isothiocyanate complex and 20 μl of anti-Leu19–phycoerythrin conjugate. Then 50 μl of mouse immunoglobulin G₁–fluorescein isothiocyanate complex and 40 μl of immunoglobulin G₁–phycoerythrin conjugate (Becton Dickinson) were added to additional aliquots of PBMC to serve as isotype control solutions. The resultant solutions were refrigerated for 20 minutes at 4°C, washed in Dulbecco phosphate-buffered saline and 1% sodium azide, and resuspended at 1 × 10⁶ PBMC/ml before cytometric analysis.

**Morphologic Studies**

A total of 5 × 10⁴ PBMC were deposited on a glass slide by using a Shandon Cytospin II (Sewickley, PA). After air drying, the slides were stained for 5 minutes with May–Grünewald stain and counterstained for another 5 minutes with Giemsa stain. The morphologic distribution was determined by evaluating 300 cells/slide.

**Single-Cell Assay**

To determine the number of tumor target-binding cells and cytotoxic tumor-binding cells, a single-cell assay was done as described previously. Briefly, nylon wool and plastic nonadherent PBMC (2 × 10⁶ cells) and K562 target cells (4 × 10⁵ cells) were mixed together in 0.3 ml of sRPMI and incubated in glass tubes for 20 minutes at room temperature. The tubes were centrifuged at 250 × g for 5 minutes at room temperature; the cell mixture was resuspended in sRPMI and 1.33% agarose and then plated onto glass slides. The agarose was allowed to solidify for 1 minute, and then the slides were incubated at 37°C for 90 minutes. After incubation, the slides were stained for 5 minutes in 0.2% trypan blue fixed by 0.3% formaldehyde solution and stored until read (up to 1 week). The percentage of tumor target-binding cells was determined by counting the number of effector–tumor conjugates in 200 PBMC. The percentage of cytotoxic tumor-binding cells was determined by assessing bound target viability observed in 200 conjugates and correcting for spontaneous (unbound) target cytotoxicity. The frequency of cytotoxic tumor-binding cells (i.e., the frequency of effector cells that killed the bound tumor within 90 minutes of incubation) was calculated as [(% tumor target-binding cells) × (% cytotoxic tumor-binding cells)] × 100.

**Analysis of Maximum Velocity of Lysis (Vₘₐₓ)**

**Calculation of Rate of Lytic Programming, and Estimation of Percent “Active” Effector Cells and Maximal Recycling Capacity**

Vₘₐₓ of lysis and rate of lytic programming was analyzed by the Michaelis–Menten kinetics model using nylon wool and plastic nonadherent PBMC. The PBMC were studied in quadruplicate at 20 target-to-effector ratios (1 × 10⁵ PBMC versus 1 × 10⁴–2 × 10⁵ K562 targets). Each ratio incorporated an additional 1 × 10⁶ chromium-51-labeled K562 target cells. The plates were incubated for 90 minutes rather than 3 hours (to ensure first-order kinetics of lysis) at 37°C in a 5% CO₂ humidified atmosphere. Vₘₐₓ was calculated using linear regressions of Hanes plots. In the Hanes plot system, Vₘₐₓ equals 1/slope of the linear-regression line, and Kₘₐₓ/Vₘₐₓ equals y intercept of the regression line. The rate of lytic programming equals Vₘₐₓ/Kₘₐₓ and the percent “active” effector cells equals (Kₘₐₓ/[PBMC]) × 100. Maximal recycling capacity was determined by dividing Vₘₐₓ by the frequency of cytotoxic tumor-binding cells, as described elsewhere.
Calcium Pulse Assay

