Hypothermia in Barbiturate-anesthetized Rats Suppresses Natural Killer Cell Activity and Compromises Resistance to Tumor Metastasis

A Role for Adrenergic Mechanisms

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Background: **Clinical studies have implicated surgery in promoting infections and compromising immune functions, including natural killer cell activity. Animal studies indicate that surgery-induced suppression of natural killer cell activity also promotes tumor metastasis. Hypothermia, a common surgical complication, has been suggested to underlie some of the deleterious consequences of surgery. This study evaluated the effect of hypothermia on the activity and number of blood natural killer cells and on host susceptibility to metastasis. The involvement of adrenergic mechanisms was also considered.**

Methods: **Fischer-344 rats remained awake in their cages (control group) or were anesthetized with 70 mg/kg thiopental and maintained for 2.5 h at core body temperatures of 30–32°C (hypothermia group) or 38°C (normothermia group). Thereafter, at several time points, blood was drawn so natural killer cell activity could be assessed, or rats were injected with syngeneic MADB106 tumor cells that metastasize only to the lungs. Lungs were removed 9 h later for assessment of lung tumor retention, or 4 weeks later for counting of metastases.**

Results: **Normothermic anesthesia reduced natural killer cell activity (lytic units at 30% specific killing, mean ± SEM) to 39 ± 6.2% of control levels and hypothermia further reduced it to 15 ± 6.6%. These changes were not accompanied by alterations in the numbers of circulating natural killer cells. Hypothermia increased tumor retention to 250% of control levels, and the number of metastases increased from 1.1 ± 0.4 to 4.7 ± 1.2. Normothermia had no significant effects on this index. Nadolol (0.4 mg/kg), a β-adrenergic antagonist, significantly attenuated the effect of hypothermia on tumor retention.**

Conclusions: **Hypothermia under thiopental anesthesia suppresses natural killer cell activity and compromises host resistance to metastatic formation, possibly *via* adrenergic mechanisms. Such suppression may place patients with metastasizing tumors or dormant viral infections at greater risk for complications after intraoperative hypothermia. (Key words: Immunity; malignancy; postoperative immunosuppression; stress.)**

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IN humans, anesthesia and surgical procedures have been reported to suppress natural killer cell activity (NKCA) and other immune functions for several days.[1–4] The biologic significance of such immune suppression is suggested by the well-established relation between surgical procedures and the outbreak of dormant infectious diseases known to be under immune surveillance (*e.g.*, tuberculosis, herpes, and cytomegalovirus).[5,6] Whether such immunosuppressive effects of surgery also accelerate tumor development in the clinical setting is largely unknown and clearly difficult to evaluate, but animal studies have provided ample evidence for this possibility.[3,4,7]

Among immune mechanisms that control viral infection and malignancies are natural killer (NK) cells, a subpopulation of lymphocytes known to spontaneously recognize and kill various virally infected and tumor cells *in vitro*.[8–10] Animal studies have shown that NK cells play an important role in controlling the development of leukemia and solid tumors,[10,11] especially during the metastatic process.[12–16] Human studies have indicated that levels of NKCA are negatively correlated with sus-

ceptibility to several types of cancer.[10] Furthermore, higher levels of NKCA at the time of tumor removal have been associated with a better prognosis after excision of breast,[17] head, or neck tumors.[18]

Intraoperative hypothermia occurs, at least to some extent, in more than 50% of surgical procedures.[19-21] Intraoperative and exposure-related hypothermia have been shown to suppress several immune measures[22-24] and to increase the risk of infections.[21,25,26] Hypothermia elicits various physiologic responses, including sympathetic discharge and adrenal release of catecholamines and, to a lesser degree, glucocorticoids.[27,28] These neuroendocrine responses have been reported to suppress immune functions, specifically NKCA.[29-31]

