Interleukin 12 Primes Macrophages for Nitric Oxide Production in Vivo and Restores Depressed Nitric Oxide Production by Macrophages from Tumor-bearing Mice: Implications for the Antitumor Activity of Interleukin 12 and/or Interleukin 2

Jon M. Wigginton,1 Douglas B. Kuhns, Timothy C. Back, Michael J. Brunda, Robert H. Wiltrout, and George W. Cox

Biological Carcinogenesis and Development Program [J. M. W., T. C. B.] and Clinical Services Program [D. B. K.], Science Applications International Corporation—Frederick and Laboratory of Experimental Immunology [R. H. W., G. W. C.], Division of Basic Science, National Cancer Institute—Frederick Cancer Research and Development Center, Frederick, Maryland 21702-1201, and Department of Oncology, Hoffmann-LaRoche, Inc., Nutley, New Jersey [M. J. B.]

ABSTRACT

Interleukin-12 (IL-12) is a recently described immunoregulatory cytokine with potent therapeutic activity in various preclinical models of infectious or malignant disease. As part of our ongoing evaluation of potential mechanisms accounting for the potent antitumor activity of IL-12, we have investigated the influence of IL-12 administration on total serum nitrate/nitrite (NO⁻) levels and the production of nitric oxide (NO) by peritoneal macrophages from normal and tumor-bearing mice. We report here that IL-12 administration to either normal or tumor-bearing mice for periods of time ranging from 7–19 days induced progressive increases in serum NO⁻ levels and primed peritoneal macrophages for NO production on subsequent exposure to lipopolysaccharide or IL-2 ex vivo. Treatment of resident peritoneal macrophages or the macrophage cell line ANA-1 with IL-12 alone or IL-12 in combination with various other stimuli failed to induce NO production, suggesting that the effects of IL-12 occurred via an indirect mechanism. Furthermore, we have shown that not only was the production of NO by macrophages from untreated long-term, tumor-bearing mice suppressed compared with control mice treated with vehicle or IL-12, but also that IL-12 administration overcame this suppression and delayed tumor growth. Lastly, we have shown that administration of weekly pulses of IL-2 in combination with IL-12 additively enhanced the priming of macrophages for NO production ex vivo and delayed tumor growth far more effectively than either agent alone. These observations and reports in the literature regarding the potential influence of NO on development of the immune response and on the regulation of tumor growth and vascularization suggest that NO may play a significant role in the antitumor activity of IL-12 and IL-2.

INTRODUCTION

IL-122 is a recently identified cytokine that possesses marked immunomodulatory activity and displays substantial therapeutic potential in various preclinical models of infectious (1–4) or malignant disease (5, 6). IL-12 is produced primarily by B-cell and/or monocyte/macrophage populations (7) in response to a range of stimuli including LPS, Gram-positive or -negative bacteria, mycobacteria, and intracellular pathogens such as Toxoplasma gondii (1, 7). In turn, IL-12 may enhance the production of cytokines, such as IFN-γ and TNF-α, by preactivated T lymphocytes and NK cells and, ultimately, may facilitate the development of dependent cellular effector mechanisms including NK/lymphokine-activated killer and CTL responses (8–14). Furthermore, IL-12 may favor development of a T-helper type 1 versus a T-helper type 2 pattern of cytokine production during the evolution of an immune response (15, 16).

The present studies were designed to evaluate the effects of chronic IL-12 administration on serum NO⁻ levels in vivo and on the production of NO by resident peritoneal macrophages ex vivo in response to various stimuli. NO is a ubiquitous and short-lived biochemical messenger and cytotoxic molecule that is produced as a consequence of enzymatic oxidation of the amino acid l-arginine by NOSs (17, 18). NOSs are a family of enzymes consisting of both constitutive and inducible (iNOS) forms. Constitutive NO synthase appears to be dependent on elevation of intracellular calcium/calmodulin and is regulated by various immunological and inflammatory stimuli and is independent of elevated calcium/calmodulin (19). NO production is induced by a variety of cellular sources, including endothelial cells and macrophages. NO production by macrophages in vitro is induced weakly by IFN-γ alone (20) and more effectively by IFN-γ in combination with LPS (21, 22), IL-2 (23), TNF-α (23, 24), or picolinic acid (25), whereas in vivo, LPS (26) or IL-2 (27) alone may induce the production of NO. Although full appreciation of the complexity of signals modulating the production of NO is clearly evolving, more recent evidence suggests that macrophage NO production may be down-regulated by mediators including IL-4 (25, 28), macrophage-stimulatory protein 1 (29), iron (30), tumor-derived IL-10, transforming growth factor β, prostaglandin E₂, or phosphatidylinositol (31, 32).