The calcium pulse assay was used to examine the programming for the lysis phase of NK cell cytotoxicity directly. We resuspended 50 μl each of effector cells (4 × 10^6 cells/ml) and chromium-51-labeled K562 target cells (1 × 10^5 cells/ml) in RPMI with 10% fetal calf serum, 1 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), and 2.5 mM MgCl₂. This suspension was placed in 96-well microplates (40:1 effector-to-target ratio). These cells were allowed to conjugate for 60 minutes at 37°C in a 5% CO₂ humidified atmosphere. Then 50 μl of 5 mM CaCl₂ in Hanks balanced salt solution without MgCl₂ was added to each well to initiate programming for lysis. Programming for lysis was terminated after 0, 5, 10, 15, 30, 60, and 90 minutes in successive triplicate wells by the addition of 50 μl of 40 mM ethylenediamine tetraacetic acid into each well.

Spontaneous-release wells were created similarly, except an additional 50 μl of sRPMI was used instead of the 50 μl of effector cells. After 3 more hours of incubation at 37°C in a 5% CO₂ humidified atmosphere, the plates were centrifuged at 500 × g for 10 minutes. Then 50 μl of supernatant was harvested to determine the NK cell cytotoxicity as in the standard chromium-51 release assay described earlier. Calcium pulse assay control samples included 3 and 5.5 hours of 40:1 effector-to-target ratio of chromium-51 release assays (no CaCl₂ pulse with sRPMI medium) and 5.5 hours of 40:1 effector-to-target ratio of chromium-51 release assays in RPMI 10% with 1 mM EGTA and 2.5 mM MgCl₂. All assays (including control assays) were done simultaneously using refrigerated preoperative and fresh postoperative effector cells.

Statistical Analysis

Student's t test for paired data was used for all statistical comparisons. Our data are presented as the mean ± the standard error of the mean.

Results

Time Kinetics of Postoperative NK Cell Impairment

We began our studies by examining the time kinetics of NK cell cytotoxicity after surgery to detect possible NK impairment. Patients with various malignant conditions had NK cell cytotoxicity determined immediately before surgery and then on days 1, 3, 5, and/or 7 postoperatively. To control for daily variability in target-cell
sensitivity to lysis in the chromium-51 release assay, each patient was paired with an individual cryopreserved normal donor who was assayed simultaneously (Fig. 1). There was variability in the rate, time kinetics, and nadir level of postoperative NK cell cytotoxic impairment. However, all patients studied had impaired NK cell cytotoxicity that could be observed within 18 hours of surgery. This decrease resulted from the effects of the surgical resection under general anesthesia per se and not from assay variability because the normal donors (tested simultaneously) did not show such declines.

Figure 2 depicts the NK cell cytotoxicity of 62 patients with solid tumors studied immediately before and 18 hours after surgery. This interval was selected for this and future studies because circulating tumor emboli would most likely successfully implant within 18 hours of surgical manipulation of the primary tumor. No significant differences were noticed between patient preoperative NK cell cytotoxicity and the cytotoxicity of cryopreserved normal donors. By contrast, the differences between preoperative and postoperative patient NK cell cytotoxicity were significant ($P < 0.0005$) at each effector-to-target ratio (mean postoperative decline at each ratio, 48.61% of preoperative value 18 hours after surgery).

NK cell cytotoxicity was analyzed stratifying for the clinical parameters of age, sex, tumor histologic findings, tumor stage, preoperative chemotherapy, preoperative radiation therapy, preoperative leukocyte differential, time between preoperative treatments and surgery, length of operation, and amount of blood transfused (Table 1). This analysis did not identify a subset of patients whose NK cell cytotoxic impairment was significantly worse than either the entire patient group or the stratification counterpart subset.

Figure 2. Effect of surgical stress on early perioperative NK cell cytotoxicity. Patients depicted ($n = 62$) include those from the time kinetic study (Fig. 1; $n = 10$), the mixing study (Fig. 4; $n = 30$), the morphology/phenotype study (Fig. 5; $n = 20$), and two patients from the $V_{\text{on}}$ studies (Fig. 8) who had sufficient cells available for a 3-hour chromium-51 release assay in addition to their other studies. Chromium-51 release assays were conducted as in Figure 1, except that the highest effector:target ratio studied in common in all 62 patients was 50:1; all patients were studied 18 hours after surgery. Cryopreserved normal donors ($n = 124$ assays) were also studied.