Because neuroendocrine responses affect the activity of individual NK cells and the distribution of NK cells among different immune compartments,[29,32-34] both indices were assessed in the current study. To assess the health implications of potential changes in NKCA, we also used a model of metastatic formation based on the MADB106 tumor line, which is known to be sensitive to NK cells. This mammary adenocarcinoma cell line is syngeneic to the inbred Fischer-344 rats used in this study. After intravenous inoculation, MADB106 cells extravasate and reside in the lungs, and surviving cells develop colonies only in this organ.[12-14] Studies have shown that these two related outcomes, lung tumor cell retention and the number of lung tumor colonies, are controlled by NK cells, primarily during the first 24 h after tumor injection.[12-14] Therefore, in addition to providing a measure of host susceptibility to metastasis, the retention and colonization of MADB106 cells in the lungs provide an indication of NK cell performance in the whole animal.

In the current study, using a rat model, we sought (1) to determine whether hypothermia under barbiturate anesthesia suppresses the activity of NK cells or affects their distribution, (2) to determine whether hypothermia facilitates metastasis of an NK-sensitive malignancy, and (3) using a β-adrenergic antagonist, to identify the extent to which these effects are mediated by adrenergic mechanisms.

Materials and Methods

Animals

Fischer-344 male rats (Harlan Laboratories, Jerusalem, Israel) were housed four in a cage with free access to food and water. Animals were acclimatized to the vivar-

ium for at least 4 weeks before the beginning of experiments and were 13–19 weeks old at that time. In any given experiment, all animals were of the same age. All experiments were approved by the Institutional Animal Care and Use Committee of Tel Aviv University.

Anesthesia, Hypothermia, and Normothermia

Anesthesia was induced by intraperitoneal injection of 70 mg/kg sodium thiopental (Abbott S.p.A, Milan, Italy). Hypothermia was induced by positioning anesthetized rats on cotton-covered ice packs. Normothermia was maintained at 38°C (the normal rat body temperature) by positioning anesthetized rats on electric heating pads. Core body temperature was monitored using a 4-cm rectal probe (400 series; YSI Inc., Yellow Springs, OH). Anesthesia lasted for approximately 6 h in the normothermia group and 7 h in the hypothermia group.

Flow Cytometric Analysis

Fluorescence-activated cell sorter analysis was used to determine the number of NK cells in the blood. These cells were identified as NKR-P1[bright] lymphocytes.[35] To determine the total number of NK cells per milliliter of blood, a fixed number of polystyrene microbeads (20 μm; Duke Scientific, Palo Alto, CA) were added to the blood samples before they were prepared for cytometric analysis. For further details, see Shakhar et al.[29]

Whole-blood Natural Killer Cell Activity Assay

The activity of NK cells was assessed using the whole-blood cytotoxicity assay described in detail elsewhere.[29] Briefly, this procedure analyzes antitumor cytotoxicity of NK cells per milliliter of blood without previous purification of peripheral-blood mononuclear cells. One milliliter of blood is drawn by cardiac puncture, plasma is replaced with culture medium, and aliquots of the washed blood are placed in a microtiter plate. To assess NK cytotoxicity at six different effector-to-target ratios, six concentrations of radiolabeled YAC-1 tumor cells were added to the blood. Spontaneous and maximal releases of radioactivity from target cells are determined by substituting blood with the culture medium or hydrochloric acid, respectively. After a 4-h incubation period, samples of supernatant are recovered from each well so radioactivity could be assessed and specific target cell lysis could be calculated. Earlier studies indicated that cytotoxicity measured using this procedure is attributable to NK cells rather than to other cell types or soluble factors.[36,37]

The MADB106 Tumor Line

MADB106 is a selected variant cell line obtained from a pulmonary metastasis of a mammary adenocarcinoma (MADB100) chemically induced in the inbred Fischer-344 rat.[14] MADB106 cells were maintained in complete medium at 100% humidity, 5% carbon dioxide at 37°C, and detached from the flask using 0.25% trypsin.

Radiolabeling of Tumor Cells and Assessment of Lung Tumor Retention

To assess lung tumor retention, DNA radiolabeling of tumor cells was accomplished by the addition of 0.4 mCi/ml of [125]iododeoxyuridine (ICN Radiochemicals, Irvine, CA) to the growing cell culture 1 day before the cells were harvested for injection.

