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Improving Survival Rates in Two Models of Spontaneous Postoperative Metastasis in Mice by Combined Administration of a β -Adrenergic Antagonist and a Cyclooxygenase-2 Inhibitor

Ariella Glasner,* Roi Avraham,^{†,‡} Ella Rosenne,* Marganit Benish,* Oded Zmora,^{§,¶} Shaily Shemer,* Hadas Meiboom,* and Shamgar Ben-Eliyahu*

Clinical practice does not consider perioperative paracrine and neuroendocrine stress responses as risk factors for cancer recurrence, although recent animal studies provided supportive evidence. Suggested mechanisms include the effects of stress-hormones on tumor cells and on host physiology. In this study, in mice undergoing primary tumor excision, we tested the survival-enhancing potential of perioperative blockade of catecholamines and prostaglandins, and studied potential mediating mechanisms. C57BL/6J mice were inoculated intrafootpad with syngeneic B16F10.9-melanoma or Lewis lung carcinoma, and the paw was amputated when a developing tumor exceeded 100 μ l. The clinically used β -adrenergic antagonist propranolol, and/or the cyclooxygenase-2 inhibitor etodolac, were administered once before amputation, and recurrence-free survival was monitored. In different studies, NK cytotoxicity, leukocytes' molecular functional markers, and vascular endothelial growth factor secretion by tumor cells were studied in the context of surgery and drug treatments. The findings indicated that the combination of propranolol and etodolac, but neither drug alone, significantly and markedly improved survival rates in both tumor models, and was as effective as established immunostimulatory agents (IL-12 and polyinosinic-polycytidylic acid). Surgery markedly reduced NK cytotoxicity and NK cell expression of Fas ligand and CD11a, reduced all circulating lymphocyte-subtype concentrations, and increased corticosterone levels. Propranolol and etodolac administration counteracted these perturbations. B16 and 3LL secreted vascular endothelial growth factor in vitro, but secretion was not affected by catecholamine agonists, prostaglandins, corticosterone, propranolol, or etodolac. Overall, propranolol and etodolac administration, which could be applied perioperatively in most cancer patients with minimal risk and low cost, has counteracted several immunologic and endocrinologic perturbations and improved recurrence-free survival rates in mice undergoing primary tumor excision. *The Journal of Immunology*, 2010, 184: 2449–2457.

Many approaches are used to reduce postoperative cancer recurrence, including chemotherapy, immunotherapy, radiation, and antihormone therapy. Nevertheless, the most prevalent cause of mortality in cancer patients is metastatic development.

Physiologic stress responses, which are common in cancer patients, have been proposed to facilitate malignant progression through several mechanisms, including excess vascular endothelial growth factor (VEGF) secretion by tumor cells and immune suppression

(1, 2). The surgical procedure, which is a necessary and crucial step in the treatment of most solid cancers, has long been suspected to facilitate the metastatic process (1), and numerous mechanisms were implicated. These mechanisms include dissemination of tumor cells during surgery (3), decreased levels of antiangiogenic factors, local and systemic increase in proangiogenic and growth factors (e.g., VEGF) (4), blood loss and transfusion (5, 6), anesthetic and analgesic drugs (7–9), and the suppression of cell-mediated immunity (CMI) (1). Generally, the more extensive the surgical procedure, the more pronounced the paracrine, neuroendocrine, and immunosuppressive effects of surgery (1, 10). Importantly, at the immediate postoperative period, the above prometastatic processes can act in synergy, markedly increasing the risk of initiation and progression of metastases (1).

Most relevant to the current study, it has recently become clear that stress responses, which characterize the immediate postoperative period, specifically the release of catecholamines (CAs; i.e., epinephrine and norepinephrine) and prostaglandins (PGs) (e.g., PGE₂), could mediate many of the adverse effects of surgery through their direct effects on the malignant tissue and through modulation of host physiology and immune competence (10, 11). For example, NK cells have been shown to kill cancerous cells in vitro and in vivo, and their perioperative activity levels were associated with long-term recurrence-free survival in patients harboring various cancers (12). The suppression of NK activity and other aspects of CMI in the perioperative period were recently suggested to result from excess secretion of CAs and PGs, which can directly act on NK cell receptors, leading to their inhibition

*Neuroimmunology Research Unit, Department of Psychology and [‡]Sakler School of Medicine, Tel Aviv University, Tel Aviv; [†]Department of Biological Regulation, The Weizmann Institute of Science, Rehovot; [§]Department of Surgery and Transplantation, Colon and Rectal Surgery, Sheba Medical Center, Tel Hashomer; and [¶]Department of Surgery, Shaare Zedek Medical Center, Jerusalem, Israel

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Address correspondence and reprint requests to Dr. Shamgar Ben-Eliyahu, Neuroimmunology Research Unit, Department of Psychology, Tel Aviv University, Tel Aviv 69978, Israel. E-mail address: shamgar@post.tau.ac.il

Abbreviations used in this paper: CA, catecholamine; CM, complete medium; CMI, cell-mediated immunity; CORT, corticosterone; COX, cyclooxygenase; E, etodolac; FasL, Fas ligand; MDSC, myeloid-derived suppressor cell; NKC, NK cytotoxicity; P, propranolol; P+E, propranolol and etodolac; PG, prostaglandin; PLSD, protected least significant differences; poly(I:C), polyinosinic-polycytidylic acid; VEGF, vascular endothelial growth factor.

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via elevated intracellular cAMP levels (13–15). In addition, CAs and PGs were shown to decrease the levels of Th1 cytokines (16–18), including IL-2, IL-12, and IFN γ , leading to reduced CMI competence. Importantly, the aforementioned stress hormones and the cytokine dysregulation seem to suppress additional aspects of CMI, including CTLs, Th cells, and dendritic cells (1). Aside from causing suppression of CMI, CAs and PGs were shown to increase tumor cell invasion capacity, elevate VEGF secretion by malignant cells (2), and enhance tumor microvascular density (19, 20). Accordingly, the blockade of CAs and PGs was indeed suggested to reduce cancer progression via multiple mechanisms (2, 20–23).

