Marginating pulmonary-NK activity and resistance to experimental tumor metastasis: suppression by surgery and the prophylactic use of a β-adrenergic antagonist and a prostaglandin synthesis inhibitor

Rivka Melamed, Ella Rosenne, Keren Shakhar, Yossi Schwartz, Naphtali Abudarham, Shamgar Ben-Eliyahu

Neuroimmunology Research Unit, Department of Psychology, Tel Aviv University, Tel Aviv 69978, Israel

Received 25 April 2004; received in revised form 7 July 2004; accepted 22 July 2004
Available online 2 September 2004

Abstract

Surgery is imperative for cancer treatment, but was suggested to suppress immunity and facilitate metastasis. Here we study the involvement of catecholamines and prostaglandins (PG) in such outcomes, and the role played by marginating-pulmonary (MP)-NK cells in controlling MADB106 metastasis. Non-operated and laparotomized F344 rats were injected postoperatively with a PG synthesis inhibitor (indomethacin, 4 mg/kg i.p.), a β-blocker (nadolol, 0.6 mg/kg s.c.), both drugs, or vehicle. Rats were then inoculated intravenously with non-immunogenic syngeneic MADB106 cells, and 24 h later lung tumor retention was assessed, or 3 weeks later lung metastases were counted. Additionally, 12 h after surgery we harvested MP-NK cells and circulating-NK cells and compared their numbers and cytotoxicity against MADB106 cells and standard YAC-1 target cells. Surgery significantly increased MADB106 metastasis. Nadolol and indomethacin reduced this effect by approximately 50% when used alone, and significantly more (75%) when used together. Only MP-leukocytes exhibited NK cytotoxicity against MADB106 cells. Surgery markedly suppressed it, and nadolol and indomethacin additively restored it. Similar effects were observed assessing MP-NK and circulating-NK cytotoxicity against YAC-1 target cells. Alterations in the numbers of NK cells were partly associated with alterations in total MP-NK activity, but not with circulating-NK activity. Last, administering naïve rats with physiologically relevant doses of a β-adrenergic agonist (metaproterenol), and/or with PGE2, additively and independently of each other promoted MADB106 metastasis, simulating the effects of surgery. These findings point at potential prophylactic measures in cancer patients undergoing surgery, and suggest a role for MP-NK cells in resisting metastasis of apparently insensitive tumors.

1. Introduction

A large proportion of cancer-related deaths are caused by metastatic recurrence, despite successful excision of the primary tumor which is imperative for eliminating the major source of mutating and metastasizing tumor cells (Shakhar and Ben-Eliyahu, 2003). Unfortunately, the surgical procedure and the removal of the primary tumor have long been suspected to facilitate the growth of preexisting micrometastases and the dissemination of tumor cells during the perioperative period (Ben-Eliyahu, 2003). Potential mechanisms for such adverse effects include a drop in the levels of anti-angiogenic factors (Zetter, 1998), shedding of tumor cells due to the physical manipulation of the malignant tissue or
its vasculature (Eschwege et al., 1995; Yamaguchi et al., 2000), local and systemic release of growth factors (Hofer et al., 1999), and suppression of cell mediated immunity (CMI) (Sietses, 1999). It has been suggested that synergy between these risk factors during the immediate postoperative period may temporarily render the patient susceptible to metastasis that would have been controlled otherwise (Shakhar and Ben-Eliyahu, 2003).

Suppression of CMI is a well-established postoperative clinical complication in major operations (Weighardt et al., 2000). Animal studies have provided direct evidence that such suppression, alone or in conjunction with other risk factors, can promote the survival and growth of minimal residual disease (MRD) after the primary tumor has been removed (Allendorf et al., 1999; Ben-Eliyahu et al., 1999; Colacchio et al., 1994; Da Costa et al., 1998; Shiromizu et al., 2000). MRD includes single tumor cells (which are found following surgery in the circulation, lymphatic system, or bone marrow of most cancer patients), as well as preexisting micrometastases. Nevertheless, many of the animal studies have been justifiably criticized for using immunogenic syngeneic tumor lines (Hewitt, 1983; Killion et al., 1998), and clinical studies have so far provided only correlative evidence supporting the notion that postoperative immunosuppression can promote cancer recurrence (Shakhar and Ben-Eliyahu, 2003). Consequently, the clinical practice does not endorse prophylactic measures against postoperative immunosuppression in cancer patients.

Animal studies have indicated that immune control over circulating tumor cells and micrometastases is carried mainly through CMI, including cytotoxic T lymphocytes (CTL), NK, NK-T cells, dendritic cells, and macrophages (Smyth et al., 2001). Specifically, molecular mechanisms of NK recognition of tumor cells have been recently revealed, and the role of NK cells in controlling leukemia and metastasis in humans has received substantial support (Brittenden et al., 1996; Cerwenka and Lanièr, 2001; Moretta et al., 2000). It is now believed that although the immune system has clearly failed to control the primary tumor in cancer patients, CMI can still eliminate MRD after the primary tumor has been removed, especially if surgery is conducted early, before elaborated tumor escape mechanisms have evolved (Shakhar and Ben-Eliyahu, 2003).

Several aspects of surgery have been proposed to underlie postoperative suppression of CMI, among them are anesthetic agents (Galley et al., 2000), hypothermia (Beilin et al., 1998; Ben-Eliyahu et al., 1999), tissue damage (Lennard et al., 1985), blood loss and transfusion (Klein, 1999), noceception and pain (Koltun et al., 1996), and perioperative distress (Cohen and Herbert, 1996; Larson et al., 2000). Several humoral factors could be suggested to mediate the immunosuppressive effects of these aspects of surgery (Ben-Eliyahu, 2003), central to the current study are prostaglandins (PGs) and catecholamines. Most of the above aspects of surgery cause activation of the sympathetic nervous system during the perioperative period (Koltun et al., 1996), and PGs are abundant following intrusive procedures due to tissue damage (Baxevanis et al., 1994). Both substances were reported to suppress various aspect of CMI in vitro and in vivo (Chambrier et al., 1996; Elenkov et al., 2000; Faist et al., 1990, 1996).

