Suppression of natural killer cell activity by surgical stress in cancer patients and the underlying mechanisms.

Hisashi Yoshihara* Noriaki Tanaka† Kunzo Orita‡
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Abstract

The influence of surgical stress on the natural killer (NK) activity of peripheral blood lymphocytes in patients with carcinoma of the lung or gastrointestinal system was studied. The peripheral blood lymphocytes of the patients showed a marked decrease in NK activity against K-562 cells as target cells 1-2 days after surgery. The activity remained lowered for 2 weeks after thoracotomy and for 1 week after laparotomy. No appreciable suppression of NK activity was observed with normal human peripheral blood lymphocytes preincubated with postoperative patient sera. Peripheral blood mononuclear cells obtained postoperatively from patients lost NK activity after ultraviolet irradiation, without any detectable loss of viability. Such irradiated mononuclear cells showed inhibition of NK activity after a 24-hour preincubation with peripheral blood lymphocytes from normal subjects. Similar suppressive activity was demonstrable in a fraction of mononuclear cells with adhesiveness to plastic petri dishes, while non-adherent cells had no such activity. When added immediately to the cytotoxicity assay system without the 24-hour preincubation, patient mononuclear cells caused no inhibition of NK activity, whereas adherent cells from normal subjects enhanced NK activity. The findings seems to indicate that, following surgical stress, plastic dish-adherent peripheral blood mononuclear cells become deprived of NK helper activity and exert suppression, thus causing postoperative depression of NK activity.

KEYWORDS: natural killing, suppressor cell, surgical stress

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Suppression of Natural Killer Cell Activity by Surgical Stress in Cancer Patients and the Underlying Mechanisms

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The influence of surgical stress on the natural killer (NK) activity of peripheral blood lymphocytes in patients with carcinoma of the lung or gastrointestinal system was studied. The peripheral blood lymphocytes of the patients showed a marked decrease in NK activity against K-562 cells as target cells 1-2 days after surgery. The activity remained lowered for 2 weeks after thoracotomy and for 1 week after laparotomy. No appreciable suppression of NK activity was observed with normal human peripheral blood lymphocytes preincubated with postoperative patient sera. Peripheral blood mononuclear cells obtained postoperatively from patients lost NK activity after ultraviolet irradiation, without any detectable loss of viability. Such irradiated mononuclear cells showed inhibition of NK activity after a 24-hour preincubation with peripheral blood lymphocytes from normal subjects. Similar suppressive activity was demonstrable in a fraction of mononuclear cells with adhesiveness to plastic petri dishes, while non-adherent cells had no such activity. When added immediately to the cytotoxicity assay system without the 24-hour preincubation, patient mononuclear cells caused no inhibition of NK activity, whereas adherent cells from normal subjects enhanced NK activity. The findings seems to indicate that, following surgical stress, plastic dish-adherent peripheral blood mononuclear cells become deprived of NK helper activity and exert suppression, thus causing postoperative depression of NK activity.

Key words: natural killing, suppressor cell, surgical stress

Many experimental and clinical studies have demonstrated depression of immune responsiveness associated with operative and accidental trauma. This decline in immune function may involve both humoral immunity and cell-mediated immunity but, in most instances, pertains primarily to the latter, i.e., depression of lymphocyte blastogenesis, polymorphonuclear leukocyte migration inhibition, delayed hypersensitive skin reaction, cell-mediated cytotoxicity and antibody-dependent cell-mediated cytotoxicity to tumor cells (1-5).

The mechanism underlying the postoperative suppression of immune responsiveness has not been well elucidated. Corticosteroids, prostaglandin E (PGE), immunosuppressive acidic protein (IAP) and other serum inhibitors are probably associated with the suppression. Participation of suppressor cells in the depression of lymphocyte blastogenesis after thermal injury and mixed lymphocyte reactions after surgical trauma has been pointed out (6, 7). Our previous report described activation of suppressor T cells among splenocytes of mice following surgical stress, as tested with reference to a local graft-vs-host reaction.
When viewed with respect to immunological surveillance against malignant tumors, the depression of cellular immunity associated with surgical stress may facilitate metastasis and growth of tumors throughout the intra- and postoperative course. Experimental evidence of increased metastasis by surgical stress has been reported (9,10). Natural killer cells are present in relative abundance in the peripheral blood and spleen and seem to prevent hematogenous spread of tumors. We have recently observed depression of natural killer (NK) cell activity after major surgery in cancer patients. This paper presents the results of experiments conducted to explore the underlying mechanism of suppression.

