Altered Expression of Interleukin 6 and Interleukin 10 As a Result of Photodynamic Therapy in Vivo

Sandra O. Golnick, Xiaonan Liu, Barbara Owczarczak, David A. Musser, and Barbara W. Henderson

Departments of Molecular Medicine [S. O. G., Radiation Biology [B. O., X. L., B. W. H.], and Dermatology [D. A. M.], Roswell Park Cancer Institute, Buffalo, New York 14263

Abstract

Photodynamic therapy (PDT), which can effectively destroy malignant tissue, also induces a complex immune response that potentiates antitumor immunity but also inhibits skin contact hypersensitivity (CHS) and prolongs skin graft survival. The underlying mechanisms responsible for these effects are poorly understood but are likely to involve mediation by cytokines. We demonstrate in a BALB/c mouse model that PDT delivered to normal and tumor tissue in vivo causes marked changes in the expression of cytokines, IL-6 and IL-10 but not tumor necrosis factor alpha (TNF). IL-6 mRNA and protein are strongly enhanced in the PDT-treated EMT6 tumor. PDT also increased IL-6 mRNA in exposed spleen and skin. These data suggest that the general inflammatory response to PDT may be mediated at least in part by IL-6. In addition, IL-6 may modulate the local antitumor immune response. In contrast, IL-10 mRNA in the tumor decreases following PDT. Most importantly, IL-10 is markedly induced in the skin of mice exposed to a PDT regime that strongly inhibits the CHS response, and the kinetics of IL-10 induction coincide with the known kinetics of CHS inhibition. We propose that the enhanced IL-10 expression plays a role in the observed suppression of cell-mediated responses seen following PDT.

Introduction

PDT has obtained regulatory approvals in the United States and elsewhere as a cancer treatment modality that can selectively destroy malignant, premalignant, and certain benign lesions in patients (1). The cytotoxic effect of this therapy is mediated primarily by the formation of singlet oxygen, which is generated by energy transfer from a light-activated, tissue-localized photosensitizer to ground state oxygen, and which can cause oxidative damage to numerous cellular components at or near the site of its generation. Tumor eradication is a consequence of direct photodamage to the malignant cells and of damage to the tumor-supporting microvasculature (2, 3). In its acute phase, the PDT response is of a pronounced inflammatory nature, characterized by the release of vasoactive, inflammatory mediators and accumulation of host immune cells at the treatment site (2–4). Long-term tumor control appears to be aided by a potentiation of antitumor immunity (4). However, preclinical evidence also demonstrates that PDT exposure can significantly suppress certain immune functions, such as the CHS response (5, 6) and murine skin graft rejection in allogeneic recipients (6, 7), possibly by interfering with the function of antigen presenting cells. It is likely that these dichotomous responses involve the mediation of immune-modulating cytokines. Here we report that PDT can alter the expression of IL-6 and IL-10 in tumor and normal tissues in vivo and propose that these changes are mechanistically involved in the observed effects of PDT on host immune responses.

Materials and Methods

Animals and Tumor System. BALB/cJ mice, obtained pathogen-free from The Jackson Laboratory (Bar Harbor, ME), were used for all experiments, either tumor-free or carrying the EMT6 mammary tumor (8). Animals were housed in microisolator cages in a laminar flow unit under ambient light. Six- to 12-week-old animals were inoculated intradermally on the shoulder with 2 ¥ 10^5 tumor cells harvested from exponentially growing cultures. Although the EMT6 cell line is highly antigenic and significantly immunogenic (9), no spontaneous tumor regressions were observed in this study. Prior to tumor inoculation and/or light treatment, all hair was removed from the prospective treatment site by shaving and depilation. Tumors were used for experimentation about 10 days after inoculation, when they had reached a size of 6–8 mm in diameter.

Photonsensitivity. Clinical-grade, pyrogen-free Photofrin (QLT PhotoTherapeutics Inc., Vancouver, British Columbia, Canada) was reconstituted to 2.5 mg/ml in pyrogen-free 5% dextrose (D5W; Baxter Corp., Deerfield, IL).

In Vivo PDT Treatment. Three treatment protocols were used:

(a) Animals were given iv. injections via the tail vein of 5 mg/kg Photofrin. Twenty-four h later, 630 nm light was delivered with an argon-dye laser system (Spectra Physics, Mt. View, CA). A treatment field 1 cm in diameter, containing either tumor or normal skin, was illuminated at 75 mW/cm^2 for a total light dose of 100 J/cm^2 (approximate TCD50). At selected time intervals following light exposure, tumors, spleens, and/or normal skin were harvested. Tissues were kept frozen at —70°C until further processed. Tumors and spleens for flow cytometric analysis were harvested and processed immediately.

(b) Animals were treated as above except that a subcurative light dose (34 J/cm^2) was given.