In our previous studies in F344 rats we have shown that following various stressful conditions, the release of adrenal catecholamines and activation of β1- and β2-adrenoceptors can suppress NK activity and increase susceptibility to experimental metastasis of the MADB106 tumor line (Ben-Eliyahu et al., 1999, 2000; Stefanski, 1994). This mammary adenocarcinoma is a non-immunogenic syngeneic tumor line that establishes metastases only in the lungs following its intravenous inoculation (Barlozzari et al., 1983, 1985; Ben-Eliyahu and Page, 1992). In addition to using stressful conditions, we were able to induce suppression of NK activity by administering a non-selective β-adrenergic agonist, or by administering PGE2 (Barlozzari et al., 1983, 1985; Ben-Eliyahu and Page, 1992; Ben-Eliyahu et al., 1996b; Shakhar and Ben-Eliyahu, 1998; Yakar et al., 2003). However, NK activity was always studied in vitro against standard xenogeneic YAC-1 target cells, because the syngeneic MADB106 line was found to be resistant to the activity of NK cells from various cell populations. These populations included splenocytes, circulating leukocytes, PBMCs (Barlozzari et al., 1983, 1985; Ben-Eliyahu and Page, 1992; Ben-Eliyahu et al., 1991), and leukocytes harvested from the bone marrow or from the lung interstitial and alveoli compartments (following dissection and collagenase digestion—unpublished data from our laboratory). This in vitro resistance of the MADB106 to NK activity, however, is in marked contrast to its in vivo susceptibility to NK cells that we and others have shown using various in vivo approaches, including selective depletion and selective replacement of NK cells (Barlozzari et al., 1983, 1985; Ben-Eliyahu and Page, 1992; Ben-Eliyahu et al., 1996b; Shakhar and Ben-Eliyahu, 1998).

Thus, the current study in rats had two major aims: (a) to assess the use of a β-blocker and of a PG-synthesis inhibitor in the postoperative context as prophylactic measures against the immunosuppressive and metastasis-promoting effects of surgery, and to study whether catecholamines and PG act independently of each other; and (b) to study the potential role of a yet unrecognized population of NK cells, those adhering to the lungs vasculature—marginating-pulmonary (MP)-NK cells—in destroying MADB106 cells, and in mediating the effects of surgery and of the studied prophylactic measures.
2. Materials and methods

2.1. Animals

Fisher 344 male and female rats 13–16 weeks old (age matched within each experiment) were purchased from Harlan laboratories, Jerusalem, Israel. Animals were housed 4 in a cage with free access to food and water on a 12:12 light:dark cycle. All studies were approved by The Institutional Animal Care and Use Committee of Tel Aviv University. Female were used only in Experiment 6, in which females and male were equally included in each experimental group.

2.2. Timing and counterbalancing of experimental procedures

Rats were acclimatized to the vivarium for at least 3 weeks prior to the beginning of experimentation, and were handled daily during the third week. The experimental manipulations (surgery or the administration of agonists) were always conducted during the first half of the light phase. The order of blood withdrawal, drug administration, and tumor injection was counterbalanced across groups in each experiment, and control animals were injected with vehicle.

2.3. Experimental laparotomy

The laparotomy procedure has been described in detail elsewhere (Page, 1993). Briefly, rats were anesthetized with 2.5% halothane and a 4-cm midline abdominal incision was made. The intestine was externalized, gently rubbed with a gauze pad, and kept moisturized for 1 h. The intestine was then returned to the abdominal cavity and the wound was sutured.

2.4. Drugs and their administration

2.4.1. Nadolol

To block β-adrenoceptor stimulation, the non-selective β-adrenergic blocker, nadolol, was administrated immediately after surgery (0.4 mg/kg, s.c in phosphate buffered saline (PBS)), and again 4 h later (s.c., 0.2 mg/kg).

2.4.2. Indomethacin

The prostaglandin synthesis inhibitor, indomethacin (Sigma, Rehovot, Israel), was dissolved in propenyl glycol and administered to the peritoneal cavity (4 mg/kg) just before suturing the abdominal muscle at the end of surgery.

2.4.3. Metaproterenol

A non selective β-adrenergic agonist with a higher affinity to β2 than to β1 receptors (Dengler and Hengstmann, 1976) was used. Metaproterenol (Sigma, Israel) was dissolved in PBS, and administrated (1 mg/kg s.c.) simultaneously with tumor inoculation.

2.4.4. Prostaglandin E-2

Prostaglandin E-2 (PGE2) (Sigma, Israel), the most abundant and studied PG, was first dissolved in ethanol, then diluted in PBS (1:8) to a concentration of 120 µg/ml. Administration (s.c 120 µg/kg) was conducted simultaneously with tumor inoculation.

2.5. Tumor cell lines

2.5.1. MADB106

MADB106 is a selected variant cell line obtained from a pulmonary metastasis of a mammary adenocarcinoma (MADB100) chemically induced in the F344 rat (Barlozzari et al., 1983, 1985). This syngeneic tumor metastasizes only to the lungs following i.v. inoculation. The number of tumor cells retained in the lungs 24 h following i.v. inoculation as well as the consequent metastases enumerated weeks later are highly dependent on NK activity (Barlozzari et al., 1983, 1985; Ben-Eliyahu and Page, 1992; Ben-Eliyahu et al., 1996b; Shakhar and Ben-Eliyahu, 1998). The MADB106 line was maintained in 5% CO2 at 37 °C in monolayer cultures in complete medium, and was used for the in vivo studies, as well as a target cell in the in vitro assessment of NK cytotoxicity.