Materials and Methods

Separation of peripheral blood mononuclear cells. Heparinized peripheral blood drawn early in the morning prior to eating was diluted twofold with physiological saline and subjected to centrifugation on Ficoll-Conray gradients (specific gravity: 1.077) at 2,000 rpm for 30 min. Lymphocyte-rich mononuclear cells suspended at the border between the plasma and Ficoll-Conray gradients were collected, washed three times with phosphate buffered saline (PBS) by centrifugation at 1,200 rpm for 5 min, and resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units of penicillin and 100 μg of streptomycin per ml, and 25 mM of N-2-hydroxyethylpiperazine N'-ethane sulfonic acid (HEPES).

Cell separation. Three to four milliliter of FBS previously inactivated by heating at 56°C for 30 min was added to plastic Petri dishes (9 cm in diameter; Falcon), which were tilted to permit the serum to cover the entire bottom surface and allowed to stand overnight at 4°C to be used as serum-coated plates. To each plate, 6 ml of the mononuclear cell suspension in RPMI-1640 medium containing 2 to 3×10⁴ cells/ml was added and incubated for 60 min at 37°C under a 95% air-5% CO₂ atmosphere. The culture dish was rinsed three to four times with the medium prewarmed at room temperature to obtain non-adherent cells. The rinsed dishes were incubated with an appropriate amount of PBS containing 0.2% EDTA and 5% FBS for 15 min at 4°C and then subjected to 4–5 pipettings with cold medium to facilitate detachment of adherent cells. The detached adherent cells were collected and washed two to three times with PBS.

The adherent cells contained more than 90% monocytes as judged by esterase staining. This fraction is hereafter referred to as "monocytes", and the non-adherent cell fraction is referred to as "lymphocytes".

Preparation of target cells. The target cells used in this study were K-562 cells, cells of a human leukemia cell strain, and cells of a measles virus-infected HeLa cell (M-HeLa) strain supplied by the Department of Pediatrics, Okayama University Medical School. K-562 cells (5×10⁶) were suspended in 0.2 ml of complete RPMI 1640 medium and incubated with 200 μCi of radioactive sodium chromate (⁶⁷Cr) for 1 h at 37°C in an atmosphere of 95% air and 5% CO₂. The cells were then washed three times with the medium, counted, rechecked for viability, and resuspended in the complete medium at a final concentration of 5×10⁴ cells/ml. The labeled K-562 cell suspension was pipetted into wells of a type II Microtest plate with 5,000 cells in 100 μl per well. M-HeLa cells were cultivated in Microtest plates for 12 h at 37°C in a 95% air-5% CO₂ atmosphere. After discarding suspended cells, the M-HeLa cells adhering to the bottom of wells were further incubated with 2 μCi of Na₂¹⁸CrO₄ in 0.2 ml of medium per well for 5–6 h. The cells were then washed twice with the medium, followed by addition of 100 μl of fresh complete medium to each well.

Cytotoxicity assay. To each well of radio-labeled K-562 and M-HeLa cell cultures, 100 μl of a lymphocyte suspension was added to yield an effector-target cell (E/T) ratio of 50 to 1 and 100 to 1, respectively. The cultures were incubated for 6 (K-562) or 12 (M-HeLa) h. At the end of the incubation period, 100 μl of supernate was aspirated from each well and radioassayed for released ⁶⁷Cr. The %⁶⁷Cr release was calculated by the formula:

http://escholarship.lib.okayama-u.ac.jp/amo/vol40/iss2/7
Experimental % release =
\[
\frac{\text{Experimental CPM} - \text{Spontaneous CPM}}{\text{Total CPM} - \text{Spontaneous CPM}} \times 100
\]

All experiments were performed in triplicate, and data were expressed as the mean.

Control cultures with lymphocytes from normal subjects were set up in each experiment, and corrections were made for experimental errors using the formula:

Corrected % cytotoxicity =
\[
\frac{\text{Experimental} \% \text{ cytotoxicity}}{\text{Control} \% \text{ cytotoxicity}} \times \text{Average} \% \text{ cytotoxicity}
\]

In this formula, % cytotoxicity is the mean of control % cytotoxicity values of 10 independently conducted experiments.