In previous studies, we found that the use of different β -adrenergic blockers and cyclooxygenase (COX) inhibitors in rats undergoing surgical procedures reduced postoperative suppression of NK activity and improved tumor cell clearance from the lungs after i.v. injection (22, 24). The combined use of the blockers often was the only useful approach, suggesting a synergistic beneficial influence in the immediate postoperative context.

The current study aims to test the effect of the above drug regimen in more clinically relevant tumor models, which assess the most important clinical outcome—recurrence-free survival rates in mice in which an orthotopic primary tumor is excised. In addition, we now use a β -blocker and a COX-2 inhibitor that are in routine clinical use, can be applied perioperatively in most cancer patients, and seem to optimize the biomedical activities of these families of drugs (see *Discussion*). A secondary aim is to explore several mechanisms that could mediate the beneficial effects of the drug regimen, including prevention of postoperative immune perturbations and alteration of VEGF secretion by tumor cells. Last, we also use acknowledged approaches of immunostimulation (polyinosinic-polycytidylic acid [poly(I:C)] and IL-12) to compare the efficacy of our drug intervention to more established approaches.

Overall, this study aims to promote the clinical testing of a new approach for reducing long-term cancer recurrence: blocking specific excessive perioperative paracrine and neuroendocrine stress responses. Such an approach has not been tested clinically thus far.

Materials and Methods

Animals and counterbalancing of procedures order

C57BL/6J male and female mice (HSD, Jerusalem, Israel) were purchased at the age of 6 wk and housed 3–4 per cage in our vivarium with ad libitum access to food and water on a 12:12 light/dark cycle at $22 \pm 1^\circ\text{C}$. Animals were used at the age of 10–14 wk and age-matched across all groups in each experiment. The order of tumor and drug administration was counterbalanced across all experimental groups, and control animals were injected with vehicle. All studies were approved by the Institutional Animal Care and Use Committee of Tel Aviv University.

Tumor cell lines

The syngeneic B16F10.9 (B16) melanoma and Lewis lung carcinoma d122 (3LL) cell lines were provided by Dr. Amiram Raz (Tel Aviv University). The SKOV3 tumor line was provided by Dr. Anil K. Sood (University of Texas, Houston, TX). Cells were grown in cultures in 5% CO₂, 100% humidity, 37°C, in complete medium (CM) (24).

Drugs and their administration

Propranolol. A nonselective lipophilic β -adrenergic antagonist (Sigma-Aldrich, Rehovot, Israel), was injected s.c. (5 mg/kg, 10 ml/kg) 30 min before surgery in an emulsion of PBS, mineral oil, and Arlacel (8:7:1) (Sigma-Aldrich). The $t_{1/2}$ of propranolol is 4–5 h in humans and is unknown in mice.

Etodolac. A semiselective COX-2 inhibitor (COX-2:COX-1 selectivity ~10:1; Taro, Haifa Bay, Israel) (25) was dissolved in corn oil and injected s.c. 30 min before surgery, (50 mg/kg, 10 ml/kg). The $t_{1/2}$ etodolac is ~8 h in humans and 16 h in mice (26).

Poly(I:C). A synthetic dsRNA (Sigma-Aldrich) was dissolved in PBS and injected i.p. (0.2 mg/kg, 10 ml/kg) 1 d before tumor excision.

IL-12. Recombinant mouse IL-12 (Cytolab, Rehovot, Israel; sp. act. $\geq 10^7$ IU/mg) was dissolved in PBS and injected s.c. (0.02 mg/kg, 10 ml/kg).

Surgical procedures

Experimental laparotomy. Mice were kept under anesthesia at 1.5–2.5% of isoflurane, the skin was shaved and sterilized, and a 1.5 cm midline abdominal incision was made. The intestine was externalized and kept moisturized for 30 min. The intestine was then returned to the abdominal cavity and the wound was sutured.

Amputation. Mice were anesthetized with 2% isoflurane, and the tumor-bearing paw was amputated with surgical scissors 2 mm above the ankle joint. The wound was sterilized and disinfected with a Polidin paste, and anesthesia was maintained for an additional 10 min.

Preparation of blood effector cells

Mice were overdosed with isoflurane, and the peritoneal and chest cavities were opened; 550 μl of blood were drawn from the right ventricle of the heart into syringes containing 75 U of preservative-free heparin, in 50 μl PBS. Blood was washed once in 2 ml PBS (centrifuged at $456 \times g$ for 10 min, then supernatant aspirated to the original volume), twice with 2 ml CM and reconstituted to 0.8 ml volume.

Preparation of plasma samples

Heparinized-plasma was collected immediately after blood harvesting and centrifugation for the assessment of corticosterone (CORT) level. All plasma samples were stored at -80°C until assayed.

Assessment of NK cytotoxicity per milliliter of blood

To assess NK cytotoxicity (NKC) at different E:T ratios, an aliquot of 300 μl washed blood (see above) was placed in the first row of a microtiter plate; 150 μl was transferred to the second row that contained 150 μl CM and successively diluted 2-fold in CM in the following rows to achieve different E:T ratios. Five thousand ^{51}Cr -radiolabeled YAC-1 target cells in 100 μl CM were added on top of the blood. Spontaneous and maximal releases of radioactivity from target cells were determined by substituting blood sample with CM or Triton-X (Sigma-Aldrich, St. Louis, MO), respectively. Plates were centrifuged ($596 \times g$ for 10 min) before 4 h incubation. After incubation, plates were again centrifuged and aliquots of 100 μl of the supernatant were recovered from each well for assessment of radioactivity in a γ -counter. Specific killing was calculated as:

$$\left[\frac{(\text{sample release} \times \text{hematocrit correction factor (HCF)} - \text{spontaneous release})}{(\text{maximal release} - \text{spontaneous release})} \right] \times 100.$$

HCF compensates for changes in the hematocrit to supernatant volume over different E:T ratios. This correction factor is included to consider the changing volume of cell-free medium into which radioactivity is released.