2.5.2. YAC-1

The standard target cell line for assessing NK cytotoxicity in vitro in rodents. The cell line was maintained in 5% CO2 at 37 °C suspension cultures in complete medium.

2.6. Radiolabeling of MADB106 tumor cells and assessment of lung tumor retention

For assessment of MADB106 lung tumor retention, DNA radiolabeling of tumor cells was accomplished by adding 0.5 µCi/ml of 125iododeoxyuridine (125-IDUR, Danyel Biotech, Rehovot, Israel) to the growing cell culture 1 day before harvesting the cells for injection. For tumor cells injection, rats were lightly anesthetized with halothane, and 105 radiolabeled cells in 0.5 ml of PBS (supplemented with 0.1% BSA) were injected into their tail vein (approximately 4 × 105/kg). Twenty-four hours later, rats were killed with halothane, and their lungs removed and placed in a gamma counter for assessment of radioactive content. The percentage of tumor cell retention was calculated as the ratio between radioactivity measured in the lungs and the total radioactivity in the injected cell suspension. Our previous studies have indicated that the levels of lung radioactivity reflect the numbers of viable tumor cells in the lungs that are expected to
form solid metastasis (Bar-Yosef et al., 2001; Ben-Eliyahu and Page, 1992; Ben-Eliyahu et al., 1996b; Shakhar and Ben-Eliyahu, 1998).

2.7. Induction and counting of tumor metastases

Rats were lightly anesthetized with halothane, and 10^7 MADB106 tumor cells (approximately 4 \times 10^7/kg) were injected into their tail vein in 0.5 ml of PBS (supplemented with 0.1% BSA). Three weeks later, rats were killed, and their lungs removed and placed for 24 h in Bouin’s 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 separately by two researchers uninformed of the origin of each lung.

2.8. Flow cytometry

Standard FACS (Fluorescence Activated Cell Sorter) analysis was used to assess the number of NK cells in the blood and lungs (Shakhar and Ben-Eliyahu, 1998). NK cells were identified as NKR-P1 \texttt{bright} (CD161 \texttt{bright}) lymphocytes using FITC-conjugated anti-NKR-P1 mAb (PharMingen, San-Diego). Specifically, the criterion for positive identification of NK cells was defined as being above a level of fluorescence intensity that distinguishes between bright and dim stained populations of CD161 positive cells, as described previously by Chambers et al. (1992). These previous studies also demonstrated that CD161 is expressed by 94% of blood LGL cells of the rat, and that the NK cytolytic activity was totally contained in the CD161 bright cell population. Polymorphonuclear (PMN) leukocytes were found to express low levels of CD161 and categorized as dim cells, and macrophages and mast cells were found to be negative (Chambers et al., 1989). In our studies, bright cells are defined as showing above 150 relative fluorescence intensity units, a level that distinguishes between the two non-overlapping populations of the dim and bright CD161 positive cells. Nonspecific binding was assessed using nonspecific IgG1 that consistently yielded 0% of brightly stained cells. To assess the total number of NK cells per \textmu l of effector cells, we added to each sample 600 polystyrene microbeads per \textmu l of sample (20 \textmu m diameter, Duke Scientific, Palo Alto, CA). Following cytometry, the formula \#CD161 \texttt{bright} \times 600/#microbeads was used to calculate the number of NK cells per \textmu l. The coefficient of variation for this method was found in our laboratory to be 6% for identical samples (Ben-Eliyahu et al., 1999).

2.9. In vitro assessment of NK cytotoxicity

The standard 4-h ^{51}Cr release assay was used to assess leukocyte anti-tumor cytotoxicity against standard YAC-1 target cells, as well as against syngeneic MADB106 cells.

2.9.1. Preparation of lung and blood effector cells

Rats were overdosed with halothane and the peritoneal and chest cavities were opened. Five ml of blood were drawn from the right ventricle of the heart into syringes containing 150 units of heparin. Adherent lung-capillary (marginating pulmonary) leukocytes were then harvested by perfusing this organ with heparinized PBS (30 units/ml): PBS was injected through the right ventricle and 30 ml collected from the left ventricle. The first 2 ml of perfusate was disposed of as it was contaminated with blood from the lungs and heart. The perfusate was then centrifugation (400g for 10 min), the supernatant removed, and the pellet was washed once again (400g for 10 min) with complete media (CM) and concentrated into 1 ml. One ml of blood was washed once with PBS and twice with complete media and reconstituted in original volume.

2.9.2. Preparation of target cells

MADB106 cells were removed from the culture flask with trypsin solution (0.25% in PBS), and were washed with complete media. Both MADB106 cells and YAC-1 cells \((5 \times 10^6 \text{ of each})\) were incubated for 1 h with 100 \textmu Ci ^{51}Cr (Danyel Biotech, Rehovot, Israel) in 100 \textmu l saline, 100 \textmu l fetal calf serum (FCS) and 75 \textmu l complete media. Following incubation, cells were washed 3 times (300g for 10 min) and adjusted to the concentration of 5 \times 10^7/ml in complete media.