Table 1. Clinical Data of Patients in Study

<table>
<thead>
<tr>
<th>Variable</th>
<th>No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>56.3 ± 1.6</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>22-76</td>
</tr>
<tr>
<td>Sex (%)</td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>54 (63.5)</td>
</tr>
<tr>
<td>Women</td>
<td>31 (36.4)</td>
</tr>
<tr>
<td>Diagnosis (%)</td>
<td></td>
</tr>
<tr>
<td>Sarcoma</td>
<td>42 (48.2)</td>
</tr>
<tr>
<td>Carcinoma</td>
<td>33 (38.8)</td>
</tr>
<tr>
<td>Melanoma</td>
<td>3 (3.5)</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>1 (1.1)</td>
</tr>
<tr>
<td>Benign</td>
<td>6 (7.0)</td>
</tr>
<tr>
<td>Stage (%)</td>
<td></td>
</tr>
<tr>
<td>Primary</td>
<td>40 (47.0)</td>
</tr>
<tr>
<td>Recurrent</td>
<td>20 (23.5)</td>
</tr>
<tr>
<td>Metastatic</td>
<td>25 (29.4)</td>
</tr>
<tr>
<td>Preoperative chemotherapy (%)</td>
<td>31/85 (33.7)</td>
</tr>
<tr>
<td>Preoperative adjuvant radiation therapy (%)</td>
<td>21/85 (24.7)</td>
</tr>
<tr>
<td>Interval between preoperative adjuvant therapy and surgery (days) (range)</td>
<td>80.1 ± 12.4 (19-310)</td>
</tr>
<tr>
<td>Preoperative leukocyte count ($\times 10^3$)</td>
<td>5.9 ± 0.4 (1.4-9.5)</td>
</tr>
<tr>
<td>Duration of operation (min) (range)</td>
<td>316 ± 17 (45-800)</td>
</tr>
<tr>
<td>Transfusion (%)</td>
<td>32/85 (37.6)</td>
</tr>
<tr>
<td>Units transfused (range)</td>
<td>3.9 ± 0.6 (1-14)</td>
</tr>
</tbody>
</table>
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Figure 3. Observed NK cell cytotoxicity impairment 18 hours after surgery is not due to decreased NK cell frequency in the peripheral blood compartment. Left panel: 20 patients had their NK cell cytotoxicity assayed immediately before and 18 hours after surgery using fresh specimens; right panel: morphologic assessment (examination of cytoplasm differentials for percentage of large granular lymphocytes) and flow cytometric phenotypic assessment were also conducted on these same individuals.

granular lymphocytes and the percentage of PBMC that were CD16+, CD56+, or CD16+/CD56+ did not change within 18 hours after surgery, although NK cell cytotoxicity in these patients was impaired sharply postoperatively (P < 0.005 for all effector-to-target ratios).

Suppressor cells are not responsible for the postoperative decline in NK cell cytotoxicity. We investigated whether the observed NK cell cytotoxic impairment was related to the presence of a rapidly generated cell that suppressed NK cell cytotoxic function. If this were the case, then mixing studies might reveal the presence of such a suppressor cell. To avoid any potential allogeneic stimulation or inhibition effects, we used autologous mixtures. To create autologous mixtures, preoperative samples were refrigerated. Figure 4 shows that 18-hour refrigeration of PBMC did not affect NK cell cytotoxicity in either patients or normal donors.