We show here that IL-12 administration not only substantially delayed tumor growth as shown previously (5, 6) but also that it induced marked increases in serum NO⁻ levels in both normal and tumor-bearing mice. We also show that IL-12 administration primed peritoneal macrophages for NO production upon subsequent triggering with LPS or IL-2 alone ex vivo. Although the production of NO by resident peritoneal macrophages from tumor-bearing mice in response to various stimuli was suppressed, this capacity was restored to normal levels in mice treated with IL-12. Lastly, we have demonstrated that the administration of weekly pulses of IL-2 in combination with IL-12 additively enhanced the priming of macrophages for NO production ex vivo and delayed tumor growth far more effectively than either agent alone.

MATERIALS AND METHODS

Mice and Tumor Cells. BALB/c mice were obtained from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research and Development Center. They were maintained in a specific pathogen-free environment and were used between 8 and 10 weeks of age. Animal care was provided in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 86–23, 1985). The tumor model utilized in these experiments was the Renca renal adenocarcinoma of spontaneous origin (33), maintained in BALB/c mice by serial i.p. passage.

Reagents. Recombinant murine IL-12 (specific activity, 7 × 10⁶ units/mg) was generously provided by Hoffmann-LaRoche (Nutley, NJ). Stock aliquots in Dulbecco’s PBS were stored at −70°C until use. For in vivo administration,
stock aliquots were diluted with PBS containing 0.1% (v/v) sterile-filtered BALB/c mouse serum and used within 48 h. Mouse recombinant IFN-γ (specific activity, ≥10^7 units/mg) was purchased from Life Technologies (Gaithersburg, MD). LPS (phenol extracted and chromatographically purified from *Escherichia coli* serotype 0111:B4), sulfanilamide, naphthylphthaleinamide dihydrochloride, and sodium nitrite were purchased from Sigma Chemical Company (St. Louis, MO). Highly purified, recombinant human IL-2 (from *E. coli*) was generously provided by Chiron Corporation (Emeryville, CA; Refs. 34 and 35).

**In Vivo Treatment Regimens.** Mice were given s.c. implants of either Renga cells (1 × 10^7/mouse) or vehicle alone. Seven days later, therapy was initiated with daily i.p. injections of IL-12 (0.5 µg in 0.2 ml PBS with 0.1% homologous mouse serum) or vehicle alone. Cohorts of mice (10/treatment group) were euthanized with CO₂ after 7 or 19 days of therapy with IL-12 (or vehicle), and specimens were obtained promptly as outlined below. For the treatment of mice with the combination of IL-12 ± pulse IL-2, mice (10–20/treatment group) were given s.c. implants of either Renga cells (1 × 10^7/mouse) or vehicle alone. Ten days later, therapy was initiated with IL-12 ± IL-2. Briefly, mice received twice daily i.p. injections of IL-2 (300,000 IU in 0.2 ml HBSS with 0.1% homologous mouse serum) or vehicle alone on days 0 and 7 of therapy. Mice received daily i.p. injections of IL-12 (0.5 µg in 0.2 ml PBS with 0.1% homologous mouse serum) or vehicle alone on days 0 through 4 and 7 through 9. On day 10, mice were euthanized, and serum and resident peritoneal macrophages were obtained as outlined below.

**Specimen Handling.** To obtain serum for NOX assay, mice were phlebotomized via subxyphoid cardiac puncture, and whole blood was placed in SST-Corvac tubes to allow clot formation. Serum was subsequently removed, spun at 2500 rpm for 15 min to sediment any residual cellular/subcellular debris, and promptly frozen at −20°C until assay. Resident peritoneal macrophages were obtained by lavage using HBSS and a Cornwall continuous pipetting outfit with a 21 gauge needle (Becton Dickinson, Rutherford, NJ).