2.9.3. Assessment of cytotoxicity

To create five different effector to target (E:T) ratios, effector cells were serially diluted five times beginning at their original concentration, and co-incubated with a fixed number of target cells. Specifically, aliquots of 150 \textmu l of effector cell suspension (from washed blood or lung perfusate) were placed in wells of a microtiter plate and serially diluted to create the 5 E:T ratios. 5000 target cells in 100 \textmu l complete media were then added to each well. Plates were centrifuged (400g 10 min) and incubated at 37 \textdegree C for 4 h. Plates were then centrifuged again and 100 \textmu l of supernatant was removed from each well and counted in a gamma counter to determine experimental release. Spontaneous release was obtained from wells receiving target cells and media only, and total release was obtained from wells receiving 1% Triton X-100. Percent cytotoxicity was calculated by the following formula: Percent cytotoxicity = 100 \times [(experimental release – spontaneous release)/(total release minus spontaneous release)]. Our previous studies (Ben-Eliyahu et al., 1996a; Page et al., 1994) indicate that cytotoxicity in this assay depends on NK cells, since their selective depletion nullified all specific killing.
2.10. Statistical analysis

One or two way ANOVAs were used to analyze lung tumor retention or numbers of metastases. Provided significant group differences were indicated by ANOVA, Fisher planned contrast were used to conduct pair wise comparisons with respect to our hypothesized effects: surgery worsens the outcome (control saline vs. surgery saline), and each of the drug treatment (and their combination) reduces the effects of surgery (surgery saline vs. surgery drug). NK cell activity was analyzed using repeated measures ANOVA (the different E:T ratios as the repeated measures), and planned contrast (as above) were used to conduct pair wise comparisons. \( p < .05 \) was considered significant in all studies.

3. Results

3.1. Experiment 1: lung tumor retention (LTR) of MADB106 tumor cells—the effect of surgery and its blockade by indomethacin and nadolol

An early index of host resistance to MADB106 lung colonization is the retention of these tumor cells in the lungs 24 h following their inoculation. These 24 h also delineate the period in which the experimental metastatic process of the MADB106 is sensitive to NK activity (Barlozzari et al., 1983, 1985; Ben-Eliyahu and Page, 1992). Thus, relative to longer indices (e.g., actual metastases), the 24 h LTR index more exclusively reflects the in vivo levels of NK activity.

3.1.1. Design and procedure

F344 male rats served as controls or underwent laparotomy. Immediately afterwards, rats were injected with the prostaglandin synthesis inhibitor, indomethacin (4 mg/kg i.p.), with the \( \beta \)-adrenergic antagonist, nadolol (0.4 mg/kg s.c.), with both indomethacin and nadolol, or with vehicle. Four hours after surgery, rats were intravenously inoculated with radiolabeled MADB106 cells, and the nadolol groups were re-injected with nadolol (0.2 mg/kg s.c.) to extend its action. Twenty-four hours later, all rats were sacrificed to assess MADB106 LTR. A total of 105 male rats were used in these 8 groups (\( n = 28 \) and 24 in the control-saline and surgery-saline groups, 13–16 in the three surgery-drug groups, and 3–4 in the three control-drug groups).

3.1.2. Results

An ANOVA revealed significant group differences (\( F_{1,97} = 8.55, \ p < .05 \) (Fig. 1A). Fisher planned contrast indicated that: (a) surgery caused a large and significant increase in LTR (a 7.5-fold, \( p < .05 \)), (b) nadolol and indomethacin significantly reduced this effect by 50% when used alone (\( p < .05 \) in both comparisons), and (c) the combine use of nadolol and indomethacin caused an even greater reduction (75%) in LTR in the operated rats, and was significantly lower compared to each of these compounds alone (\( p < .05 \) for both comparisons). These last two comparisons were also conducted using \( t \) tests for independent samples, as the variance within the surgery groups were markedly larger than in the control groups, violating the ANOVA assumption of homogeneity of variance. In this specific condition, the \( t \) tests are more conservative than the planned contrasts, and they too indicated that the com-

![Fig. 1. The effects of surgery and their attenuation by the \( \beta \)-adrenergic blocker, nadolol, by the prostaglandin synthesis inhibitor, indomethacin (indo), and by the combined used of these drugs (indo and nadolol) (mean ± SEM). Surgery increased lung tumor retention of the MADB106 tumor (A), and increased the number of experimental MADB106 lung metastases counted 3 weeks later (B). Each of the blockers attenuated these effects (A), and their combined use almost completely abolished them (A and B). ¥ indicates a significant effect of surgery (difference between the control saline and surgery saline groups), and * indicates a significant attenuation of this effect by drug treatment (difference between the surgery saline group and the surgery drug group). The combined treatment seen in A was significantly lower than each treatment alone (indicated by two *). A total of 105, and 57 male rats were used in A, and B, respectively.](image-url)
bined use of nadolol and indomethacin reduced LTR in the operated rats more than nadolol or indomethacin alone ($t_{(26)} = 5.3,$ and $t_{(27)} = 5.2,$ respectively, $p < .05$ for both comparisons).

No significant drug effects were evident in non-operated rats.

3.2. Experiment 2: lung colonization by MADB106 tumors—promotion by surgery and blockade by the combined use of indomethacin and nadolol

To study a more clinically related index, this study assessed the actual development of metastases three weeks after MADB106 inoculation.

3.2.1. Design and procedure

The exact same procedure as in Experiment 1 was used, with the exception that only the combination of drugs was employed in order to save rats—the index of number of metastases has much greater variance than LTR, and the combination of drugs was found most efficient in the previous study. Thus, using a $2 \times 2$ design, rats served as controls or underwent laparotomy. Immediately afterwards, rats were injected with saline or with both indomethacin (4-mg/kg i.p. in propylene glycol) and nadolol (0.4-mg/kg s.c.). Four hours after surgery, rats were intravenously inoculated with MADB106 cells and given another bolus of nadolol (0.2-mg/kg s.c) to maintain its effects. Three weeks later rats were sacrificed and pulmonary metastases were counted. A total of 57 male rats were used in these 4 groups ($n = 12–16$ per group).