Loss of NK activity after ultraviolet irradiation. A mononuclear cell suspension in RPMI-1640 medium at a concentration of \(1 \times 10^6\) cells/ml was placed in plastic Petri dishes (6 cm in diameter; Falcon), 5 ml per dish, and irradiated with a 15-watt ultraviolet lamp held about 40 cm above the dishes. As shown in Fig. 1, the NK activity of cells diminished to 58.4%, 42.5% and 8.8% of control (non-irradiated) cells 10, 20 and 30 min after irradiation, respectively. The cells irradiated for 30 min showed 100% viability 1 h after irradiation, and an 80% viability after 6 h that are required to complete the assay of NK activity (Figs. 1, 2).

Results

Influence of surgical stress on NK activity. Peripheral blood lymphocytes were serially followed for postoperative changes in NK activity in patients having undergone laparotomy or thoracotomy. The mean duration of the operations was 244 min with an average blood loss of 437 ml in the laparotomy group, and 173 min with an average blood loss of 410 ml in the thoracotomy group.

The NK activity of peripheral blood lymphocytes, as measured using M-HeLa cells as the target cells, diminished markedly about 1–3 days after surgery. The activity returned virtually to the initial level within 7 days of the operation in the laparotomy

Fig. 1 Loss of NK activity after ultraviolet irradiation.

Fig. 2 Influence of ultraviolet irradiation on the viability of mononuclear cells.

Fig. 3 Influence of surgical stress by thoracotomy on NK activity tested against K 562 cells at an effector-to-target cell ratio of 50 : 1.
group, whereas in the thoracotomy group, it remained depressed after 7 days.

When assessed with K-562 cells as the target cells, peripheral blood lymphocytes showed remarkably depressed NK activity in both groups 7 days after surgery. The activity returned to the preoperative level by the 14th postoperative day in the laparotomy group, while there was a noticeable delay in its recovery in the thoracotomy group (Figs. 3, 4).

**Effect of postoperative patient serum on the NK activity of lymphocytes from normal subjects.** Peripheral blood lymphocytes (2 × 10^6 cells) of normal subjects were preincubated with heat-inactivated patient serum (1 ml) obtained on the 3rd postoperative day at 37°C for 1 h in a 95% air-5% CO₂ atmosphere, and tested for NK activity to find out whether the postoperative patient serum might influence lymphocyte cytotoxicity. The tests revealed no significant difference among the control group (preincubated with AB serum), the patient serum-treated group and the non-serum-treated group (Table 1).

**Suppression of NK activity by UV-irradiated mononuclear cells from postoperative patients.** To the peripheral blood lymphocyte suspension of a normal subject, UV-irradiated mononuclear cells from postoperative patients or normal subjects were added at a cell population ratio of 1 to 1, and the cell mixtures were incubated in a moist atmosphere of 95% air and 5% CO₂ for 20 h at 37°C. The cultures were then tested for NK activity. In 4 of the 5 cases studied, the cultures preincubated with UV-irradiated patient mononuclear cells (P.UV. PBMC) showed NK activities 30–51% lower than in the cultures preincubated with UV-irradiated normal donor mononuclear cells (non-U.V. PBMC) (Table 2).

**Table 1** Effects of postoperative patient serum on NK activity*

<table>
<thead>
<tr>
<th>Normal lymphocytes</th>
<th>Percent cytotoxicity to M-HeLa cells at</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50 : 1^b</td>
</tr>
<tr>
<td>Medium</td>
<td>19.3 ± 11.3</td>
</tr>
<tr>
<td>Normal AB serum^c</td>
<td>17.0 ± 11.9</td>
</tr>
<tr>
<td>Patient serum</td>
<td>17.2 ± 11.9</td>
</tr>
</tbody>
</table>

^a: Results are expressed as means ± SE of 8 individuals. ^b: Effector-to-target cell ratio. ^c: Blood type: AB positive.

**Table 2** Effects of preincubation with UV-irradiated mononuclear cells on the NK activity of normal lymphocytes

<table>
<thead>
<tr>
<th>Exp. no.</th>
<th>Percent cytotoxicity against K 562 cells of normal lymphocytes^a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UV-irradiated postoperative mononuclear cells added</td>
</tr>
<tr>
<td>1</td>
<td>8.9</td>
</tr>
<tr>
<td>2</td>
<td>10.6</td>
</tr>
<tr>
<td>3</td>
<td>11.1</td>
</tr>
<tr>
<td>4</td>
<td>9.0</td>
</tr>
<tr>
<td>5</td>
<td>15.4</td>
</tr>
</tbody>
</table>