For tumor cell injection, rats were lightly anesthetized with halothane (Trofield Surgicals, London, UK), and 4×10^5/kg [125]iododeoxyuridine-labeled MADB106 tumor cells in approximately 0.5 ml phosphate-buffered saline (supplemented with 0.1% bovine serum albumin) were injected into their tail veins. Nine hours later, rats were killed with halothane, and their lungs were removed and placed in a gamma counter for assessment of radioactive content. The percentage of tumor cells retained was calculated as the ratio of radioactivity measured in the lungs to total radioactivity in the injected tumor cells. Our previous studies have indicated that the level of lung radioactivity reflects the number of viable tumor cells in the lungs (for more information on this, see Ben-Eliyahu *et al.*[13]).

Induction and Counting of Tumor Metastases

Rats were lightly anesthetized with halothane, and 10^5 MADB106 tumor cells were injected into their tail veins in 0.5 ml phosphate-buffered saline. Four weeks after tumor inoculation, rats were anesthetized, and lungs were removed and placed for 24 h in Bouin solution (72% saturated picric acid solution, 23% formaldehyde [37% solution], and 5% glacial acetic acid). After being washed in ethanol, visible surface metastases were counted by a researcher blinded to the donor of each lung. When the rats were killed, no signs of respiratory distress, body weight loss, or any noticeable illness-related behavior were observed. With the anticipated number of lung metastases, rats are not expected to show such signs before 8 weeks.[13]

Design and Procedure

In a preliminary study, we developed the procedure for inducing hypothermia. Core temperature was monitored continuously until rats emerged from anesthesia. No immune measures were studied in this initial phase.

All further experiments were devoted to the study of immune measures related to NK activity. Rat temperatures were taken only three times: immediately after anesthesia and 1 and 2.5 h into the procedure. These measurements were conducted to verify adherence to the expected course of changes in core temperature.

In all experiments, rats were allocated to one of three groups: control, normothermia, and hypothermia. Rats from the control group remained awake and undisturbed in their home cages. Rats from the normothermia group were anesthetized and kept for 2.5 h at normal body temperature, as described before. Rats from the hypothermia group were anesthetized and their temperatures were reduced for 2.5 h, as described before. After this 2.5-h period, rats from the latter two groups were returned to their home cages. All immune- and tumor-related measures were taken from this time point (T_0) and onward, and their timing is recorded in relation to T_0.

In the first experiment, blood was drawn by cardiac puncture at 2 or 6 h to assess the activity and numbers of circulating NK cells (n = 54; 9–14 per group).

In the second experiment, rats were injected with radiolabeled MADB106 tumor cells at T_0, or at 3 h. Lungs were removed 9 h after tumor injection so tumor retention could be assessed (n = 48; 9–15 per group).

In the third experiment, rats were injected intravenously with MADB106 cells at 2 h; 4 weeks later, lungs were removed and the number of metastases was counted (n = 57; 19 per group).

In the fourth experiment, each of the three experimental groups was subdivided and treated with saline or nadolol, a β-adrenergic antagonist.[38] A first subcutaneous injection of the drug (0.4 mg/kg) or saline was given at the induction of anesthesia (−2.5 h), and a second identical injection was given together with radiolabeled MADB106 cells at 2 h. Lungs were removed so that tumor retention could be assessed 9 h after tumor injection (n = 73; 8–18 per group).