Patients were studied for the presence of suppressor cells (Fig. 5). Increasing aliquots of fresh 18-hour postoperative effector cells (up to threefold increases) were mixed with a fixed aliquot of refrigerated preoperative effector cells (equivalent to the 50:1 effector-to-target ratio). In these mixtures, NK cell cytotoxic impairment caused by postoperative suppressor cells might be masked by the cytotoxicity of NK cells in the postoperative specimens. To eliminate this possibility, we removed NK cells from postoperative PBMC using anti-CD16 monoclonal antibody plus complement. Fifteen patients had sufficient numbers of 18-hour postoperative PBMC to examine all the effector-to-target and

Figure 4. Effect of refrigeration on normal and patient NK cell cytotoxicity. Patients (n = 10) and healthy donors (n = 10) had NK cell cytotoxicity assessed using fresh specimens, an aliquot of which was then refrigerated at 4°C for 18 hours before reassay the following day.
mixture ratios and also have an aliquot of PBMC treated with anti-CD16 monoclonal antibody plus complement (pretreatment percent of CD16− PBMC, 7.86 ± 1.59; posttreatment percent CD16+ PBMC, 0.80 ± 0.49; P < 0.05).

As increasing numbers of 18-hour postoperative PBMC were added to the fixed number of preoperative PBMC, levels of cytotoxicity resulted that were in between the cytotoxicity of preoperative and 18-hour postoperative cell number control samples, suggesting the possible presence of a suppressor cell. However, removal of CD16+ cells by pretreatment of the 18-hour postoperative PBMC with anti-CD16 monoclonal antibody plus complement before mixture resulted in 1:3 mixture cytotoxicity that was equivalent to the 1:0 mixture (1:3 mixture, 26.08% ± 3.03; 1:0 mixture, 24.13% ± 1.72). These results suggest that, after removal of CD16+ 18-hour postoperative cells, the remaining CD16− 18-hour postoperative cells (when placed in a mixture with preoperative cells) were functioning as filler cells (i.e., neither augmenting nor suppressing preoperative NK cell cytotoxicity).

It was still possible, however, that the CD16+ 18-hour postoperative cells were themselves functioning as suppressor cells. Several lines of evidence suggest that this was not the case. First, NK cell cytotoxicity was observed to increase at least twofold at the 200:1 compared with the 50:1 effector-to-target ratio using either preoperative or 18-hour postoperative effector cells. If the CD16+ cells were functioning as suppressor cells, then decreased or at least steady-state NK cell cytotoxicity would be anticipated as the number of CD16+ putative suppressor cells was increased at the higher effector-to-target ratios.

This line of analysis was extended to four patients with sarcoma whose preoperative and 18-hour postoperative effector cells were enriched for CD16+/CD56− content using serial Ficoll-Hypaque centrifugation, nylon wool filtration, plastic-plate adherence, and sheep erythrocyte rosetting (Table 2). This treatment resulted in at least a sevenfold increase in CD16+ cells when using either preoperative or 18-hour postoperative specimens. When the NK cell cytotoxicity of these enriched specimens was examined, markedly augmented (rather than suppressed) NK cell killing was observed. Moreover, despite comparable levels of NK cell enrichment, the cytotoxic gap between preoperative and 18-hour postoperative specimens remained (P < 0.05). Taken together, these results suggest that the surgical-stress impairment of NK cell cytotoxicity was not related to the generation of either CD16+ or CD16− suppressor cells.

Surgical-stress impairment of NK cell cytotoxicity involves decreased lytic Vmax and the rate of programming for lysis. We next considered the possibility that surgical stress exerted direct "toxic" effects on NK cells. Such effects might be manifested at one or more steps in the NK cell lytic cycle (i.e., at the level of tumor-target binding, programming for lysis, detachment, or recycling). To separate these possibilities, we used the single-cell assay in conjunction with the Michaelis–Menten model kinetic assay. We evaluated NK cell tumor binding and lytic activity, kinetics of total killing (Vmax), rate of lytic programming, and maximal recycling capacity. In the Michaelis–Menten kinetics model, effector cells are target saturated, and the assay time is short. These conditions ensure that effector cell killing has not reached a plateau and therefore follows first-order kinetics. As demonstrated in Figure 6, these requirements were satisfied using 1 × 105 effectors in a 90-minute chromium-51 release assay. Figure 7 illustrates the target saturation Vmax assay for a representative patient; the data are displayed in Hanes plots.