3.2.2. Results

Surgery caused a 3-fold increase in the number of metastases, and drug treatment reduced this increase by more than 50% (Fig. 1B). Two way ANOVA indicated a significant interaction between the effect of surgery and the effect of drug treatment ($F(1,53) = 5.05,$ $p < .05$), and planned contrast indicated that drug treatment significantly reduced the number of metastases in operated rats ($p < .05$). No significant drug effects were evident in non operated rats.

3.3. Experiment 3: the effects of surgery, indomethacin and nadolol on number and cytotoxicity of circulating and MP-NK cells

Here we studied the potential involvement of NK cells in mediating the above in vivo effects of surgery, nadolol, and indomethacin on MADB106 metastasis (evident in Experiments 1 and 2, Figs. 1A and B). To this end we assessed the number and activity of NK cells 12 h after surgery, as this time point is within the time frame most potently affecting MADB106 LTR (0–24 h after tumor administration).

3.3.1. Design and procedure

Using the same $2 \times 4$ design and drugs treatments as in Experiment 1, MP-leukocytes were harvested from the vasculature of the lungs, the target organ for MADB106 metastasis, by selective perfusion of this organ, and their cytotoxicity was assessed in vitro against the MADB106 tumor line and against the standard xenogeneic YAC-1 target cell. Additionally, we assessed cytotoxicity of circulating leukocytes against both tumor lines. FACs analysis was used to identify and count NK cells. A total of 38 male rats were used in these 8 groups ($n = 4–5$ per group).

3.3.2. Results

(A) NK Cytotoxicity per ml blood and per MP-leukocytes. No appreciable NK cytotoxicity against MADB106 was observed in circulating leukocytes. In contrast, MP-leukocytes exhibited marked NK cytotoxicity against the MADB106, increasing from 5% to 25% in the control group (Fig. 2, E vs. F). Surgery markedly and significantly suppressed this cytotoxicity, and the combined administration of nadolol and indomethacin abolished this suppression (see details regarding significance below). In non-operated rats, these drugs had no significant effects on NK cytotoxicity (Fig. 2F). Similar findings were observed with respect to MP-NK cytotoxicity against YAC-1 cells, with the following two exceptions. Indomethacin alone significantly reduced the suppressive effect of surgery (Fig. 2B). In the blood, surgery significantly suppressed NK activity against YAC-1 target cells, and no other comparison was significant (Fig. 2A). ANOVA indicated significant group differences in the three assays that showed appreciable NK cytotoxicity (MP-NK cytotoxicity against MADB106 and YAC-1, and circulating-NK cytotoxicity against the YAC-1: $F_{(7,120)} = 2.4, 5.4, 2.9,$ respectively, $p < .05$ in all). Planned contrast indicated that the surgery-saline group was significantly lower than the control-saline in each of these three assays ($p < .05$). The combination of indomethacin and nadolol significantly reduced the suppression by surgery in MP-NK cells, tested against both MADB106 and YAC-1 target cells ($p < .05$). In the blood, this reduction was not significant.

(B) Numbers of NK cells per ml blood and per MP-leukocytes. In the blood, no significant group differences were evident in the number of circulating blood NK cells (Fig. 2C). On the other hand, there were significant group differences in the numbers of MP-NK cells ($F_{(7,30)} = 4.4,$ $p < .05$) (Fig. 2D). Planned contrasts revealed that while surgery itself did not significantly affect numbers of MP-NK cells (control-saline vs. surgery saline), within the surgery groups, indomethacin (alone or together with nadolol) significantly increased the numbers of MP-NK cells ($p < .05$). We did not plan to conduct pairwise contrasts within the non operated groups, and post hoc comparisons did not indicate any significant differences within these groups.
Fig. 2. The effects of surgery and drug treatments on the numbers of blood (C) and marginating-pulmonary (MP)-NK cells (D), and on their respective NK activity against standard YAC-1 target cells (A and B) and syngeneic MADB106 target cells (E and F). Rats either served as control or underwent surgery, and were further subdivided to be treated with the β-adrenergic blocker, nadolol, with the prostaglandin synthesis inhibitor, indomethacin (indo), with both drugs (indo and nadolol), or with saline (mean ± SEM). NK cytotoxicity (A, B, E, and F) is indicated at 5 different effector to target (et) ratios. et1 is the number of NK cells in each group, seen in C and D, to a constant number of target cell (each consequent et ratio is 2-fold lower). While circulating leukocyte failed to exhibit appreciable NK cytotoxicity against MADB106 target cells, MP-leukocyte showed marked cytotoxicity (E vs. F) despite having lower numbers of NK cells (C vs. D). Surgery significantly suppressed NK activity of both blood and MP leukocytes (A, B, and F), and the combined use of the blockers significantly attenuated this effect in MP cells (B and F), but did not reach significance in the blood (A). While there was no effects on the number of blood NK cells (C), within MP cells, drug treatments significantly increased the numbers of NK cells in the surgery conditions (D) (indicated by * compared to the surgery saline group). Together, the findings in A–D also suggest that, per NK cell, NK activity of MP leukocytes against the standard YAC-1 target cell (B) is at least 4-times greater than that of blood leukocytes (A), as less than half the number of MP NK (D vs. C) cells killed at least twice as many target cells. A total of 38 male rats were used in this study.
(C) Comparison between MP-NK activity and circulating-NK activity per NK cell. Because both MP-NK cells and circulating-NK cells were tested simultaneously against the YAC-1 target cell, and because we also recorded the numbers of NK cells in each population tested for cytotoxicity, we can make a preliminary comparison of the relative levels of NK activity per NK cell between these populations. Looking at the control-saline group at Fig. 2, and taking into consideration both cytotoxicity and numbers in each immune compartment, approximately 4-fold greater numbers of circulating-NK cells are needed to achieve the same levels of cytotoxicity (e.g., 30% cytotoxicity levels) compared to MP-NK cells. Looked at differently, approximately half the number of NK cells in the MP samples killed at least twice as many target cells. It is noteworthy that this comparison was not conducted in purified NK cells, but rather in the context of additional leukocytes from the respective immune compartments which could have affected cytotoxicity levels (see Section 4).