Preparation of target cells

Five $\times 10^6$ YAC-1 cells were incubated for 1 h with 100 μCi of [^{51}Cr] (Danyl Biotech, Rehovot, Israel) in 100 μl saline, 100 μl heat-inactivated FCS, and 220 μl CM. After incubation, cells were washed three times ($335 \times g$ for 10 min) and adjusted to the concentration of 5×10^4 cells/ml in CM.

Comparing cytotoxicity per NK cell

Cytotoxicity levels of the different effector-cell concentrations were used for each sample to generate an extrapolated cytotoxicity curve, based on the regression exponential fit method (27). Then, based on the number of NK cells unique to each sample, each curve was shifted horizontally by mathematical transformation of its extrapolated formula and positioned in a cytotoxicity (y) by E:T ratio (x) plot based on its individual NK:target ratio. Thus, cytotoxicity levels in different samples are compared based on the same number of NK cells.

Flow cytometry

FACS was used to assess leukocyte numbers and cellular marker expression levels. Fifty microliters of whole blood was incubated for 20 min at room temperature with the following anti-mouse Abs: FITC-conjugated NK1.1, PE-conjugated CD95Ligand (CD178), PE-conjugated TRAIL (CD253) (eBioscience, Kfar Saba, Israel), PE conjugated CD3e, PEcy5 conjugated CD3e, PEcy5 conjugated CD69, and PE-conjugated CD11a (Biolegend, Petach-

Tiqva, Israel). Next, samples were incubated at room temperature for 12 min with 1 ml FACS lysing solution (BD Biosciences, San Jose, CA) containing 15,000 polystyrene microbeads (300 microbeads per microliter blood sample; Duke Scientific, Palo Alto, CA), centrifuged for 5 min at $1026 \times g$, and lysate aspirated. Cells were then washed again (5 min, $1026 \times g$) with 1 ml PBS containing 2% FCS and 0.1% NaN_3 (PBS++) and resuspended in 500 μl PBS++ for flow cytometric analysis using the FACScan (BD Biosciences).

Cell identification

Granulocytes and lymphocytes were identified based on size and granularity. Within the lymphocyte population, NK cells were identified as $\text{NK-1.1}^+\text{CD3}^-$, T cells as $\text{NK-1.1}^- \text{CD3}^+$, and B cells as $\text{NK-1.1}^- \text{CD3}^-$. Absolute number of cells per microliter of blood was quantified using the formula: number of positive events / number of microbeads \times 300. Expression level of cellular markers was indicated as the median fluorescence emission in cells that were found positive to the marker.

In vitro VEGF secretion by tumor lines and the impacts of various agonists

We followed the exact procedures used by Lutgendorf et al. (2) for assessing the in vitro effect of various hormones on VEGF secretion by the B16 and 3LL tumor lines. We used norepinephrine, the β -adrenergic agonist metaproterenol, the β -adrenergic antagonists nadolol and propranolol, and the COX-2 inhibitor etodolac (as well as the combination of propranolol and etodolac) at the concentrations of 0 (control), 10^{-5} , 10^{-6} , and 10^{-7} M, and PGE_2 and CORT at the concentrations of 0 (control), 10^{-6} , 10^{-7} , and 10^{-8} M, and tested their effects on VEGF secretion at 3 and 6 h after in vitro drug exposure. For a positive control, we used the ovarian carcinoma SKOV3 tumor line that was studied by Lutgendorf et al. (2), similarly exposing it to norepinephrine and metaproterenol. All cell lines were tested in all conditions in triplicate, and in the presence or absence of FCS. Supernatant levels of VEGF were quantified using ELISA kits (R&D, Kfar Saba, Israel), based on manufacture protocols.

Procedure for all survival studies

Each mouse was injected with 5×10^4 B16F10.9 melanoma cells or d122 Lewis lung carcinoma (in 20 μl PBS containing 0.1% BSA) intrafootpad, and tumors were visually inspected daily. Once a tumor reached 100–150 μl in volume, the mouse was anesthetized with 2% isoflurane and underwent a specific drug treatment, and the tumor was excised by paw amputation 30 min later. Mice in which the designated tumor volume was achieved were assigned to a specific drug treatment group based on a predetermined counterbalanced order, thus ensuring equal distribution of tumor size and tumor age at excision time between the different drug groups. The experimenter conducting the amputation was unaware of the drug treatment group. Mice were subsequently monitored for morbidity signs on a daily basis for an 80 d period (and no less than 2 wk following the last morbidity incidence). Mice that showed sickness behavior or manifested cancer recurrence were overdosed with isoflurane and autopsied to determine malignant foci. Sickness behavior was defined by slow body movements, irresponsiveness to environmental stimuli, significant weight loss, or tremor.

Statistical analysis

One-way, two-way, or repeated measures ANOVA were used to identify significant group differences. Provided that group differences were indicated, Fisher's protected least significant differences (PLSDs) contrasts were used to test specific pairwise comparisons with respect to a priori hypotheses. To assess survival rates, the Kaplan-Meier model was used, followed by the Tarone-Ware test for pairwise group comparisons. Values of $p < 0.05$ were considered significant in all studies, and all p values were two-tailed.

Results

Experiment 1: the effects of amputation and of laparotomy on NKC and on survival rates

NK numbers and cytotoxicity per milliliter of blood. NK numbers and cytotoxicity were assessed in blood samples taken from naive (control), amputated, laparotomized, or amputated and laparotomized animals ($n = 19$). Amputation ($p = 0.0112$), laparotomy ($p = 0.0003$), and amputation and laparotomy ($p < 0.0001$) each significantly suppressed NKC relative to control levels (Fig. 1A). Amputation alone was significantly less suppressive than laparotomy and amputation ($p = 0.0253$). NK cell numbers per mil-