**Nitrate Assay-Serum.** Total serum NOX was determined by the conversion of NOX to NOX using powdered cadmium as a catalyst and subsequent detection using a Griess reagent (36). Briefly, serum samples (360 µl) were heated at 56°C for 10 min prior to the addition of 16% ZnSO₄·7H₂O to precipitate the protein. The samples then were spun at 4°C for 30 min at 12,000 × g, and the clarified supernatants were harvested for analysis. Each sample (50 µl) of the protein-free supernatants was added to duplicate wells of a Multiscreen-GV 96-well filtration plate (0.22 µm hydrophilic polyvinylidene fluoride, low-protein binding; Durapore; Millipore Corp., Bedford, MA) that contained a lawn of acid-washed cadmium powder (20 mg/ml, 100 mesh; Aldrich Chemical Company, Inc., Milwaukee, WI) and ammonium chloride-sodium borate buffer (50 µl/well, 1 M NH₄Cl, and 50 mM Na₂B₄O₇·10 H₂O, pH 8.5). The plate was covered with adhesive tape and rotated for 2 h at room temperature. After the incubation, vacuum was applied to the plate using a vacuum manifold (Millipore Corp.), and the samples were collected in a standard, flat-bottomed, 96-well microplate (Nunc, Inc., Evanston, IL). Each well of the filtration plate was washed once with ammonium chloride-sodium borate buffer (50 µl) and the wash was collected by vacuum in the 96-well microplate.

Griess reagent (100 µl of a freshly prepared solution containing equal parts of 1% sulfanilamide dihydrochloride in 5% H₃PO₄ and 0.1% 1-naphthyl-ethylenediamine in distilled water) was added to each well and mixed for 5 min; the absorbance at 540 nm was read on a microplate reader (Molecular Devices Corp., Menlo Park, CA). Standard curves included both sodium NOX and sodium NOX standards and ranged from 10–300 µM. A normal serum sample spiked with 50, 100, and 150 µM NOX was analyzed on each plate and exhibited >80% recovery of the added spike.

**Nitrite Assay-Culture Supernatants.** The accumulation of NOX in culture supernatants was utilized as a measure of NO production (36). Fifty microliters of sample were incubated with 50 µl of Griess reagent (1% sulfanilamide, 0.1% naphthylphthaleinamide dihydrochloride, and 2.5% H₃PO₄) at room temperature for 10 min, and the absorbance at 550 nm was determined in a Dynatech MR 3000 microplate reader. The concentration of NOX was determined from a least squares linear regression analysis of a sodium nitrite standard curve generated with each experiment.

**Macrophage Cultures.** Resident peritoneal cells were obtained as described above. Cytospin preparations were stained with Diff-Quik, and the percentage of macrophages was determined. Approximately 2 × 10⁶ macrophages were aliquoted in triplicate wells in a 96-well, round-bottomed microtitr plate. After 2–3 h, nonadherent cells were removed by washing with warm DMEM (Whitaker Bioproducts, Walkersville, MD) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, UT), 2 mM L-glutamine, 100 unit/ml penicillin, and 100 µg/ml streptomycin (GIBCO/Life Technologies, Grand Island, NY; complete medium). Test reagents or media were added in 50-µl volumes to give a final volume of 200 µl/well. Fifty microliters of culture supernatant were removed from each well after approximately 48 h and assayed for nitrite accumulation as outlined above. The mouse macrophage cell line ANA-1 was established and characterized in our laboratory (37). ANA-1 macrophages were routinely cultured in complete medium at 37°C in a humidified incubator containing 5% CO₂ in air.

**RESULTS**

IL-12 Administration Causes an Elevation of Serum NOX Levels in Both Normal and Tumor-bearing Mice. As part of our evaluation of the immunomodulatory properties of IL-12 *in vivo* and to investigate potential mechanisms underlying the antitumor activity of IL-12, we measured serum NOX concentrations in normal and tumor-bearing mice treated with IL-12 or vehicle alone. As shown in Fig. 1, substantial increases in serum NOX levels were seen in both normal and tumor-bearing mice treated with IL-12 compared with mice treated with vehicle alone. These differences were observable after as little as 7 days of IL-12 administration and were even more pronounced after 19 days of therapy with IL-12. The serum NOX levels in normal versus tumor-bearing mice treated with IL-12 did not differ significantly at the time points examined.