Figure 8 depicts the results of the kinetic experiment. NK cell cytotoxicity was impaired significantly 18 hours after surgery at all effector-to-target ratios (P

Table 2. Enrichment for CD16+/CD56− Cells Leads to Cytotoxic Augmentation Rather Than Suppression

<table>
<thead>
<tr>
<th>% CD16+/CD56− phenotype</th>
<th>NK cell cytotoxicity (E:T ratio)*</th>
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<tbody>
<tr>
<td></td>
<td>3:1</td>
</tr>
<tr>
<td>Preoperative</td>
<td>7.38 ± 2.96</td>
</tr>
<tr>
<td>Preoperative enriched</td>
<td>55.38 ± 0.98</td>
</tr>
<tr>
<td>Postoperative</td>
<td>6.57 ± 2.47</td>
</tr>
<tr>
<td>Postoperative enriched</td>
<td>49.58 ± 3.35</td>
</tr>
</tbody>
</table>

NK: natural killer; E:T: effector to target.
*P < 0.05 for differences between preoperative enriched and postoperative enriched NK cell cytotoxicity.

Four patients with sarcoma had their preoperative and 18-hour postoperative NK cell cytotoxicity assessed in 3-hour chromium-51 release assays.

Enrichment for CD16+/CD56− cells was accomplished by serial Ficoll-Hypaque centrifugation, nylon wool filtration, plastic-plate adherence, followed by sheep erythrocyte resetting.
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Figure 6. Demonstration of first-order kinetics of killing in target saturation chromium-51 release assays. Normal and patient donors were assayed for 30, 60, 90, and 120 minutes using 1 x 10^6 and 5 x 10^6 effector cells versus radiolabeled K562 targets. A representative normal donor experiment is shown; patient assays demonstrated equivalent kinetics. Black circle: 90-minute assay using 5 x 10^6 effectors; black square: 30-minute assay using 5 x 10^6 effectors; X: 120-minute assay using 1 x 10^6 effectors; white circle: 90-minute assay using 1 x 10^6 effectors; white square: 30-minute assay using 1 x 10^6 effectors.

< 0.01) even though the assay time was only 90 minutes (by contrast with the 3-hour cytotoxicity studies). This cytotoxic impairment apparently was not caused by possible postoperative defects in tumor-binding properties or the frequency of lytic NK cells because the percent tumor target-binding cells, the percent cytotoxic tumor-binding cells, the percent of "active" effectors, and the percent of large granular lymphocytes were not affected by surgery (Table 3).

When the effector cells were free to kill under first-order kinetic conditions (Fig. 8), marked impairment in the 18-hour postoperative V_max was observed (P = 0.008). In addition, the calculated rate of lytic programming under these conditions also decreased markedly postoperatively (P = 0.004). These alterations were apparently not the result of maximal recycling capacity; this was not affected by surgery.

Surgical stress impairment of NK cell cytotoxicity involves surgically induced defects in calcium-dependent programming for lysis. Tumor binding and recycling capacity after tumor lysis were intact postoperatively, whereas V_max and calculated rate of lytic programming were impaired sharply. This suggests that the surgically induced NK cell defect might be found in the intermediate calcium-dependent programming for lysis step of the lytic cycle. This possibility was addressed directly using the calcium pulse assay (Fig. 9).