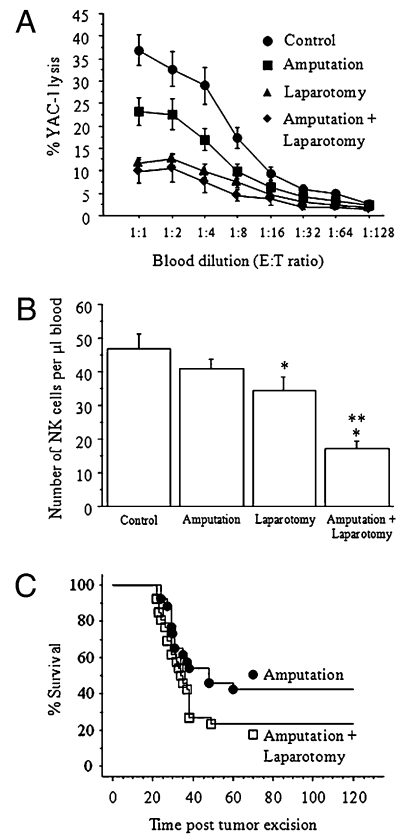


FIGURE 1. A, NKC per milliliter of blood against YAC-1 target cells (mean \pm SEM): amputation alone, laparotomy alone, and both procedures, each significantly suppressed NKC compared with control animals. The suppressive effects of amputation alone were significantly smaller than those evident in the two groups undergoing laparotomy. B, NK cell numbers per microliter of blood (mean \pm SEM): NK numbers were significantly reduced by laparotomy, with or without amputation, in comparison with the control group (*). NK number in mice undergoing both procedures was significantly reduced compared with either procedure alone (**). C, Survival rates following B16 tumor excision: laparotomy and amputation reduced survival rates compared with amputation alone, but this effect was not statistically significant ($p = 0.092$).

liliter of blood were also affected. Laparotomy ($p = 0.0255$), or amputation and laparotomy ($p < 0.0001$), significantly reduced NK cell numbers (Fig. 1B).

Survival rates. Amputation is necessary for removing the primary tumor in this survival model, otherwise all mice will die. To determine whether conducting laparotomy in amputated mice will reduce survival rates, B16 tumor-bearing mice either underwent amputation alone or amputation and laparotomy (total $n = 52$). Although mice also undergoing laparotomy showed worse survival rates, no statistically significant difference was revealed between mice that underwent amputation and laparotomy compared with mice that underwent amputation alone ($p = 0.0921$; Fig. 1C).

Experiment 2: the effects of propranolol and etodolac in laparotomized and in amputated mice

B16 tumor bearing mice were administered once either with propranolol (P), etodolac (E), both drugs (P+E), or vehicle (neither drug) (total $n = 234$). Thirty minutes later, each group was subdivided to undergo amputation alone, or amputation and laparotomy, and was monitored for morbidity thereafter. Similar to the survival outcome in experiment 1 (Fig. 1C), subjecting amputated mice to laparotomy did not significantly worsen survival rates in any of the drug conditions, nor across conditions

(Fig. 2A). Irrespective of the surgical procedure (only amputation or amputation plus laparotomy), the combined P+E treatment significantly increased survival rates ($p = 0.0345$), whereas neither drug alone had an effect (Fig. 2B).

Experiments 3: only the combined administration of propranolol and etodolac increases survival rates

This study was conducted to verify the previous surprising finding that only the combined drug treatment was effective. Because in the previous two studies laparotomy did not significantly worsen survival rates in amputated mice, all B16 tumor-bearing mice underwent amputation only. Mice were administered once with propranolol, etodolac, P+E, or vehicle (total $n = 190$) 30 min before tumors were excised. P+E administration significantly increased survival rates (Fig. 3; $p = 0.0315$). As in the previous experiment, neither drug alone improved survival rates.

Experiment 4: comparing and combining poly(I:C) immunostimulation with the drug treatment

We compared the efficacy of P+E as a treatment for either poly(I:C) alone, or to poly(I:C) and P+E. One day before amputation, B16 tumor bearing mice were administered with poly(I:C) or vehicle, and 30 min before amputation each group was further subdivided to receive either P+E or vehicle (total $n = 171$). P+E administration ($p = 0.0200$) and P+E and poly(I:C) ($p = 0.0131$) significantly increased survival rates (Fig. 4). Poly(I:C) alone did not reach statistical significance in improving survival ($p = 0.2489$), nor did it further improve the effects of P+E (Fig. 4).

Experiment 5: comparing and combining IL-12 immunostimulation with the drug treatment in the Lewis lung carcinoma tumor model

One day before amputation, 3LL tumor-bearing mice were administered with either IL-12 or vehicle, and 30 min before am-

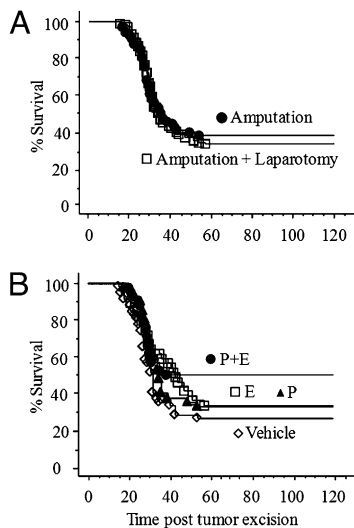


FIGURE 2. Survival rates following B16 tumor excision: Drugs (P, propranolol; E, etodolac) were administered once, 30 min before tumor excision. Half the mice in each drug group underwent laparotomy and amputation, and the other half only amputation. A, No significant difference was evident between amputated mice that were also laparotomized and mice that were only amputated (in each drug separately and across all drug treatments, as seen in the figure), suggesting a ceiling effect of amputation. B, As the addition of laparotomy to amputation did not affect survival rates or the effects of drug treatment, mice that underwent both surgical procedures and those only undergoing amputation are presented together. Survival rates in the P+E condition were significantly higher than in the control group. Neither drug alone was effective.