^a: Effector-to-target cell ratio was 50 : 1.
were separated into monocytes and lymphocytes by adhesiveness to plastic Petri dishes and tested for their suppressor activity toward NK cells of normal subjects. In these experiments, adherent cells were used without any pretreatment as they were devoid of NK activity (data not shown), while non-adherent cells were pretreated by UV-irradiation to eliminate NK activity. These cells were added as regulators to a suspension of lymphocytes from a normal donor at a ratio of 1:1. The mixtures were preincubated for 20 h, and then tested for cytotoxicity. In 4 of the 5 postoperative cases studied, significant suppression of NK activity of normal lymphocytes by addition of postoperative monocytes was observed, whereas no such suppression occurred with postoperative lymphocytes (Table 3).

To discover whether or not the preculture of suppressor monocytes and NK cells is required for suppression, we tested postoperative monocytes for NK activity suppression without preincubation. The addition of monocytes from postoperative patients to a mixture of effector and target cells did not inhibit NK activity in 3 of the 4 cases studied. Unexpectedly, enhancement of NK activity was observed in the presence of monocytes from normal donors (Table 4).

**Discussion**

It has been demonstrated that depression of NK cell activity results in increased incidence of metastasis in experimental tumor-bearing beige mice (11, 12). Since the NK activity is prominent in the peripheral blood and spleen, and is low in the lymph nodes and neoplastic lesions (13), it appears that NK cells play a part in impeding hematogenous metastasis by way of destruction of tumor cells that have entered the bloodstream.

Most patients with malignant tumors show low levels of NK cell activity, which is further depressed over 1–2 weeks following surgical stress (11). Surgical manipulations tend to cause malignant cells to enter peripheral circulation (14), and, therefore, the postoperative marked depression of NK activity may facilitate hematogenous metastasis of cancer. Particularly, tumors of the lungs, where blood perfusing the organ flows directly into the general circulation without filtration through the liver or any other organs, have greater chance for metastasis via the blood stream. It seems to be of vital importance to clarify the mechanism whereby NK activity declines following surgical stress.

Certain serum factors are thought to be associated with the suppression of NK activity. It has been reported that sera from
tumor-bearing mice and those of cancer patients showed suppression of NK activity (15, 16). Hidig reported inhibition of human natural cytotoxicity by macromolecular antiproteases such as antitrypsin (17). Occurrence in postoperative patient sera of a circulating polypeptide with the ability of inhibiting PHA-induced lymphocyte blastogenesis has also been described (18). In the present series, patient serum did not show any appreciable inhibition of NK activity. This discrepancy may be attributed to the difference in preincubation times.

Besides humoral factors, there have been studies demonstrating the presence of cells which suppress NK cell activity. Such suppressor cells are induced among splenocytes of mice injected with Corynebacterium parvum, carrageenan or hydrocortisone (19, 20). The suppressor cells induced with carrageenan or hydrocortisone are thought to be macrophage-like cells, and those seen in infant mice and in radiation-treated mice to be null cells (20). Uchida noted the occurrence of monocytes with NK suppressor activity among peripheral blood mononuclear cells from patients after surgery for breast cancer (21).

A technical difficulty inherent in experiment to demonstrate the presence of NK suppressor cells is the unavoidable contamination by NK cells in suspensions of cells collected as suppressor cells. It was found in this investigation that ultraviolet irradiation of the cell suspension for a brief period almost completely deprived cells of NK activity without affecting their viability. Results of experiments employing this technique indicated the presence of NK suppressor cells in the postoperative peripheral blood mononuclear cell population, with profound suppressive activity in the fraction of cells with adhesiveness to plastic petri dishes.

It was essential to preincubate the suppressor cells with the effector cells for 20 h to obtain a detectable inhibition of NK cell activity in the experimental system described here. No appreciable inhibition occurred upon mere addition of the suppressor without such preincubation. In contrast, enhancement of NK activity was observed following addition of monocytes from normal subjects into the assay system. Several reports have described that macrophages have the ability to augment NK activity (22). Accordingly, it is speculated that macrophages may either inhibit or enhance NK activity, depending upon the condition in which they are placed.

Concerning mechanisms of activation of suppressor cells after operative or accidental trauma, there have been reports demonstrating that histamine activates suppressor T cells with consequent depression of antibody formation and that immunosuppressive acidic protein exerts an influence upon the synthesis of prostaglandin E by macrophages (6,7,23,24). However, the mechanism of suppressor cell activation against NK cell activity is yet to be elucidated.

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