IL-12 Administration Primes Peritoneal Macrophages for Cytokine or Endotoxin-induced NO Production *ex Vivo*. As shown in Fig. 2, resident peritoneal macrophages from normal or tumor-bearing mice treated with vehicle alone for 7 days produced little or no NO constitutively or in response to IFN-γ, IL-2, or LPS alone *ex vivo*. However, over the course of 7 days of administration, IL-12 exerted a potent priming effect *in vivo*. Resident peritoneal macrophages from normal or tumor-bearing mice treated with IL-12 produced large quantities of NO on subsequent triggering *ex vivo* with either LPS or IL-2 alone. Long-term administration (*i.e.*, 19 days) appeared to further accentuate the *in vivo* priming effect...
of IL-12 (Fig. 3). Resident peritoneal macrophages from either normal or tumor-bearing mice treated with IL-12 produced extremely high levels of NO on subsequent triggering with LPS or IL-2, compared with undetectable or very low NO production by macrophages from mice that had been treated with vehicle alone. Furthermore, unlike the earlier time point, macrophages from normal and tumor-bearing mice treated with the 19-day course of IL-12 produced substantial amounts of NO spontaneously or upon exposure to IFN-γ alone ex vivo. Again, no differences were noted in comparing the normal and tumor-bearing groups under the respective conditions.

IL-12 Does Not Directly Activate Macrophages for NO Synthesis in Vitro. We next evaluated whether treatment of macrophages in vitro with IL-12 alone or in combination with other biological response modifiers could directly induce the production of NO. Although resident peritoneal macrophages from untreated normal and long-term, tumor-bearing mice produced substantial quantities of NO upon exposure in vitro to IFN-γ plus LPS; little or no NO was produced in response to medium, IFN-γ, LPS, or IL-12 alone (data not shown). Furthermore, treatment of these macrophages with IL-12 in combination with LPS, IL-2, or IFN-γ induced minimal or no production of NO (data not shown). Similar results were found with treatment of the macrophage line ANA-1 with IFN-γ, LPS, or IL-2 in combination with concentrations of IL-12 as high as 500 units/ml in vitro (data not shown). Thus, IL-12 did not appear to directly influence the production of NO by macrophages in vitro. Furthermore, it appeared unable to directly induce the production of any macrophage products capable of acting as co-stimuli for NO production with IFN-γ, LPS, or IL-2.

IL-12 Administration Delays Tumor Growth and Overcomes the Suppression of Macrophage NO Production in Long-Term, Tumor-bearing Mice. Daily administration of IL-12 to mice bearing established Renca significantly delayed tumor growth (Fig. 4). Tumor volumes at day 19 were significantly smaller among mice treated with IL-12 compared with mice treated with vehicle alone ($P = 0.0033$). The median tumor volume at day 19 was 307 mm$^3$ in the IL-12 and 1728 mm$^3$ in the vehicle-alone treatment groups, respectively. Peritoneal macrophages from untreated, long-term, tumor-bearing mice...
produced less NO in response to either IFN-γ plus LPS or IFN-γ plus IL-2 compared with macrophages from nontumor-bearing mice treated with IL-12 or vehicle alone (Fig. 3). In contrast, upon exposure to either IFN-γ plus LPS or IFN-γ plus IL-2 ex vivo, macrophages from long-term, tumor-bearing mice treated with IL-12 produce levels of NO comparable with those produced by macrophages from nontumor-bearing mice treated with IL-12 or vehicle alone. There was little or no difference in the production of NO in response to either IFN-γ plus LPS or IFN-γ plus IL-2 by macrophages from nontumor-bearing mice treated with IL-12 compared with those treated with vehicle alone.