(c) Animals were injected as above with 10 mg/kg Photofrin. Six h later, their shaved backs were illuminated with a bank of blue fluorescent lights (Sylvania F15TS/B) emitting between 350 and 650 nm (with a maximum at ~450 nm) at a distance of 18 cm. A 2-mm glass plate was placed 10 cm above the mice to remove any UV radiation. The animals received a light dose of 1.3 J/cm^2 (0.745 mW/cm^2 for 30 min). Mice were sacrificed at selected time points after illumination, and treated skin was removed, frozen in liquid nitrogen, and stored at —70°C until processed. All experiments included control animals without treatment, with photosensitizer only, and with light only.

In Vitro PDT Treatment. EMT6 cells were grown in basal media eagle (BME) supplemented with 15% FBS and antibiotics (all from Life Technologies, Inc., Grand Island, NY) in a humidified atmosphere of 5% CO2 in air. Exponentially growing cells were exposed to 10 μg/ml Photofrin in complete medium for 24 h, followed by exposure to drug-free complete medium for 3 h. Cells were removed from culture plates with trypsin-EDTA (Life Technologies, Inc.), washed once with complete medium, and placed in Petri dishes to avoid attachment. They were then exposed to laser light as above for a total light dose of 0.75 J/cm^2 (=LD50). At selected time intervals thereafter, cells were collected. Cell viability was assessed by trypan blue exclusion and ranged from 50 to 70%. Cells were spun down, and pellets were kept frozen at —70°C.

RNA Isolation and RT-PCR. Total RNA was isolated from either cells or tissues with Ultraspec (Biotex Laboratories, Inc., Houston, TX) using a modification of the method of Chomczynski and Sacchi (10). RNA was quantified spectrophotometrically and diluted to a final concentration of 2
RT-PCR was performed as described earlier, with slight modifications (11). Briefly, first-strand cDNA synthesis was performed with 2 μg of total RNA in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 500 μM deoxynucleotide triphosphates (Perkin-Elmer, Foster City, CA), 50 μg/ml random hexamer (Promega Corp., Madison, WI), and 10,000 units/ml Super-Script II reverse transcriptase (Life Technologies, Inc.) in a total of 20 μl for 1 h at 37°C. Following first-strand synthesis, the reaction was placed on ice, and 5 μl were used for PCR analysis of cytokine RNA. PCR analysis was done using 1 μM of cytokine-specific primers (Stratagene, La Jolla, CA) in 10 mM Tris-HCl, 50 mM MgCl₂, 0.2 mM deoxynucleotide triphosphates, 0.1 μCi [α-32P]dCTP, and 2 units of Taq polymerase (Boehringer Mannheim, Indianapolis, IN). Following a 5-min denaturation step at 95°C and a 5-min annealing step at 60°C, a total of 20–30 cycles was performed with each cycle consisting of 1.5 min at 72°C, 45 s at 94°C, and 45 s at 60°C, with a final extension of 10 min at 72°C. All amplifications were done in the linear range of the assay; actin and TNF-α reactions were amplified for 20 cycles, whereas IL-6 and IL-10 reactions were amplified for 30 cycles. The reaction products were separated on a 6% acrylamide gel, dried, and exposed to Kodak X-OMAT film. Control PCR reactions were performed with “cDNA” synthesized in the absence of reverse transcriptase. Reagents were routinely checked for enzymatic digestion of the excised tumor (8), and IL-6 protein was determined as follows. Cell suspensions were lysed in 0.2% NP40, 10 mM HEPES (pH 7.9), 1 mM EDTA, 1 mM KCl, 1 mM DTT, 5 μg/ml each peptatin, aprotinin, and leupeptin for 10 min on ice (all from Life Technologies, Inc.). A total of 2.5 μg of protein per sample was separated on a 12.5% SDS polyacrylamide gel in the presence of β-mercaptoethanol according to Laemmli (12). Following transfer to nitrocellulose, Western analysis was performed using biotinylated antisem RNA (PharMingen, San Diego, CA) and developed using an Immuno-Stain streptavidin-biotin-alkaline phosphatase kit according to the manufacturer’s instructions (Bio-Rad, Hercules, CA). Results were confirmed in at least three replicate experiments.

**Western Analysis.** Single-cell suspensions of tumors were prepared by enzymatic digestion of the excised tumor (8) and IL-6 protein was determined as follows. Cell suspensions were lysed in 0.2% NP40, 10 mM HEPES (pH 7.9), 1 mM EDTA, 1 mM KCl, 1 mM DTT, 5 μg/ml each peptatin, aprotinin, and leupeptin for 10 min on ice (all from Life Technologies, Inc.). A total of 2.5 μg of protein per sample was separated on a 12.5% SDS polyacrylamide gel in the presence of β-mercaptoethanol according to Laemmli (12). Following transfer to nitrocellulose, Western analysis was performed using biotinylated antisem RNA (PharMingen, San Diego, CA) and developed using an Immuno-Stain streptavidin-biotin-alkaline phosphatase kit according to the manufacturer’s instructions (Bio-Rad, Hercules, CA). Results were confirmed in at least three experiments.

