Surgical Stress-mediated Suppression of Murine Natural Killer Cell Cytotoxicity

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ABSTRACT

Natural killer cell-mediated cytotoxicity (NKCC) is one of several possible immune defense mechanisms that may protect against the development of solid-tumor metastases. We have demonstrated that in vitro NKCC can be significantly impaired by both surgical stress and progressive tumor burden. Female C57BL/6 mice received a hindfoot amputation under anesthesia with Nembutal i.p. Twenty-four hr later, amputated and control groups were sacrificed, spleens were harvested, and cytotoxicity assays were performed using 51Cr-labeled Yac-1 lymphoma target cells. In amputated animals, in vitro NKCC was significantly impaired at four effector:target ratios, decreasing by as much as 59%. Nembutal treatment alone caused no significant changes in in vitro NKCC compared to untreated controls. Tumor burden was studied by inoculating the hindfoot pads of C57BL/6 mice with 5 x 10^5 Lewis lung tumor cells. Animal groups were sacrificed 24 hr, 1 week, and 2 weeks after tumor inoculation, and the 51Cr release assay was performed. One day and 1 week of tumor burden mildly stimulated NKCC in vitro; after 2 weeks of tumor burden, when lung metastases were detectable, in vitro NKCC was almost totally suppressed compared with non-tumor-bearing controls. Animals bearing tumor for 1 week and then given amputations showed significantly impaired NKCC in vivo. In vivo, identical animals bearing tumor for 1 week and then given amputations on sacrifice 1 week later were found to have a 71% incidence of lung metastases compared with 38% tumor-bearing unstressed controls. Surgical stress and progressive tumor burden independently and codependently impair NKCC in vitro; this may possibly contribute to the hypermetastatic response observed after surgical stress in this in vivo animal model.

INTRODUCTION

Attention recently has focused on a possible role for natural killer cells as a factor influencing solid-tumor metastasis. The ability to eliminate circulating tumor emboli is closely associated with the level of host NKCC3 (7–10). Low NKCC results in increased tumor cell survival in the blood stream and metastatic pulmonary lodgment (7). Tumor cells from metastatic sites tend to be more resistant to NKCC in vitro than are cells from the primary tumors from which they are derived (6). These considerations assume additional practical importance since many experimental models have demonstrated that surgical stress increases the size, rate, and number of metastases in tumor-bearing animals relative to unstressed tumor-bearing controls (13, 14, 16, 21). Surgical stress has also been shown to increase circulating corticoids (16); to inhibit macrophage function (22), leukocyte migration and cytotoxicity (3, 26), and phthologagglutinin-induced lymphocyte transformation (17, 20); to decrease B-cell function as measured by pokeweed mitogen stimulation (12); and to decrease NKCC (25, 26) and decrease the number of circulating T-cells (23).

The impact of surgical stress and tumor burden on NKCC as an independent and codependent variables has not been established. The important issue of this potential relationship is whether surgical stress induction of tumor metastasis, alluded to in the cited models, may possibly be due in part to the surgical impairment of NKCC. Results of this study clearly demonstrate that surgical stress significantly interferes with NKCC in tumor-bearing and tumor-free animals as measured in vitro. Enlarging tumor burden likewise causes a significant depression of NKCC in vitro. These in vitro effects may correlate with increased metastatic activity in surgically stressed tumor-bearing animals in vivo relative to unstressed tumor-bearing controls.

MATERIALS AND METHODS

Animals. Specific-pathogen-free female C57BL/6 mice were obtained from the Charles River Breeding Laboratories, Wilmington, MA. Animals 6 to 10 week old, housed 8/cage, were allowed food and water ad libitum. All animals were acclimatized for 10 days prior to experimentation.

Surgical Stress. General anesthesia was induced by i.p. injection of 0.1 ml Nembutal (5 mg/ml) per 10 g body weight; hind limb amputation was performed above the knee. The amputated stumps were hemostatically secured with chromic suture.

Preparation of Effector Cells. Animals were sacrificed by cervical dislocation followed by cellotomy and splenic harvesting. Spleen cells were retrieved by forceful injection of HBSS into the splenic capsule. The resultant suspension was then centrifuged at 800 x g for 10 min, and the supernatant was discarded. RBC were removed by hypotonic lysis with distilled water.

After being washed in HBSS, the remaining cells were resuspended in RPMI 1640 (Grand Island Biological Co., Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (Irving Scientific, Santa Ana, CA), 20 mm N-2-hydroxypiperazine-N’-2-ethansulfonic acid (Research Organics, Cleveland, OH), and antibiotics (penicillin and streptomycin). This mixture was then passed through a 200 mesh stainless steel screen, and the unpassed debris was discarded. The viability of the spleen cells was assessed by trypan blue exclusion to assure greater than 95% viability.