(D) Small and large NK cells. Based on forward scatter of FACS analysis, we have classified NK cells into two populations of small vs. large NK cells, which showed little overlap. These populations differ in size (small cells are 8–15 μm and large cells are 16–25 μm), but show almost no difference in CD161 fluorescence intensity. Within MP-NK cells (from each of the experimental groups) large NK cells constituted 30–35% of all NK cells. This percentage was significantly greater than in the blood of the same animals, in which only 9–11% of NK cells were categorized as large (e.g., t(4) = 4.8, p < .05 vehicle no-surgery group). There were no significant group differences (e.g., surgery-saline vs. control-saline) in the percentage of large NK cells in the circulation or within MP leukocytes.

3.4. Experiment 4: simulating the effects of surgery on MADB106 lung tumor retention by administration of a β-adrenergic agonist and of PGE2

3.4.1. Design and procedure

To assess whether stimulation of β-adrenoceptors or of prostaglandin receptors is sufficient to reduce host resistance to MADB106 metastasis, we injected rats with the β-adrenergic agonist metaproterenol (1 mg/kg, s.c), with PGE2 (120 μg/kg, s.c), with both compounds, or with vehicle. The doses of metaproterenol and of PGE2 used were chosen to be physiologically relevant doses, based on previous studies assessing the induced levels of PGE2 in the serum (Yakar et al., 2003), and an increase in heart rate following metaproterenol administration (Shakhar and Ben-Eliyahu, 1998). Immediately after drug administration, rats were intravenously inoculated with radiolabeled MADB106 cells, and 24 h later, rats were sacrificed to assess LTR. A total of 39 males were used in these 4 groups (n = 9–11 per group).

3.4.2. Results

An ANOVA indicated significant group differences (F(3,35) = 22.9, p < .05). PGE2 and metaproterenol, each significantly increased MADB106 LTR compared to the control group (p < .05) (Fig. 3A). LTR in animals treated with both metaproterenol and PGE2 was the highest, significantly higher than each of the treatments alone (p < .05 for both).

Fig. 3. The effects of a β-adrenergic agonist, metaproterenol (mp), prostaglandin E2 (PGE2), and their respective blockers (nadolol and indomethacin) on lung tumor retention (LTR) of MADB106 cells (mean ± SEM). (A) PGE2 and mp, each significantly increased LTR compared to the saline group (*), and together had an additive effect significantly larger than each treatment alone (two *). (B) The prostaglandin synthesis inhibitor, indomethacin, did not attenuate the significant (*) effects of the β-adrenergic agonist (mp), and (C) the β-adrenergic blocker, nadolol, did not attenuate the significant (*) effects of PGE2. B and C suggest that the effects of the β-adrenergic agonist and of PGE2 are independent of each other. A total of 39, 32, and 40 rats were used in A, B, and C, respectively.
3.5. Experiments 5 and 6: the effects of β-adrenoceptor stimulation and of prostaglandin receptor stimulation on MADB106 lung tumor retention—are they co-dependent?

In this study we assessed whether the promotion of MADB106 LTR by stimulating β-adrenoceptors or by stimulating prostaglandin receptors (caused by surgery in Experiments 1 and 2, and by administration of agonists in Experiment 4), are independent of each other’s ligand/receptor system. To this end, we attempted to block the effects of each agonist by the other ligand’s antagonist.

3.5.1. Design and procedure

In Experiment 5, using a 2 × 2 design, rats were injected with indomethacin (4 mg/kg i.p.) or with vehicle 1 h before the injection of the β-adrenergic agonist metaproterenol (1 mg/kg, s.c.) or saline. A total of 32 males were used in these 4 groups (n = 9–10 in all groups, except in the control-indomethacin group in which four rats were used as no effect was evident in this group in previous experiments). In Experiment 6, rats were injected with nadolol (0.4 mg/kg s.c.) or saline, then with PGE2 (120 μg/kg, s.c.) or saline. In this experiment 16 males and 24 females were used (a total of 40 rats), and were equally divided in the 4 experimental groups (n = 6 females and 4 males in each group). In both experiments rats were intravenously inoculated with radiolabeled MADB106 cells immediately after the agonist administration (the second of the 2 injections), and sacrificed 24 h later to assess LTR.

3.5.2. Results

In Experiment 5, an ANOVA indicated significant group differences (F(3,28) = 5.8, p < .05). Planned contrasts indicated that the β-adrenergic agonist (metaproterenol) significantly increased MADB106 LTR compared to the control group (p < .05), and the prostaglandin synthesis inhibitor (indomethacin) did not reduce this effects (Fig. 3B). In Experiment 6, in which both females and male were used, we conducted a two way ANOVA with sex and drug treatment as independent variables. Significant drug effect was evident (F(3,32) = 15.2, p < .05), but no significant sex or sex by drug interaction were evident. Planned contrasts indicated that PGE2 significantly increased MADB106 LTR compared to the control group (p < .05), and the β-adrenergic antagonist (nadolol) did not reduce the effects of PGE2. Because females and male had very similar levels of LTR in the four drug conditions, and because the focus of the study does not include sex differences, the findings from females and males are graphically presented together (across sex) for the sake of clearer presentation (Fig. 3C).