Programming for lysis depended absolutely on calcium, as demonstrated by the lack of target cell lysis in control 5.5-hour assays conducted in medium containing EGTA and MgCl_2 without CaCl_2 using preoperative or 18-hour postoperative effector cells. At each of the seven assay times after calcium pulsation, major differences between preoperative and 18-hour postoperative NK cell cytotoxicity were observed, suggesting that surgical stress induced an immediately detectable and persistent defect in NK cell programming of tumor targets for lysis (P = 0.002 for 0, 5, 10, and 15-minute calcium pulses; P < 0.005 for 30, 60, and 90-minute calcium pulses).

Discussion

Surgical stress impairs perioperative NK cell cytotoxicity in various human tumors, including those in patients undergoing resections for gastrointestinal tumors, colorectal tumors, ovarian carcinoma, breast carcinoma, and other malignant lesions, such as sarcomas. Comparable NK cell derangements also were observed in patients undergoing surgery for nonmalignant conditions, such as living-related kidney donation, total hip replacement, and cardiopulmonary bypass procedures. However, the mechanism underlying the phenomenon of surgical stress-mediated im-

Figure 7. Target saturation V_max assay incorporating Hanes plot analysis. Left panel: NK cell cytotoxicity at 20 target:effector ratios; center panel: velocity of lysis/hour at a given target:effector ratio, calculated as percent NK cell cytotoxicity times number of targets available at that ratio; right panel: Hanes plot transformation of the data (T versus T/ν); r² = 0.94 ± 0.01 for the 32 kinetic assay linear-regression lines of Figure 8.
impairment of human NK cell cytotoxicity has remained obscure.

Our studies found a consistent pattern of rapid and massive loss of NK cell cytotoxicity in the immediate perioperative period. This loss occurred in patients regardless of age, sex, benign versus malignant diagnosis, histologic findings of malignancy, stage of tumor, length of operation, amount of blood loss or transfusion, or the use of preoperative therapy.

The nature of this early perioperative surgical stress-induced NK defect did not involve either rapidly emerging suppressor cells or decreased NK cell frequency in the peripheral blood compartment. Our findings localized the defect in the NK cell cytolytic process that was induced by surgical stress. Tumor target identification and binding, the first step in NK cell cytotoxicity, was not affected by surgery. Likewise, the final NK cell cytotoxicity step of recycling to new tumor targets also was unchanged by surgical stress. However, the intermediate step of NK cell programming tumor for lysis (delivery of lethal hit) was impaired at both the rate and total tumor programming levels. The magnitude of the impairment at this intermediate programming level could explain the markedly decreased total tumor target killing observed in both the target saturation rate assay and the standard chromium-51 release assay, particularly because postprogramming NK cell maximal recycling capacity was equivalent before and after surgery. Our studies did not address directly the possible defects in NK cell detachment from dying tumor targets (NK cell-independent lysis). However, the lack of surgical effects on both the frequency of NK cells (as assessed morphologically, phenotypically, and functionally) and NK cell maximal recycling capacity make problems with detachment unlikely.

The potential clinical relevance of this NK cell dysregulation was suggested by a long-standing clinical awareness that tumor embolization can occur during surgery and the immediate perioperative period.23 It is possible that these embolic factors may be related to clinical problems of local tumor recurrence, tumor implantation or outgrowth in surgical wounds, and early distant metastatic implantation secondary to surgical impairment of regional visceral immunity. In the rat, impaired blood stream clearance and increased pulmonary localization of circulating tumor emboli has been noticed as early as 1 hour after surgical stress.23 In the 85-patient cohort we studied, 4 of the 41 patients with sarcomas (and none of the other patients) had local or distant recurrence within 2–3 months of a putatively curative resection.