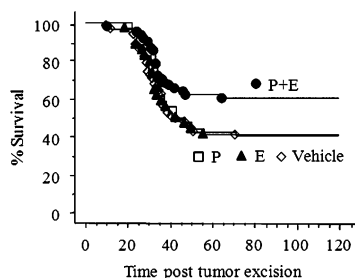


FIGURE 3. Survival rates following B16 tumor excision (amputation only); drugs (P, propranolol; E, etodolac) were administered once, 30 min before amputation. A significant effect was indicated for the combined drug treatment, as survival rates in the P+E group were notably higher than in the control group. Neither drug alone was efficient in elevating survival rates.

putation each group was further subdivided to receive P+E or vehicle (total $n = 218$). All treatments significantly improved survival rates compared with vehicle treatment (Fig. 5; IL-12, $p = 0.023$; IL-12 and P+E, $p = 0.024$; P+E, $p = 0.047$), and showed similar effect sizes (Fig. 5).

Experiment 6: the effects of laparotomy and of the combined drug treatment on NK cell number and activity, cellular surface markers, and plasma CORT level

Because this study aimed to assess the effects of a surgical procedure, and because the animals did not bear a primary tumor, we used laparotomy as the experimental manipulation. Laparotomy is a more robust surgical procedure than amputation in tumor-free animals (see experiment 1), and it does not maim the animals. Animals received either P+E or vehicle and 30 min later were further subdivided to undergo either laparotomy or no surgical procedure (total $n = 108$). Twelve hours later, animals were sacrificed for blood withdrawal by cardiac puncture.

NK cell number and cytotoxicity per milliliter of blood. The indices of NKC per milliliter of blood and blood concentration of NK cells showed the same patterns of effects, and are thus presented together. Repeated measures ANOVA revealed a main effect for surgery (NKC, $p < 0.0001$; NK number, $p < 0.0001$), a main effect for drug treatment (NKC, $p = 0.0003$; NK numbers, $p = 0.0130$; Fig 6A, 6B), and no interaction. Specifically, laparotomy reduced NKC and NK numbers, and the drug treatment elevated these indices in both operated and nonoperated animals.

NKC per NK cell. To assess the degree to which NKC was affected irrespective of reduced NK cell numbers, NKC in the different groups was compared per equal number of NK cells (see *Materials*

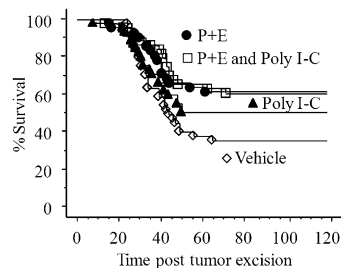


FIGURE 4. The effects of immunostimulation and of the combined drug administration on survival rates following B16 tumor excision: poly(I:C) or vehicle was administered 1 d before tumor excision, and the two drugs (P, propranolol; E, etodolac) were coadministered 30 min before tumor excision. Survival rates in the P+E and the P+E and poly(I:C) groups were significantly higher than in the control group. The effects of poly(I:C) alone did not reach statistical significance.

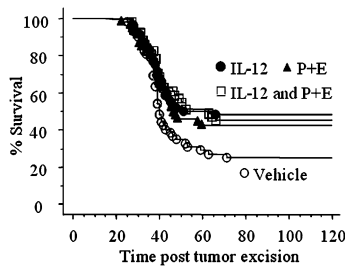


FIGURE 5. The effects of immunostimulation and the combined drug administration on survival rates following 3LL tumor excision: IL-12 or vehicle was administered 1 d before tumor excision, and the two drugs (P, propranolol; E, ethodolac) were coadministered 30 min before tumor excision. Survival rates in all groups were significantly higher than in the control group.

and Methods). Repeated measures ANOVA revealed main effects for laparotomy ($p = 0.0005$) and for drug treatment ($p < 0.0002$) and no interaction (data not shown). PLSD indicated that laparotomy significantly reduced NKC on a per NK cell basis ($p = 0.0002$), and P+E administration increased it on a per NK cell basis, in both operated and nonoperated animals ($p = 0.0001$).

Blood concentration of other leukocytes. Laparotomy significantly decreased overall lymphocyte numbers ($p < 0.0001$). Specifically, B cell number significantly decreased by 3-fold ($p < 0.0001$) and T cells by 2-fold ($p < 0.0001$). Laparotomy nearly doubled granulocyte numbers ($p < 0.0001$). The combined P+E administration did not significantly affect any non-NK leukocyte numbers (Table I).

Within the NK cell population, a distinction was made between the small NK and the large NK subpopulations, because in previous studies we have repeatedly noticed molecular and functional differences between these populations (24, 28). See Table I for detailed findings.

Proportion and expression levels of cell surface markers

The TNF-related apoptosis inducing ligands CD253 (TRAIL) and CD95L (Fas ligand [FasL]), the CD69 early activation marker, and the adhesion molecule LFA-1 (CD11a) expression levels were assessed in each of the aforementioned leukocyte populations. Histogram plots for assessment of expression levels of each marker were gated only on marker-positive cells. Table I quantitatively summarizes significant effects caused by laparotomy and by the drug treatment (i.e., P+E) on blood concentrations of different leukocyte subpopulations and the expression levels of these markers. Given the vast number of outcomes, below is a detailed description of the results in regard to NK cells.

FasL. Laparotomy caused a 5-fold decrease in CD95L⁺ NK numbers per milliliter of blood, a 2-fold decrease in the proportion of CD95L⁺ NK cells (within the NK cell population), and a small but statistically significant decrease in CD95L⁺ expression levels on NK cells. P+E administration significantly increased expression levels of CD95L on NK cells (Fig. 6C).

LFA-1. Laparotomy caused a 2-fold decrease in CD11a⁺ NK numbers, and a small but statistically significant decrease in expression level. P+E administration significantly increased CD11a⁺ NK numbers in both laparotomized and control animals (Fig. 6D).