4. Discussion

Our findings demonstrate that a β-adrenergic blocker (nadolol), and a prostaglandins synthesis inhibitor (indomethacin), each attenuated the metastasis-promoting effects of surgery when used alone, and together almost completely abolished them. The combination of drugs was also effective in preventing the NK-suppressive effects of surgery on lung NK activity against the standard YAC-1 target line and against the syngeneic MADB106 line. Administration of physiologically relevant doses of either PGE2 (the most abundant PG) or of a β-adrenergic agonist, was each sufficient to reduce host resistance to MADB106 experimental metastasis independent of the other ligand system, and together simulated the metastasis-promoting effects of surgery. Taken together, these findings suggest that the release of catecholamines and prostaglandins, caused by the surgical procedure used herein, are sufficient and necessary to render the host more susceptible to metastasis of the MADB106 tumor, seemingly through inducing suppression of NK activity. These findings do not exclude a potential role for other mediators (e.g., glucocorticoids or opioids), but stress the importance of catecholamines and prostaglandins in the current paradigm.

An important finding of the current study is the unique ability of marginating-pulmonary (MP)-NK cells to destroy MADB106 tumor cells, and their potential role in mediating the suppressive effects of surgery on resistance to experimental MADB106 metastasis. The MADB106 tumor line is known to be controlled in vivo by NK cells: selective in vivo depletion of NK cells markedly reduces the in vivo disintegration of MADB106 cells, and causes an approximately 100-fold increase in MADB106 lung retention and in the numbers of lungs metastases developed weeks later (Barlozzari et al., 1983, 1985; Ben-Eliyahu and Page, 1992; Ben-Eliyahu et al., 1996b; Shakhar and Ben-Eliyahu, 1998). Nevertheless, in our previous studies and in other studies, circulating leukocytes and splenocytes show no appreciable NK cytotoxicity against MADB106 cells using the standard 4-h in vitro Cr51 release assay (Barlozzari et al., 1983, 1985; Ben-Eliyahu and Page, 1992; Ben-Eliyahu et al., 1991). We were also unable to see such cytotoxicity using leukocytes harvested from the interstitial and alveoli compartments of the lungs (unpublished findings). In the current study, while circulating leukocytes again failed to show appreciable NK cytotoxicity against MADB106 tumor cells, half the number of NK cells from the MP pool exhibited remarkable cytotoxicity against this tumor. Additionally, using standard YAC-1 target cells, MP leukocytes exhibited approximately 4-fold higher NK cytotoxicity per NK cell than circulating leukocytes. The MP population also contained approximately 3-fold higher percentage of large NK cells (greater than 16 μm), a
morphology consistent with activation of cytotoxic cells. Thus, NK activity within the MP-leukocyte population is clearly unique in its potency, especially in its ability to destroy the syngeneic MADB106 line. Because in the current study we assessed NK cytotoxicity in the presence of other leukocytes (from the MP population or from the blood), our findings are not sufficient to determine if this high NK cytotoxicity depends on the presence or absence of other leukocytes, or is contained within MP-NK cells themselves. Our ongoing studies aim at characterizing purified MP-NK cells in terms of specific activation markers and cytotoxicity against various tumor lines, and seek to determine whether other populations of NK cells, such as marginating hepatic NK cells, also exhibit unique potency against the MADB106 line.

Additional support for the critical role played by MP-NK cells in the in vivo elimination of MADB106 cells can be derived from the parallel effects we observed in the in vitro cytotoxicity studies of MP-NK cells against MADB106 target cells. These parallel effects occurred with respect to the suppressive effects of surgery, and to their attenuation by nadolol and indomethacin (Figs. 1A–C). Additionally, we have previously visualized direct NK-MADB106 interactions in the lungs by in situ immunostaining (von Horsten et al., 2000), but the NK subpopulation or the exact lung compartment of this interaction were not studied.

Because in the current study NK cytotoxicity was assessed per ml blood or per the MP population, rather than on a per NK cell basis, alterations in the numbers of NK cells in the samples tested for cytotoxicity can contribute or underlie our findings. In the blood, however, no changes in the numbers of NK cell occurred, thus the effects of surgery and drug seem independent of changes in NK cell concentration. In the MP population, the effects of surgery and drugs on NK cytotoxicity are somewhat associated with their effects on numbers of NK cells. However, given the magnitude of the NK-suppression induced by surgery (the horizontal shift in the cytotoxicity curves) (Fig. 2B), an approximately 3-fold reduction in the number of NK cells should have occurred to completely explain the effect of surgery, while only a 30% reduction in the number of NK cells was evident (Fig. 2D). Thus, it seems that changes in numbers of MP-NK cells only partially contributed to the observed differences in lung cytotoxicity. To better disentangle the impact of altered numbers and altered cytotoxicity per individual NK cells, one need to a priori adjust the E:T ratio in the cytotoxicity assay based on the actual numbers of NK cells in the tested sample. Even better, one may purify NK cells and then adjust their concentration. However, these procedures are time consuming and/or involve manipulations of the effector cells. Given that the impact of surgery on cytotoxicity per NK cell is expected to dissipate with time and in the absence of the in vivo inducing milieu, it is questionable whether the impacts of surgery will persist through such lengthy procedures. The approach we took in the current study—assessing NK cytotoxicity per total lung MP population or per ml blood, and simultaneously assessing the number of NK cells in each sample tested for cytotoxicity—is a compromise. It seems to enable detection of alteration in cytotoxicity per NK cell (as clearly evident in the blood), to provide data that is directly related to the in vivo resistance of the total lung MP compartment to metastasis, but to lack vigor with respect to a per NK cell inferences.

Many tumors excised from cancer patients are considered immune-insensitive, as circulating autologous leukocytes fail to show appreciable cytotoxicity against them (NK or non-NK mediated) (Brittenden et al., 1996). However, if MP-NK cells similarly exist in human, then metastasizing cells originating from tumors that are considered insensitive, may actually be controlled by the patient MP-NK cell population. This population, although seems to constitute only a small portion of the host NK cell population, may be important given its unique ability to destroy some autologous tumor cells, and given its strategic location: the lungs vasculature filters all circulating cells, and the physical dimensions of its capillaries force close contact of circulating cells with MP leukocytes. Notably, the lungs are one of the primary organs for metastases of various types of tumors.