**IL-12 and Pulse IL-2 Interact Additively in the Priming of Macrophages for Subsequent NO Production ex Vivo and Combined Administration of IL-12/Pulse IL-2 Delays Tumor Growth More Effectively Than Either Agent Alone.** Given the ability of IL-12 to delay tumor growth and prime peritoneal macrophages for NO production upon subsequent triggering with IL-2 ex vivo, we next evaluated the synthesis of NO by resident peritoneal macrophages obtained from normal mice or tumor-bearing mice treated with either the combination of IL-12 ± IL-2, IL-2 or IL-12 alone, or vehicles alone (Fig. 5). We found that compared with IL-12 or IL-2 alone, the administration of IL-12 in combination with IL-2 can enhance the production of NO by resident peritoneal macrophages. Macrophages from mice treated with IL-12/pulse IL-2 showed an additive increase in the production of NO in response to IL-2 treatment ex vivo, compared with macrophages from mice treated with either agent alone. Furthermore, macrophages from mice treated with IL-12/pulse IL-2 produced low levels of NO spontaneously and more substantial levels upon treatment with IFN-γ alone ex vivo. In contrast, macrophages from mice treated with vehicle, IL-2, or IL-12 alone produced negligible amounts of NO spontaneously or after treatment with IFN-γ ex vivo.

From a therapeutic standpoint, administration of the combination of IL-12 and pulse IL-2 led to marked delay in tumor growth and, in several cases, complete regression of established s.c. Renca (Fig. 6). Comparison of tumor volumes revealed a highly significant reduction in tumor volume among mice treated with either IL-12 alone (P = 0.0099) or IL-12/pulse IL-2 (P = 0.0001) compared with control mice, whereas tumor volumes among mice treated with IL-2 alone were not significantly different than controls (P = 0.96). Furthermore, tumor volumes among mice treated with the combination of IL-12/pulse IL-2 were significantly smaller than those among mice treated with either IL-2 (P = 0.0038) or IL-12 alone (P = 0.024). Median tumor volumes for the respective
treatment groups were as follows: control, 392 mm³; IL-2, 368 mm³; IL-12, 138 mm³; and IL-12/pulse IL-2, 5 mm³. Furthermore, after 10 days of therapy, 4 of 10 mice in the group treated with IL-12/pulse IL-2 had experienced complete regression of their tumors, compared with 1 of the 10 mice treated with IL-12 alone and none of the mice treated with IL-2 or vehicle alone.

**DISCUSSION**

We have investigated the impact of IL-12 on the production of NO in vivo and have demonstrated that IL-12 induced marked increases in serum NO³⁻ levels in both normal and tumor-bearing mice, particularly with long-term administration. More modest increases have been noted in normal mice treated with a short (5-day) course of IL-12 (5). Increased serum NO³⁻ levels have been demonstrated in human cancer patients treated with IL-2 (27), where these increases have been thought to correlate with the occurrence of clinical toxicity (38). Although based in a murine system, our findings suggest that chronic IL-12 administration is very well tolerated in both normal and tumor-bearing mice, in spite of the induction of high serum NO³⁻ levels. In
investigating sources for this production of NO, we have focused our efforts on the interaction of IL-12 with macrophages and have not evaluated other potential sources of NO such as endothelial cells. We have shown that IL-12 administration primed macrophages for NO production in response to endotoxin or cytokine treatment ex vivo. The observation that in vitro treatment of either resident peritoneal macrophages or the ANA-1 macrophage cell line with IL-12 alone or in combination with LPS, IFN-γ, or IL-2 failed to induce NO production suggests that the induction of NO production in vivo by IL-12 may occur via an indirect mechanism. IL-12 is a potent inducer of IFN-γ production by T and/or NK cells, and in combination with LPS (21, 22) or IL-2 (23), IFN-γ can induce macrophages to produce large amounts of NO. Thus, we speculate that the ability of IL-12 to prime peritoneal macrophages for NO production upon subsequent triggering with LPS or IL-2 ex vivo may, at least in part, be accounted for by exposure of these macrophages in vivo to IFN-γ produced by T and/or NK cells in response to IL-12 administration. IL-12 also could exert its observed priming effect by enhancing the expression of receptors such as CD14, IL-2 receptor, and/or IFN-γ receptor on the surface of macrophages.