Statistical Analyses

Natural killer cell activity was analyzed using repeated-measures analysis of variance for the different effector-to-target ratios and an α error of 0.05. In all other cases, analysis of variance was not used because markedly different variances characterized the different groups, thereby violating an analysis-of-variance assumption. Instead, we used unpaired Student *t* tests to compare
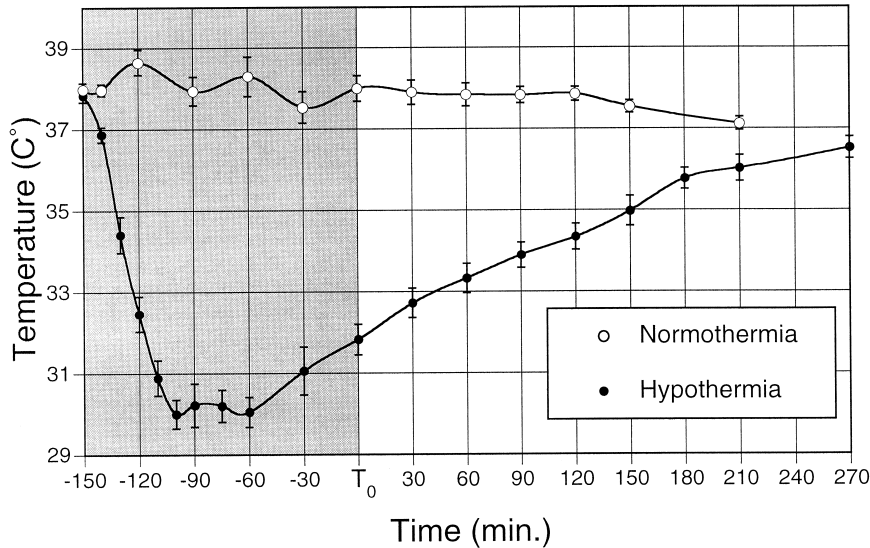
Fig. 1. Body temperatures (mean ± SD) of anesthetized rats maintained at normothermia or hypothermia. Anesthesia was induced by intraperitoneal injection of 70 mg/kg sodium thiopental. The gray area denotes the 2.5-h period in which body temperature was controlled.

groups and the Bonferroni α-correction procedure to compensate for multiple comparisons. Thus, $\alpha_{corrected}$ was set at 0.015, allowing for as many as three independent comparisons ([1 − $\alpha_{corrected}$]^3 > 95%). P values indicate the significance levels of each t test. Lytic units were calculated using the formula 100/ET$_{30}$, where ET$_{30}$ is the effector-to-target ratio needed to lyse 30% of target cells. The regression exponential-fit method[39] was used to infer ET$_{30}$ from the data.

Results

Changes in Body Temperature

In the hypothermia group, the average target core temperature of 30°C was achieved within 50 min and began to increase 40 min later, to 32°C by T$_0$. Normal body temperature was regained gradually within 5 h of T$_0$ (fig. 1). The body temperature of normothermic animals remained at approximately 38°C before T$_0$ and typically decreased thereafter by as much as 1°C until rats had emerged from anesthesia completely. Hypothermic treatment was uniform in all subsequent experiments and resulted in very similar changes in body temperature.

Activity and Numbers of Blood Natural Killer Cells

At 2 h and 6 h, hypothermia significantly suppressed NKCA compared with levels in the control (P < 0.0001) and the time-matched normothermic (P < 0.05) groups (fig. 2A). Normothermia significantly suppressed NKCA at 6 h (P < 0.05), but not at 2 h (P = 0.068). Because NKCA at the two time points was similar in each group (fig. 2A), they were combined for further analysis using lytic units. Normothermia suppressed NKCA (expressed as the mean lytic units at 30% specific killing [LU$_{30}$] ± SEM) to 39% of control levels (from 49.30 ± 10.30 in the control group to 19.08 ± 3.03; P < 0.003). Hypothermia further suppressed NKCA to 15% of control levels (to 7.41 ± 3.24; P < 0.015) (fig. 2B).

At the two time points when blood was drawn to assess NK activity, NK cell numbers were similar in all groups, ranging from 305 to 350 NK cells/μl (fig. 2C).

Lung Tumor Retention of MADB106 Cells

After tumor injection at T$_0$, normothermia increased the percentage of MADB106 tumor cells retained in the lungs from 0.40 ± 0.05% in the control group to 0.64 ± 0.09% (P = 0.024). This effect completely dissipated within 3 h (fig. 3A). Hypothermia, on the other hand, caused more than a twofold increase in lung tumor retention at T$_0$ (as much as 1.01 ± 0.23; P < 0.004) and at 3 h (as much as 0.94 ± 0.16%; P < 0.001). The increase caused by hypothermia compared with normothermia was also significant at T$_0$ and 3 h (P < 0.01 and P < 0.002, respectively).