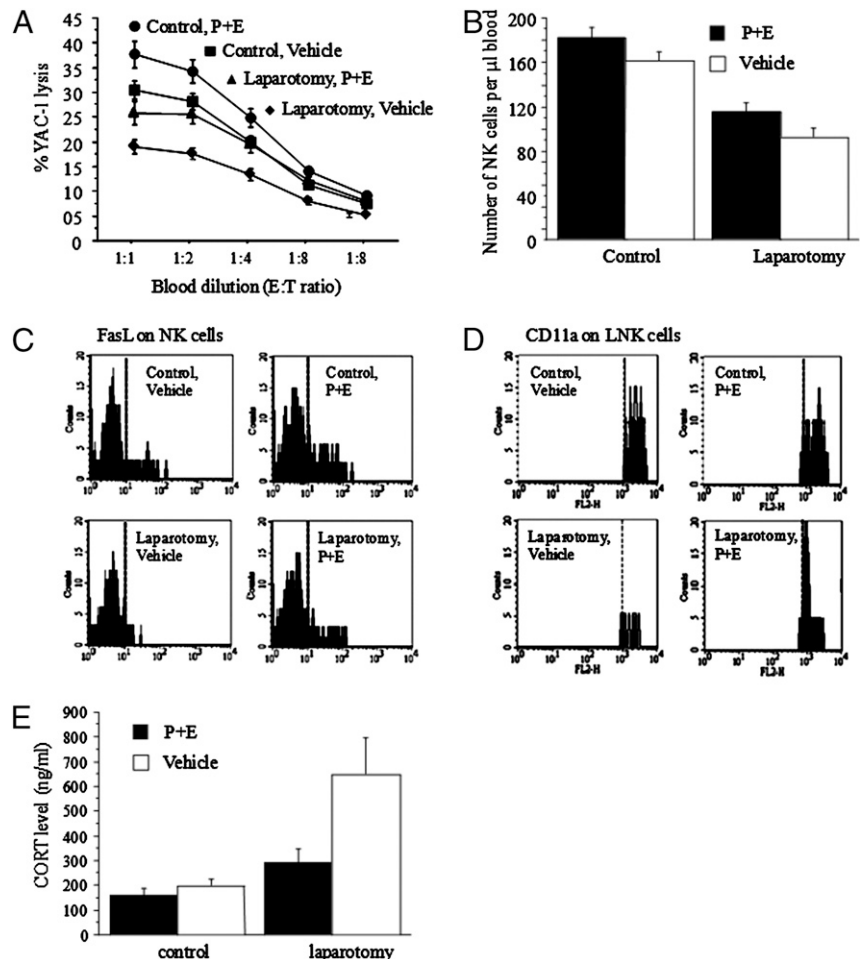


FIGURE 6. The effects of laparotomy and drug administration on immune and endocrine indices (mean \pm SEM): NKC (A) against YAC-1 target cells, and numbers of NK cells per milliliter of blood (B) were significantly suppressed by laparotomy, and the combined drug treatment of P+E increased it both in laparotomized and in non-laparotomized animals. C, Laparotomy decreased the number of CD95⁺ (FasL⁺) NK cells, and expression levels of CD95 in NK⁺ cells. P+E administration increased both indices in laparotomized and in nonlaparotomized animals. D, Laparotomy caused a marked decrease in the numbers CD11a⁺ NK numbers, and P+E administration restored this index. E, CORT plasma levels increased 12 h following laparotomy, and the combined P+E treatment significantly reduced this effect.

Table 1. The effects of laparotomy and drug treatment with P+E on blood concentrations of different leukocyte subpopulations and their expression of FasL, TRAIL, CD11a, and CD69 surface markers

Condition	Population ^a	Blood Concentration	CD95L (FasL)		CD253 (TRAIL)		CD11a (LFA-1)		CD 69	
			No. per ml Blood	%	No. per ml Blood	%	No. per ml Blood	%	No. per ml Blood	%
Laparotomy P+E	Granulocytes	+p < 0.0001	+p = 0.0033	-p < 0.0011	-p < 0.0001	-p < 0.0001	+p < 0.0001	-p < 0.0001	-p < 0.0001	+p < 0.0001
Laparotomy P+E	Lymphocytes	-p < 0.0001	-p < 0.001	+p = 0.0015	+p < 0.0001	+p < 0.0001	+p < 0.0001	+p = 0.0211	-p = 0.0005	+p < 0.0001
Laparotomy P+E	T cells	-p < 0.0001	-p < 0.0001	+p < 0.0002	-p < 0.0001	-p < 0.0001	+p < 0.0001	+p < 0.0001	+p < 0.0001	+p < 0.0001
Laparotomy P+E	B cells	-p < 0.0001	-p < 0.0001	-p < 0.0001	-p < 0.0001	-p < 0.0001	+p < 0.0001	-p = 0.0038	-p < 0.0001	+p = 0.0020
Laparotomy P+E	NK	-p < 0.0001	-p < 0.0011	-p < 0.0011	-p < 0.0001	-p = 0.0030	-p < 0.0001	-p < 0.0001	-p < 0.0001	+p = 0.0059
Laparotomy P+E	Small NK	+p = 0.0056	-p < 0.0001	-p < 0.0001	+p = 0.0267	-p = 0.0412 ^b	+p = 0.0021	-p < 0.0001	-p < 0.0001	-p < 0.0001
Laparotomy P+E	Large NK	-p < 0.0001	-p < 0.0001	-p < 0.0001	-p = 0.0324	+p < 0.0001	-p < 0.0001	-p = 0.0022	-p < 0.0001	-p < 0.0001
Laparotomy P+E		-p < 0.0001	-p < 0.0001	-p < 0.0010	-p = 0.0142	+p = 0.0078	-p < 0.0001	-p < 0.0001	+p < 0.0001	+p < 0.0001
Laparotomy P+E		+p = 0.0562					+p = 0.0425	+p = 0.0246 ^b		

The table indicates whether a significant effect of laparotomy or of P+E was evident with respect to blood concentration of different leukocyte subpopulations (first column), and their cellular markers (CD95L, CD253, CD11a, CD69; following columns).

^aThe magnitude of the changes in cells' blood concentrations (e.g., 3-fold increase) are detailed in the Results section.

^bA significant effect only within laparotomized animals.

+, represents a significant increase in the levels of each index; -, represents a significant decrease in the levels of each index; %, indicates proportion of positive cells within this cell population; E, expression level of the CD within CD positive cells.