Spleen cells were resuspended in supplemented RPMI to a concentration of 2.0 x 10^6 viable cell/ml for use as effector cells.

Labeling of Target Cells. Yac-1, a T-cell lymphoma of A/Sn origin, served as target cells. After centrifugation at 800 x g for 10 min, 5 x 10^6 Yac-1 cells were suspended in 0.5 ml supplemented RPMI and labeled with 200 μCi 51Cr at 37° for 90 min. After incubation, the labeled
Tumor. 3LL syngeneic to the C57BL/6 mouse was used because of its propensity to metastasize to the lungs. This tumor line is maintained by intrascapular trocar transplantation into C57BL/6 mice. Upon removal from a carrier, tumor was homogenized in a Ten-Broek homogenizer and suspended in supplemented RPMI. The suspension was centrifuged at 800 x g for 10 min and then resuspended in supplemented RPMI. Greater than 95% viability was ascertained using trypan blue, and the cells were then diluted in supplemented RPMI to a concentration of 1 x 10^7 viable 3LL cells/ml. The hind footpad of each animal was then inoculated with 0.05 ml of this suspension, using a 1.0-ml syringe and a 27-gauge needle.

Counting Metastases. At designated times, animals were sacrificed by cervical dislocation. Lungs were removed by sharp dissection and placed in a Petri dish containing HBSS. Using a dissecting microscope at x15, each lung lobe was dissected free, and all surfaces were scrutinized for the presence of metastases.

Statistical Analysis. The Student's t test was used for all quantitative data and is reported with the standard error of the mean. \( \chi^2 \) with Yates correction for 1 d.f. was used for qualitative data analysis. Each experiment was performed repeatedly to assure statistical significance and avoid the effect of experimental artifact. On a given day, each experiment included at least one control and one amputated animal (in addition to other experimental groups) to further minimize daily minor variation inherent in the use of an \textit{in vitro} bioassay. For each animal, the \(^{51}\text{Cr} \) release assay was performed in triplicate, and the results were averaged for each animal prior to any statistical analysis.

RESULTS

\textit{In Vitro} Studies

Effect of Surgery. The effect of surgical stress on NKCC was studied in non-tumor-bearing animals. C57BL/6 mice were divided into experimental and control groups, and hind limb amputation was performed on the experimental group. After 24 hr, spleens of experimental and control animals were removed. Effector spleen cells and Yac-1 target cells were prepared as described, and the \(^{51}\text{Cr} \) release assay was performed. Surgically stressed animals displayed a significantly lower level of NKCC than controls. This experiment thus allowed a comparison between animals only bearing tumors, tumor bearing with amputation, and amputation alone for their respective effects on NKCC (Table 1).

NKCC values for tumor with amputation tend to fall midway between tumor alone and amputation alone values. It is intriguing
that, when these almost linear single variable relationships are combined into a normalized 2-variable framework, intermediate and also seemingly linearly related NKCC values result (Table 2).

**Table 1**

<table>
<thead>
<tr>
<th>Effector: Target Ratio</th>
<th>% 7 days after tumor inoculation (X = 10)</th>
<th>% 7 days after tumor inoculation and 24 hr after amputation (X = 10)</th>
<th>% 24 hr after amputation (X = 29)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50:1</td>
<td>16.5 ± 1.4 b</td>
<td>18.0 ± 2.5 p</td>
<td>9.4 ± 1.1</td>
</tr>
<tr>
<td>25:1</td>
<td>16.5 ± 1.0</td>
<td>12.8 ± 1.8</td>
<td>7.9 ± 1.1</td>
</tr>
<tr>
<td>12.5:1</td>
<td>11.4 ± 0.8</td>
<td>7.8 ± 1.1</td>
<td>4.9 ± 0.9</td>
</tr>
<tr>
<td>6.25:1</td>
<td>6.8 ± 0.5</td>
<td>4.2 ± 0.8</td>
<td>2.7 ± 0.7</td>
</tr>
</tbody>
</table>

* Student t-derived p value for significance of observed differences.