We also have shown that not only did IL-12 administration overcome the suppression of macrophage NO production in long-term, tumor-bearing mice and delay tumor growth, but also that the combination of IL-12 and IL-2 interacted additively in the priming of tumor-bearing mice and delay tumor growth far more effectively than either IL-2 or IL-12 alone. NO can participate in the regulation of a broad range of physiological processes, including mediation of the cytotoxic activity of activated macrophages directed against infectious pathogens and tumor targets (39, 40). Activated macrophages produce a variety of factors that may contribute to the destruction of tumor cells in vivo, including proteases, reactive oxygen intermediates, TNF-α, and NO (39, 41–44). In mice bearing advanced malignancies, peritoneal exudate macrophages have impaired ability to kill xenogeneic, allogeneic, syngeneic, or autologous tumor targets (45–47), and although such macrophages produce levels of TNF-α and hydrogen peroxide comparable with normal mice (47), they appear to have a depressed capacity to produce NO, as we have observed in the present studies. In turn, macrophage-derived NO has been shown to induce apoptosis of tumor cells (48), and several reports suggest that NO is a key mediator of the tumoricidal activity of macrophages (21, 41, 49–51). Furthermore, the induction of regression of M5076 reticulum cell sarcoma metastases by administration of the lipopeptide CGP 31362 has been shown to correlate with induction of iNOS expression and local production of NO in the tumor bed (52), and transfection of iNOS gene into the K-1735 murine melanoma line induces tumor apoptosis, suppresses tumorigenicity, and abrogates metastasis in vivo (53). In contrast, tumor-derived NO may suppress lymphocyte proliferative responses (54). Furthermore, NO derived from monocytes (55) or tumor cells engineered to continuously produce NO (56) may promote angiogenesis in vivo, and administration of inhibitors of NO production to tumor-bearing mice can retard tumor neovascularization and growth in vivo (57, 58). Furthermore, spleen cells from iNOS knockout mice that are infected with Leishmania major display markedly greater lymphocyte proliferative responses and IFN-γ production and substantially less IL-4 production upon exposure to concanavalin A or L. major antigen (59). It appears that the overall role of NO in the destruction of individual tumor cells during an immune response and in the regulation of tumor vascularization and growth may be very complex.

IL-12 possesses significant single-agent antitumor activity in a range of murine tumor models (5, 6). More recently, we have reported that a regimen consisting of IL-12 administered in combination with weekly doses of IL-2 (pulse IL-2) possesses antitumor activity superior to that of either single agent alone (60). In those studies, we demonstrated that up to 88–100% of treated mice bearing established primary and/or metastatic murine renal carcinoma achieved complete and durable tumor regression. Furthermore, we found that as many as 56% of the mice cured of their original tumor failed to reject a rechallenge with the same tumor, suggesting a significant role for non T-cell-dependent mechanisms in the activity of this regimen. As mentioned, we have shown that not only can the administration of IL-12 overcome the suppression of NO production by macrophages seen in long-term, tumor-bearing mice, but also IL-12 may prime macrophages to produce extremely large amounts of NO upon subsequent exposure to IL-2. Having made these observations, we subsequently found that treatment of mice with the combination of IL-12 and pulse IL-2 not only delays tumor growth far more effectively than either single agent alone but also that the regimen further enhanced the production of NO by macrophages obtained from these mice. The present studies provide further evidence that the combination of IL-12 and pulse IL-2 can induce striking regression of established tumor and provide preliminary insight into one non T-cell-mediated mechanism by which IL-12 and the combination of IL-12/pulse IL-2 could exert their potent antitumor effects. Given the ability of IL-12 and/or IL-2 to induce the production of large quantities of NO in vivo and the potentially complex relationship between NO, the antitumor immune response, and the overall regulation of tumor growth, further investigation of the role of NO in the potent antitumor activity of IL-12/pulse IL-2 appears warranted. These studies and further evaluation of the mechanisms by which IL-12 induces NO production is currently under investigation.

ACKNOWLEDGMENTS

We thank Drs. John R. Ortaldo and William Fogler for their critical review of the manuscript, Joyce Vincent for typing and editing, Charles Riggs for statistical support, and Eileen Grays for assistance with graphics.

REFERENCES


Interleukin 12 Primes Macrophages for Nitric Oxide Production \textit{in Vivo} and Restores Depressed Nitric Oxide Production by Macrophages from Tumor-bearing Mice: Implications for the Antitumor Activity of Interleukin 12 and/or Interleukin 2


Updated version Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/56/5/1131

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/56/5/1131. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.