TRAIL. The percent of CD253⁺ NK cells significantly increased following laparotomy, whereas expression levels of CD253 on NK decreased. P+E administration halved the increase in proportion in laparotomized animals only.

CD69. Laparotomy significantly decreased numbers of CD69⁺ NK cells, but significantly increased expression levels of CD69 on CD69⁺NK cells. P+E administration had no effect in laparotomized or control animals.

Postoperative CORT plasma levels

CORT plasma levels were measured using RIA (MP Biomedicals, Rehovot, Israel). The assay was conducted using plasma samples from 50 animals that were randomly sampled from the 108 mice used in the above study (blood taken 12 h following laparotomy). Laparotomy significantly increased CORT levels ($p = 0.0036$) and P+E significantly reduced them ($p = 0.0442$) (Fig 6E). PLSD indicated that P+E treatment significantly decreased CORT levels in operated animals only ($p < 0.0119$).

Experiment 7: in vitro effects of various drugs on VEGF secretion by B16, 3LL, and SKOV3 tumor lines

All three tumor lines (B16, 3LL, and SKOV3) secreted VEGF, and VEGF supernatant levels significantly increased from 3–6 h in all lines in both the absence and presence of FCS (e.g., in the absence of FCS, 79 ± 3 to 152 ± 8 , 338 ± 55 to 509 ± 72 , and 217 ± 8 to 512 ± 33 pg/ml for B16, 3LL, and SKOV3, respectively). In the positive control SKOV3 line, norepinephrine and metaproterenol significantly increased VEGF levels in a dose-dependent manner both in the presence and absence of FCS ($p < 0.05$), as previously reported (2). In contrast, norepinephrine, metaproterenol, nadolol, PGE₂, and CORT did not affect VEGF secretion at any of the time points and doses (see *Materials and Methods*) tested in the B16 and 3LL lines with or without FCS. Etodolac, propranolol, and P+E also did not affect VEGF secretion. These negative findings were confirmed in a second replication of the study.

Discussion

The primary aim of this study is to promote the clinical testing of a combined blockade of excess release of CAs and PGs in the perioperative period in cancer patients undergoing tumor excision. Therefore, we studied the most important clinical outcome—namely, long-term recurrence-free survival rates after the excision of the primary tumor. Our findings indicate that the blockade of β -adrenoreceptors simultaneously with the inhibition of PG synthesis (using P+E) significantly improve recurrence-free survival rates following 3LL or B16 primary tumor excision. We used these tumors given that, like most human cancers (29), both lines are poorly immunogenic, expressing no MHC-II and low levels of MHC-I (30). The B16 primary tumor was also implanted and developed orthotopically.

Notably, only the combined administration of P+E had a significant prophylactic effect in the survival studies, suggesting synergistic effects of these drugs through various mechanisms. One possible explanation for this synergism is that during the postoperative period both PG and CA levels are high, and each factor alone can interfere with NKC by activating its membrane receptors on NK cells, which, through the same intracellular mechanisms elevate intracellular cAMP levels and cause suppression of NKC (14, 15). Similar mechanisms regulate CTL and NKT activity, and CAs and PGs have analogous immunosuppressive effects on many aspects of CMI (1). It is therefore reasonable to assume that only simultaneous blockade of both receptor systems will eventually prevent the suppression of CMI by surgery, as we reported in rats undergoing laparotomy (22). Similarly, both CAs and PGs were

reported to affect angiogenic-related processes (2, 19, 20), which can synergistically affect tumor progression.

To further estimate the efficacy of the combined drug regimen, we compared it to poly(I:C) and to IL-12 administration, two well-established experimental interventions reported to control tumor progression (31). The combined drug regimen using P+E was at least as effective as these treatments in our studies. Importantly, whereas such biologic response modifiers act through boosting immune competence, propranolol and etodolac could operate both via augmenting perioperative immune competence, as well as through several nonimmunologic pathways, as discussed below.

Two observations in the current study support our working hypothesis presented in the introduction, that the immediate postoperative period is critical in determining long-term tumor recurrence rates, and that clinical interventions during this critical period may have substantial long-term benefits. First, the various treatments in this study were given once (i.e., a single administration of drugs), in conjunction with tumor excision. Given that such short interventions doubled long-term survival rates, the perioperative period seems to exert a disproportionately large impact on the metastatic process and on long-term survival rates. Second, in all studies the survival rates of the different groups started to differ from each other abruptly, and not before the second half of the survival monitoring time (on approximately day 35 after amputation, Figs. 2–5). Presumably, during this later phase mortality is a consequence of processes that have occurred during the immediate postoperative period, when the surgery and drug treatments exerted their effects. In contrast, along the earlier phase, mortality is a consequence of metastatic processes that have consolidated before subjecting the animals to surgery and drug treatment. These observations suggest that the perioperative period is critical for determining long-term survival, as are the drug treatments given in this setting.

In addition to demonstrating improved survival rates by the combined drug regimen, we started to explore the many potential underlying mechanisms. For example, CAs have been shown to upregulate VEGF secretion by several tumor cells through activation of tumor β -adrenoreceptors (2, 21). In the current study, we replicated the findings by Lutgendorf et al. (2), indicating that norepinephrine increases VEGF secretion by the SKOV3 tumor line. Alternatively, although both the B16 and the 3LL tumor lines secreted VEGF in culture, epinephrine, norepinephrine, the β -agonist metaproterenol, PG, or CORT did not modulate this secretion across a range of doses and time intervals tested, nor did propranolol or etodolac (alone or in combination). Therefore, the beneficial effects of propranolol and etodolac seem not to be mediated by directly affecting B16 or 3LL secretion of VEGF, nor by reducing stress-hormone-induced increase in VEGF secretion by these tumor cells. Nevertheless, *in vivo* tumor responses may differ from the *in vitro* setting, and Sood et al. have also shown that CAs can increase MMP2 and MMP9 secretion by tumor cells and accelerate tumor invasion capacity (20). Therefore, other direct effects of stress hormones on tumor cells, which were not tested herein, could clearly be involved and mediate the beneficial effects evident in this study.