**Table 2**

<table>
<thead>
<tr>
<th>E:T</th>
<th>Control</th>
<th>Tumor</th>
<th>Amputation</th>
</tr>
</thead>
<tbody>
<tr>
<td>50:1</td>
<td>2.00</td>
<td>2.53</td>
<td>2.76</td>
</tr>
<tr>
<td>25:1</td>
<td>1.93</td>
<td>2.53</td>
<td>1.96</td>
</tr>
<tr>
<td>12.5:1</td>
<td>1.44</td>
<td>1.75</td>
<td>1.21</td>
</tr>
<tr>
<td>6.25:1</td>
<td>1.00</td>
<td>1.01</td>
<td>0.64</td>
</tr>
</tbody>
</table>

**Table 3**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of animals with metastases</th>
<th>% of animals with metastases</th>
<th>Total no. of metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8/21</td>
<td>38</td>
<td>26</td>
</tr>
<tr>
<td>Day 0 amputation</td>
<td>15/23*</td>
<td>65</td>
<td>41</td>
</tr>
<tr>
<td>Day 7 contralateral amputation</td>
<td>15/21b</td>
<td>71</td>
<td>66</td>
</tr>
<tr>
<td>Day 7 ipsilateral amputation</td>
<td>2/21c</td>
<td>9</td>
<td>3</td>
</tr>
</tbody>
</table>

* Control versus Day 0 amputation $\chi^2 = 3.08; p < 0.05$.

**DISCUSSION**

Promotion of solid-tumor metastasis by surgery is reproducible experimentally; unfortunately, it is also thought to occur occasionally in patients who undergo extirpative solid tumor resection (4). It is important to understand the immunological consequences of surgical intervention in the tumor-bearing host because of its potential role in host defense at this time. Further knowledge of such events may suggest means for immunomodulation in the perioperative period and the possible prevention of subsequent metastases.

This study has examined surgical stress and tumor burden as independent and codependent factors impinging on NKCC. Several conclusions can be drawn. The systemic stress of a hind limb amputation in the C57BL/6 mouse model significantly decreases NKCC in the perioperative period. A possible underlying mechanism of the systemic stress of surgery is a postoperative hypermetabolic response that includes but is not limited to an increase in the adrenal secretion of corticosteroids (16). An increase in corticosteroids could suppress NKCC cytotoxicity, as has already been demonstrated in humans *in vitro* (11), and could also promote metastases experimentally (19).
The impact of tumor bearing on NKCC was assessed by study at 3 time points representative of increasing tumor burden. Significant suppression of NKCC occurred as the primary tumors grew to a size at which lung metastases were detectable. The mechanisms by which progressive tumor burden inhibits NKCC are not clear (24); perhaps increasing elaboration of circulating suppressive factors by the tumor (2) or activation of suppressor cells by large tumor may play a role (5, 27). When tumor bearing and surgical stress were studied codependently, intermediate and linearly related cytotoxicity values resulted.

In vivo, amputation 24 hr before tumor inoculation increased the numbers of detectable metastases and increased the percentage of animals with metastasis, although not as dramatically as a contralateral amputation after 1 week of tumor bearing. While it could be expected from the in vitro control-versus-amputation cytotoxicity studies (Chart 1) that a significant suppression of NKCC had occurred 24 hr after amputation, it may be that the suppression is transitory, as has been suggested for other immune systems (15). Suppression could also be offset by the subsequent NKCC stimulation that tumor inoculation 24 hr later provides (Chart 2).

Amputation of the tumor-bearing limb after 1 week of tumor bearing protected against the development of detectable pulmonary metastases. The presence of ongoing tumor burden may be an important prerequisite for metastasis in this model. This could possibly be due to the NKCC suppressive effect of progressive tumor burden delineated in the in vitro studies (Chart 2) or perhaps be due to ongoing shedding of tumor cells with decreased NKCC immunosurveillance in the bloodstream. However, the presence of detectable metastases in 2 of 21 animals treated with tumor limb amputation might also be construed as evidence that some pulmonary seeding occurs by rapid intravascular dissemination at the time of tumor inoculation (7) and that awith stress the already present pulmonary foci escape surveillance and begin to grow.

Amputation of the non-tumor-bearing limb resulted in significant enhancement of metastases. This in vivo tumor-bearing/amputation group was identical to the in vitro tumor-bearing/amputation group (Table 1) that demonstrated significant periorperative impairment of NKCC. We are not claiming that a direct cause-effect relationship has been established. Our work to date has examined only one immune parameter studied in only one spontaneous tumor model; the concept of immune surveillance implies the interaction of many immune (and nonimmune) mechanisms functioning to protect the tumor-bearing host from the development of metastases. This model does provide a possible framework in which to temporally if not causatively correlate in vitro and in vivo findings; however, the ultimate in vivo significance of natural immunity in protecting against the development of metastases has not yet been conclusively delineated.

In conclusion, surgical stress and progressive tumor burden can be shown independently and together to suppress NKCC in a mouse model in vitro. These in vitro suppressions happen at the very time that enhancement of solid tumor metastasis can be shown to occur secondary to the application of surgical stress in the same mouse model in vivo. Our data suggest that a delicate balance exists within the immune response; this balance can be greatly influenced by tumor burden and surgical stress. It is hoped that methods to reverse the periorperative stress-induced impairment of NKCC may help to forestall the development of metastases in this model; such studies are currently under way.

REFERENCES

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