The role of NK cells in the control of human non-lymphoid solid tumors is still not clear. However, hu-

![Figure 8. Mechanism of surgical NK cell impairment involves decreased maximal velocity of lysis (V_max) and rate of lytic programming. Sixteen patients (ten with sarcomas) had simultaneously conducted 90-minute chromium-51 release assays (50:1–6.25:1 effector:target ratios), kinetic assays, and single-cell assays using preoperative and 18-hour postoperative effector cells. Effector cells were enriched using sequential Ficoll-Hypaque centrifugation, nylon wool filtration, and plastic adherence. Left panel: preoperative and postoperative NK cell cytotoxicity; right panel: preoperative and postoperative V_max rate of lytic programming, and maximum recycling capacity.](image-url)
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Figure 9. Calcium pulse assays show defect in NK cell programming of targets for lysis. Seven patients (five with sarcoma) were studied before and 18 hours after surgery. After 60 minutes of effector:target conjugation in EGTA and MgCl₂ (40:1 effector:target ratio), calcium pulses of various length were applied and then terminated by the addition of ethylenediamine tetraacetic acid. After an additional 3 hours of incubation time, percent NK cell cytotoxicity was determined as above. Bars on right are NK cell cytotoxicity time controls: Standard 3-hour and 5.5-hour chromium-51 release assays and 5.5-hour chromium-51 release assay for all preoperative and postoperative specimens in calcium depleted medium.

man studies suggest that a broad range of solid tumors are sensitive to NK cell lysis if fresh autologous tumor targets and highly enriched autologous NK cells are studied. In animal models, NK cell control of intravascular tumor dissemination has been delineated. In other spontaneous metastasis animal models, surgical stress has been associated with increased metastatic tumor growth of Lewis lung carcinoma and other tumor systems. We also examined the nature of surgical-stress impairment of murine NK cell cytotoxicity; a complex multifactorial process was elucidated.

The surgical milieu is a rapidly changing host environment where acute hormonal and metabolic perturbations occur, including markedly increased glucocorticoid, catecholamine, and prostaglandin secretion and turnover. Complex cytokine alterations involving interferons, tumor necrosis factors, interleukin-1, interleukin-2, interleukin-6, epidermal growth factor, and transforming growth factor beta also have been described during the perioperative period. All these acute stress responses are imposed on a host concomitantly burdened with temporary anesthetic-induced reticuloendothelial impairment, marked surgical wound inflammatory responses, and severe central nervous system-mediated pain responses that usually require narcotic analgesia. Currently, it is not clear how these factors, singly or together, may contribute to the observed impairment of NK cell cytotoxicity. However, many of these host perturbations can decrease NK cell cytotoxicity in vitro and in vivo.

The calcium-dependent programming for lysis defect may be the result of alterations in signal transduction across the NK cell surface by molecules such as CD2, CD11a/CD18, CD16, the p70-75 interleukin-2 receptor, or NKR-P1. Intracellular biochemical processes (such as inositol phosphate hydrolysis, protein kinase C, or protein tyrosine kinase activation, or alterations in intracellular calcium concentrations) also may be relevant. Alternatively, surgical stress may impair the quantity or lytic effectiveness of perforins or granzymes produced by the NK cell, a process that is itself calcium dependent. Research is ongoing to determine the underlying molecular and biochemical events contributing to the surgical stress-mediated programming for lysis defect in NK cells we identified.

Our study was based on the premise that the perioperative period may be important as a focus for future immunotherapeutic trials because the tumor burden is decreased maximally. As an additional incentive, increased quantities of tumor emboli may be discharged mechanically by perioperative manipulation, precisely at a time of acute host immunosurveillance impairment. Patients with sarcoma may be a particularly important population because of the aggressive and almost exclusive pattern of intravascular metastasis especially to the lungs and the radical and physiologically stressful surgical resection techniques usually required for sarcoma extirpation. Because of the importance of NK cells as progenitors in lymphokine-activated killer cell schemes, future in vivo activation treatment programs used during the perioperative period may need to address the cytotoxic impairments we identified. Such future efforts may generate therapeutic interventions that will help patients undergoing tumor resection.

References

1. Barlozzari T, Reynolds CW, Herberman RB. In vivo role of natural killer cells: involvement of large granular lymphocytes in the