Although in cancer patients the immune system had failed to control the primary tumor, some researchers believe that for numerous reasons antimalignant immunity is significant in restricting postoperative metastasis (1, 2). Specifically related to the current study, various forms of immunotherapy were shown to reduce B16 and 3LL spontaneous metastasis through CTL- and NK-dependent responses (32, 33), and experimental metastases of B16, but not 3LL, were reported to be under NK control when tumor cells were administered *i.v.* at low numbers (34). NK cells and CTLs induce cytotoxicity through two main mechanisms, namely the perforin/granzymes pathway and the Fas/FasL pathway. Both pathways re-

quire cell-to-cell contact and rely on surface receptors for activation (35). Several studies report death receptor-mediated tumor cell apoptosis by NK cells (36), and physiologic stress was shown to suppress FasL-mediated NK activity (37, 38). Indeed, the current study showed that the expression of FasL on NK cells decreased following surgery and that our drug treatment counteracted this effect. Similarly, CD11a expression was implicated in initiating activation signals in NK cells and was suggested to induce important signaling in human NKC (39). We have demonstrated a marked decrease in NK cell expression of CD11a following laparotomy and a corresponding increase following the drug treatment. Last, laparotomy induced a significant suppression of NKC, and the administration of propranolol and etodolac increased NKC both in operated and nonoperated animals.

Hypothalamus-pituitary-adrenal axis activation and the production of CORT are rapid reactions not only to adrenocorticotrophic hormone (ACTH) release, but also to a variety of humoral factors, including PGs, CAs, and several cytokines (40, 41). Postoperative increase in CORT levels was shown to correlate with CMI suppression (42). CORT is known to suppress NKC through regulating transcription factors (including AP-1, NF- κ B, and STAT5) (43), and several animal studies associated elevated CORT levels with increased cancer progression (44). In the current study, elevated postoperative CORT levels were reduced by the combined drug treatment, constituting another potential mechanism for the herein evident improved survival rates. Another immune function that could mediate the effects of surgery is myeloid-derived suppressor cells (MDSCs) (expressing CD11b/Gr-1 markers) that were shown to invade the spleen following traumatic stress and cause T cell dysfunction by an arginase-mediated mechanism (45). Indeed, in renal cell carcinoma patients, PGE₂ produced by the tumor was suggested to induce arginase-I expression in MDSCs, and depletion of MDSCs restored IFN- γ production and T cell proliferation (46).

Supporting the potential clinical use of these drugs in cancer patients undergoing surgery, both propranolol and etodolac are routinely used in the clinical setting, and both are applied either in the context of surgery or/and in the context of cancer treatment. COX-2 inhibitors, which unlike COX-1 inhibitors do not increase the risk of bleeding, are used as pain relievers during surgery (47) and have been used for the prevention and treatment of malignancies (48). Furthermore, in a previous study we found that COX-2, but not COX-1 inhibition, attenuated the metastasis-promoting effects of surgery, and etodolac was more effective than the highly COX-2 selective celecoxib (22). The perioperative administration of propranolol and other β -blockers have been frequently used to prevent surgery related cardiac complications and mortality (49). We used a nonselective β -antagonist (propranolol), as human leukocytes and human tumor cells express both β -1 and β -2 adrenoreceptors (50, 51), and as our previous study indicated that the blockade of both receptor systems was more effective than each alone in preventing stress-induced promotion of metastasis (52). Importantly, in the clinical setting of oncologic surgery, the drug regimen used in this study may be even more efficient if initiated a few days before surgery, because etodolac could reduce PGs release by primary tumors (14); propranolol could reduce anxiety (53) and antagonize the excess release of CAs owing to preoperative physiologic and psychologic stress responses (54, 55). Together, preoperative use of these drugs can attenuate preoperative suppression of CMI in cancer patients awaiting surgery (10, 56). Postoperatively, the drugs may be used until the common phenomena of surgery-induced endocrine responses and immunosuppression dissipate (1, 10). The doses should be gradually reduced along several postoperative days, as the excess release of PGs, cortisol, and CAs decrease respectively.

The survival studies presented herein causally linked the drug treatment to improved long-term survival rates. Alternatively, all other effects of the drug treatment reported in this study (e.g., increased postoperative NKC) are merely associated with the observed improved survival rates and thus merit further research. The wide range of immunologic indices we assessed seems to provide an approximation of CMI status, its systemic postoperative compromise, and attenuation of these deleterious effects by the drug treatment. However, the current study cannot causally implicate the immune system in mediating the effects of surgery or the beneficial effects of drug administrations; some of the potential nonimmunologic mediators discussed above may be involved. Regardless of the relative weight of each potential mediator, and considering that host-tumor interactions are multifaceted and that animal tumor models are intrinsically imperfect, we believe that only clinical studies in cancer patients would prove or refute the potential benefits demonstrated by this drug regimen in our current and previous animal studies. Because the drugs used in this regimen are routinely used clinically for other indications, such clinical trials seem feasible, relatively inexpensive, and safe.

Specifically, patients with colon, breast, or prostate cancers, undergoing open or laparoscopic tumor excision with curative intent and with no known metastases, would be suitable and prevalent candidates for such studies. Surgeries in these patients involve significant neuroendocrine responses, immune suppression, and alleged metastasis-promoting effects of surgery (1, 47, 57). In preparation for such a study in colon cancer patients, we have recently established that the use of etodolac and/or propranolol in rats does not interfere with colon healing following anastomosis (O. Hazut, B. Benjamin, L. Shaashua, M. Benish, N. Zmora, I. Barshack, O. Zmora, and S. Ben-Eliyahu, submitted for publication). To implicate perioperative immunity or specific neuroendocrine responses in improved long-term survival rates in the clinical setting, all those indices should be recorded using a within-patient design, and a sufficiently large sample should be recruited to test potential enhancement of 3-y recurrence-free survival rates. Such studies may indicate, for the first time, whether reducing excessive perioperative stress responses and/or immunosuppression in cancer patients would also reduce recurrence rates.

Disclosures

The authors have no financial conflicts of interest.

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