MADB106 Lung Metastases

The average number of metastases identified in the hypothermia group was 4.68 ± 1.27, which is more than three times greater than in the control and normothermia groups, in which the metastases numbered 1.10 ± 0.4 and 1.47 ± 0.39, respectively (one-tailed test; P < 0.006 and P < 0.011, respectively) (fig. 3B).

Importantly, only 21% of the hypothermic animals were free of metastases, whereas in the normothermic and control groups, 36.8% and 52.6%, respectively, were free of metastases.

Effects of β-Adrenergic Blockade on Lung Tumor Retention

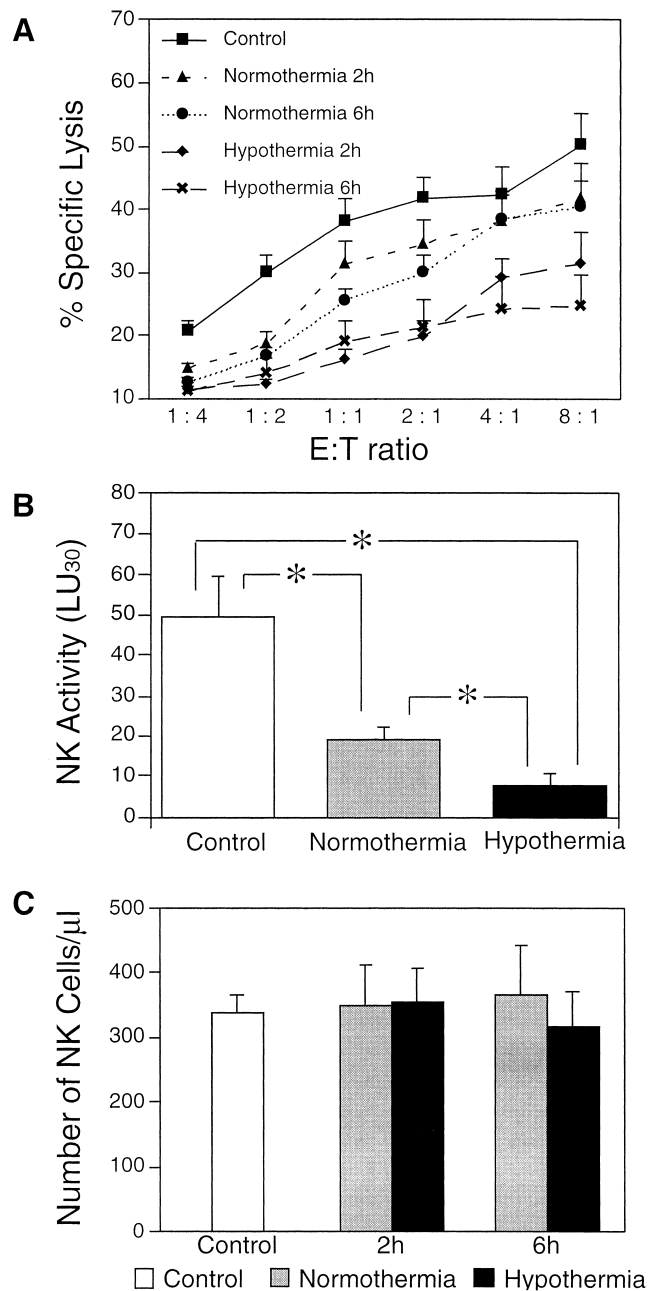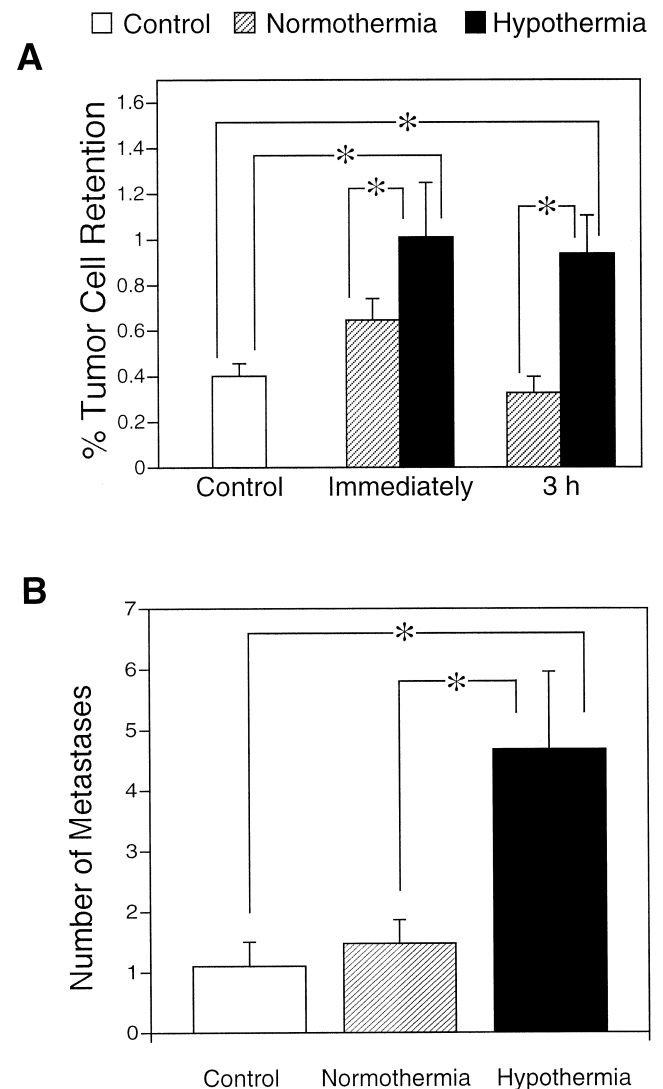Normothermia and hypothermia significantly increased lung tumor retention, from $0.53 \pm 0.05\%$ in the