Although there are reports that high catecholamine levels can cause the release of prostaglandins (Ueda et al., 1994), it seems that in the current study catecholamines and prostaglandins act independently of each other in reducing host resistance to MADB106 experimental metastasis. The metastasis-promoting effect of PGE_{2} was not reduced by the β-adrenergic antagonist (nadolol), and the similar effect of the β-adrenergic agonist was not reduced by pretreatment of rats with the prostaglandin synthesis inhibitor (indomethacin). Additionally, the combined use of both antagonists blocked the effects of surgery on MADB106 LTR markedly and significantly more than each of the antagonists alone (notably, each antagonist was used in optimal concentrations based on our previous experience in similar in vivo conditions (Shakhar and Ben-Eliyahu, 1998; Yakar et al., 2003)). At the cellular level, although catecholamine and PGs activate different receptor systems on NK cells, both increase the levels of intracellular cAMP that eventually leads to suppressed cytotoxicity (Malygin et al., 1993; Whalen and Bankhurst, 1990). Therefore, clinical intervention through one receptor system only may bear no therapeutic advantage, especially when higher levels of catecholamines or prostaglandins are released in more severe surgeries.

The suppressive impact of catecholamines and of prostaglandins on NK cytotoxicity may be carried directly
through their receptors on NK cells, or indirectly via various mechanisms. In vitro activation of β-adrenoceptors or of prostaglandin receptors were shown to directly suppress NK activity (Malygin et al., 1993; Whalen and Bankhurst, 1990). Because the in vitro concentrations used in such studies were commonly 10-fold higher than the high systemic levels of these ligands following surgery, and because it is hard to compare in vitro and in vivo concentrations, it is still unclear whether systemic physiological levels of catecholamines or of prostaglandins are sufficient to cause such direct effects on NK cells in vivo (see additional discussion of this issue in (Shakhar and Ben-Eliyahu, 1998; Yakar et al., 2003)). Notably, both ligands are released locally at much higher concentrations; catecholamines are released in sympathetic-innervated immune organs (e.g., spleen (Feltén et al., 1985)), and prostaglandins in the sites of tissue damage. Not excluding direct effects, it is feasible that catecholamines or prostaglandins act indirectly by regulating the levels of other factors that modulate NK activity. Specifically, both ligands were reported to shift cytokine production from Th1 to a Th2 mode (Elenkov et al., 2000; Faist et al., 1996), a condition which is associated with suppressed NK activity (Elenkov et al., 2000; Faist et al., 1996). Noteworthy, levels of both factors may increase weeks before oncological surgeries: catecholamines are released due to psychological distress that commences with the uncovering of cancer, and prostaglandins are secreted by many primary tumors (or by macrophage they recruit), presumably as an escape mechanism against immune destruction (Menetrier-Caux et al., 1999; Wojtowicz-Praga, 1997). Thus the use of prophylactic measures against excessive release of catecholamines and prostaglandins may be beneficial before surgery as well.

The current study models only some aspects of the more complex metastatic process, specifically focusing on resistance to circulating tumor cells during the postoperative period. In the clinical setting, oncological patients bear tumors while undergoing surgery, and in many patients the metastatic process has been initiated well before surgery. Thus, the prophylactic measures we propose here should also be tested in models of spontaneous metastasis to more adequately assess their clinical relevance. This study is also limited in assessing only one index of CMI, namely NK activity. Nevertheless, it is feasible that the findings have broader implications. Host defense against metastasis is believed to heavily depend on several components of CMI, notably CTLs, dendritic cells, macrophages, NK-T and NK cells (Smyth et al., 2001). Catecholamines and prostaglandins were shown in vitro to suppress most aspects of CMI (Elenkov et al., 2000; Faist et al., 1996). Similarly the Th1/Th2 cytokine balance, known to be sensitive to catecholamines and PGs and to be shifted toward a Th2 mode following surgery (Elenkov et al., 2000; Faist et al., 1996), is believed to contribute to a general decrease in levels of CMI following surgery (Elenkov et al., 2000; Faist et al., 1996). This suggests that the current finding may have relevance to non-NK sensitive tumors and to the more complex and diverged clinical settings. Indeed, using a different model to study postoperative resistance to MRD, we are currently recording survival following administration of 60 syngeneic leukemia cells per F344 rat. Our preliminary results show similar protective effects of the blockers in the post surgical condition (Inbar et al., 2003).

It has become clear that cellular immunity interacts with the malignant tissue and can control circulating cancer cells and residual disease, even if it is ineffective against the primary tumor (Shakhar and Ben-Eliyahu, 2003). Therefore, preventing immunosuppression during the immediate post-surgical period could be crucial: this period constitutes a high risk for spreading and seeding of tumor cells and for flare-up of preexisting micrometastases, but also presents a transient opportunity for the immune system to eradicate cancer by destroying MRD. If remaining neoplasm is not eliminated shortly after surgery, it might develop into established metastases and become resilient to immunocytes, as was the primary tumor. It seems that only prospective clinical trials that successfully target mechanisms of immunosuppression would be instrumental in resolving the question of whether suppression of CMI is indeed a significant risk factor for long-term recurrence in cancer patients. Given the current findings, substances that can block β-adrenoceptors or that can reduce COX2-mediated production of prostaglandins could be considered as prophylactic measures in such clinical trials, and their use in oncological patients may be beneficial before surgery as well. Such clinical studies remain to be undertaken.

References