Fig. 2. Animals were subjected to thiopental anesthesia in either normothermic or hypothermic conditions or served as controls. Blood was drawn 2 or 6 h after T_0. (*A*) The percentage of specific lysis (mean ± SEM) at different effector-to-target ratios (natural killer:YAC-1 tumor cells). Natural killer cell activity in the two hypothermic groups was significantly less than in the control group and in the time-matched normothermic groups. (*B*) Natural killer cell activity expressed as lytic units at 30% specific killing (LU_{30}; mean ± SEM). The two time points were joined because no differences were evident. *A significant difference ($P < 0.02$). (*C*) The numbers of natural killer cells ($NKR-P1^{bright}$) per microliter of blood (mean ± SEM). No significant differences were evident.

Fig. 3. (*A*) The percentage of lung tumor retention (mean ± SEM). Animals were anesthetized and subjected to either normothermic or hypothermic conditions or served as controls. MADB106 cells were injected at T_0 or at 3 h. *A significant difference ($P < 0.02$). (*B*) The number of lung tumor metastases (mean ± SEM) counted 4 weeks after MADB106 inoculation in normothermia, hypothermia, and in control animals. Tumor cells were injected at 2 h. *A significant difference ($P < 0.02$).
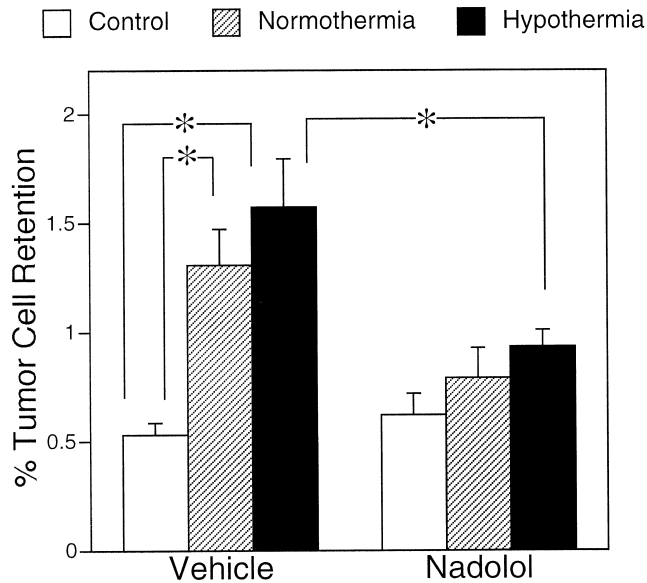
Fig. 4. The percentage of lung tumor retention (mean ± SEM) in animals inoculated with MADB106 cells. Rats were treated with nadolol or vehicle and assigned to a condition of either control, normothermia, or hypothermia. * A significant difference (*P* < 0.02).

control group to 1.30 ± 0.16% and 1.57 ± 0.22%, respectively (fig. 4). Although the effect of hypothermia was larger than that of normothermia, this difference was not statistically significant in this experiment. Nadolol attenuated the effects of hypothermia, keeping tumor retention at 0.93 ± 0.07% (hypothermia saline *vs.* hypothermia nadolol; *P* = 0.0055). This attenuation was not statistically significant in the normothermia condition (normothermia saline *vs.* normothermia nadolol; *P* = 0.0329).

Discussion

To the best of our knowledge, this is the first study to systematically assess the effect of hypothermia under anesthesia on NK activity or tumor metastasis. Our findings show that 2.5 h of hypothermia during thiopental anesthesia markedly suppressed NK activity and promoted metastasis. Specifically, normothermic rats showed less suppression of NKCA than did hypothermic rats. This suppression occurred at the level of individual NK cell activity, because the numbers of NK cells per milliliter of blood were unaffected. Correspondingly, the retention of MADB106 tumor cells in the lungs and the number of lung metastases were significantly increased by hypothermia compared with rats in the control and

the normothermia groups. The effects of normothermia (thiopental anesthesia alone) were smaller and reached significant levels only on one of the four occasions that the MADB106 was used. Finally, nadolol, a β-adrenergic antagonist, significantly attenuated the effect of hypothermia on lung tumor retention.

Overall, these findings suggest that the suppression of NKCA by hypothermia is robust and long-lasting and has consequences for the health of the animal. Specifically, NKCA was still markedly suppressed at 6 h, and lung tumor retention, reflecting cumulative clearance of tumor cells from 3 to 12 h, was greatly increased. This latter effect seems resistant to factors that influence tumor development at later stages, because lung metastases, counted 4 weeks after hypothermia, were four times as numerous in the rats in the hypothermia group compared with those in the control or normothermia groups. Considering the high sensitivity of the MADB106 to NKCA[12-14] and our previous findings of parallel effects of various manipulations on NKCA and on resistance to MADB106 metastasis,[29,40] the suppression of NKCA by hypothermia is likely to underlie the increase in MADB106 metastases.

The suppression of NKCA by hypothermia seems unrelated to the cooling of NK cells themselves or to the cooling of other leukocytes. The blood samples from all groups were kept at room temperature (23°C; *i.e.*, "hypothermia") for approximately 3 h during the preparation for the assessment of NKCA. Natural killer cell cytotoxicity was then assessed in all samples at 37°C. Therefore, it appears that physiologic responses secondary to hypothermia are involved in the suppression of NKCA. Possible candidates are the various hormones and cytokines released after hypothermia, most prominently catecholamines, and to a lesser degree glucocorticoids and other hormones.[27,28]

It is our hypothesis that adrenergic discharge plays an important role in the suppression of both NKCA and resistance to MADB106 metastasis observed in the current study. Anesthesia and surgery have been found to increase epinephrine levels. Similarly, hypothermia, under surgical conditions and in nonanesthetized persons, has been reported to increase norepinephrine levels compared with normothermia.[27,28] *In vitro* suppression of NKCA by catecholamines has been demonstrated by various groups,[41-43] and we recently reported that *in vivo* β-adrenergic stimulation markedly suppresses NKCA per NK cell and consequently promotes tumor metastasis of the same tumor used in the current study.[29] Correspondingly, β-adrenergic antagonists have been re-

ported to block the suppression of both NKCA and tumor resistance caused by various manipulations.[44,45] Finally, in the current study the β-adrenergic antagonist, nadolol, almost completely blocked the effects of thiopental anesthesia and hypothermia on the retention of the MADB106 tumor cells. Although several studies in humans have suggested that adrenergic stimulation can enhance NKCA,[34,46] there are indications that this increase is attributable to a transient elevation in the number of circulating NK cells, and that the long-term effect of catecholamines on NK activity is suppressive.[33,34]

Suppression of NKCA per NK cell, when induced *in vivo*, is likely to dissipate with time during preparations for the *in vitro* assessment of NKCA.[13] When blood is withdrawn, serum is replaced with an artificial medium, and factors such as cytokines and hormones are removed. *In vitro* studies using human blood have shown that the suppressive effects of catecholamines dissipate after hormone removal.[43] Therefore, a prolonged delay before the assessment of NKCA might conceal the effects of hypothermia. In the current study, only 3 h elapsed between blood withdrawal and assessment of NKCA. The whole-blood assay, or other timesaving procedures, may be considered to obviate these obstacles in future clinical studies.

Although several surgical procedures in humans are characterized by levels of hypothermia similar to or deeper than those reached here,[47] most are not. To clarify the clinical applicability of the current findings, a wide range of hypothermic levels and durations should be tested to identify the conditions in which hypothermia begins to suppress NKCA. Simultaneous assessment of neuroendocrine responses could suggest potential mediators. These important elaborations are beyond the scope of the current study and should be addressed in experimental and clinical conditions. An additional unresolved issue in this study is the possible effect of hypothermia on the pharmacokinetics of thiopental metabolism. Because this anesthetic had effects of its own, some of the effects of hypothermia could be attributed to elevated plasma concentrations of thiopental. Nevertheless, we estimate this factor to be of minor significance: Hypothermia prolonged the duration of anesthesia by only 15% but typically had a much greater effect than normothermia on the immune measures, some of which (*e.g.*, the number of metastases) were not affected at all by normothermia.

Recent studies have suggested the molecular mechanisms that make NK cells important for preventing metastasis. By the time malignant cells evolve to a metasizing stage, they have usually suppressed the expression of functional major histocompatibility complex class I molecules, making them invulnerable to cytotoxic T cells. The mechanism by which NK cells recognize their targets, however, involves the detection of aberrations in, or the absence of, these molecules.[48] This, and the fact that a large proportion of NK cells reside in the blood and the tissues targeted by metastasizing cells (*e.g.*, the lung and liver), make NK cells most important at this stage.[49,50] Apart from lysing single tumor cells dispersed from the primary tumor, NK cells have been shown to infiltrate and diminish established micrometastases.[16]

Our findings suggest that hypothermia may contribute to the clinically well-documented observation that surgery suppresses NKCA.[1-4] In the current study in rats, hypothermia and suppression of NKCA were also associated with decreased resistance to metastasis. Relations of this kind would be difficult to assess clinically, because the delay between tumor dissemination and detection of possible related metastases is long and variable. In contrast, suppression of immunity by surgery or hypothermia may lead to an outbreak of latent or opportunistic infections within days, a phenomenon that is indeed well documented clinically.[5,21] In sum, it is as yet unclear to what extent the morbidity and immunosuppression observed after surgery is caused by hypothermia. Nevertheless, given the current findings, exposing patients to intraoperative hypothermia should be considered carefully when there is a high risk of viral infection or metastatic spread. Of particular clinical relevance is the removal of metastasizing tumors in procedures that tend to cause hypothermia, such as open abdominal surgeries. Because hypothermia is imperative in several lifesaving procedures, prophylactic measures, such as preoperative stimulation of the immune system or the perioperative use of β-blockers, should be considered.

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