**ELISA Assays.** IL-10 protein in PDT-treated skin was determined by ELISA as recommended by the manufacturer (PharMingen). Briefly, skin samples were frozen in liquid nitrogen, mechanically ground, and prepared for protein extraction according to Liu et al. (13). Protein was extracted as described above. Wells of a 96-well microtiter plate were coated with 2 μg/ml capture antibody (rat antisem IL-10, JES5–16E3; PharMingen) in 0.1 M NaHCO₃ (100 μM/well) and incubated overnight at 4°C. Plates were washed twice, 5 min/wash, with 0.02% Tween 20 in PBS and blocked for 2 h at room temperature with 10% FCS in PBS (blocking buffer). Duplicate samples (10 μg of total protein) or standards (recombinant IL-10) were added in 100 μl of blocking buffer and incubated overnight at 4°C. Plates were washed twice and incubated with 2 μg/ml detection antibody (biotinylated rat antisem IL-10, SXC-1; PharMingen), 100 μl/well, and incubated for 2 h at room temperature. Plates were washed four times, and 100 μl/well streptavidin–horseradish peroxidase (Bio-Rad) was added for 30 min. Plates were washed six times, and 100 μl/well substrate (2,2′-azino-di[3-ethylbenzthiazoline sulphonate(6)] diaminonium salt; Boehringer Mannheim) were added. Absorbance was read at 495 nm. The assay was carried out at least twice, using duplicate wells.

**Flow Cytometry.** The cell populations present in EMT6 tumors and spleens before and after PDT were characterized by FACS analysis, using Hoechst 33342 staining to assess ploidy (all EMT6 cells are >diploid) and allow for tissue to be harvested at delayed time points. IL-6 mRNA expression was done by quantitative RT-PCR and allow for tissue to be harvested at delayed time points. IL-6 protein was detected by Western blotting in untreated control tumors or tumors exposed to Photofrin alone in vivo (Fig. 2). A weak IL-6 band can be observed for the sample exposed to light treatment only and harvested 24 h later. Strong induction of IL-6 protein was evident by 3 h after PDT and persisted for at least 48 h but had disappeared by 6 days after PDT.

**Results**

PDT Alters Cytokine mRNA Expression in EMT6 Cells *in Vitro*. EMT6 Tumors, and Spleen. Initial analysis showed that of the cytokines examined in EMT6 cells *in vitro* (IL-1, IL-3, IL-6, IL-10, IFN-γ, granulocyte/macrophage-colony-stimulating factor, TNF-α, and transforming growth factor β; data not shown) and in tumors *in vivo* (same cytokines as above, IL-2 and IL-4; data not shown), mRNA levels for IL-6 and IL-10 were most markedly changed by PDT. Therefore, this work was focused primarily on IL-6 and IL-10. TNF-α was investigated in parallel because it is a recognized IL-6 inducer and acts synergistically with IL-6 to induce anti-tumor responses in mice (14, 15).

Fig. 1 shows the mRNA expression of IL-6, IL-10, and TNF-α as a function of time after PDT. EMT6 cells *in vitro* responded with a transient enhancement of IL-6 mRNA expression, which peaked at 1–3 h after PDT exposure (Fig. 1A). IL-10 mRNA was not expressed in the cells *in vitro*, either constitutively or PDT induced. In contrast to IL-6, TNF-α mRNA remained largely unchanged after PDT *in vitro*.

In the tumor, IL-6 mRNA was not or was minimally present in untreated control tumors but was found to be slightly enhanced after exposure to photosensitizer alone (Fig. 1B). Between 1 and 6 h after treatment (100 J/cm²), IL-6 mRNA expression was strongly and consistently enhanced, and this enhancement lasted for at least 24 h. The results with IL-10 were more variable, with occasional control samples not expressing any IL-10 mRNA. The majority of tumors, however, showed IL-10 mRNA decreasing with time after PDT, as shown in Fig. 1B. TNF-α mRNA was expressed at high levels in control tumors but showed no changes with time after PDT. Very similar results were obtained after subcurative treatment (data not shown).

Spleen cell populations harvested from animals after exposure to Photofrin only or PDT treatment of their shoulder tumors also showed weak, transient IL-6 mRNA induction (Fig. 1C). IL-10 mRNA was also transiently induced by PDT (100 J/cm² to shoulder tumor), peaking at 3 h after treatment. Again, TNF-α mRNA was unchanged.

Very similar results were obtained in spleens from animals after PDT was given to a 1-cm diameter area of skin at the shoulder without the presence of a tumor (data not shown). When a comparable area of skin at the right hind thigh was exposed to PDT, no cytokine induction was observed in the spleen.

**PDT Induces IL-6 Protein in EMT6 Tumors.** These experiments used subcurative treatment (34 J/cm²) to avoid rapid tumor destruction and allow for tissue to be harvested at delayed time points. IL-6 protein was not detectable by Western blotting in untreated control tumors or tumors exposed to Photofrin alone *in vivo* (Fig. 2). A weak IL-6 band can be observed for the sample exposed to light treatment only and harvested 24 h later. Strong induction of IL-6 protein was evident by 3 h after PDT and persisted for at least 48 h but had disappeared by 6 days after PDT.
Changes in Tumor and Host Cell Populations in EMT6 Tumors after PDT. Because both tumor cells and tumor-infiltrating host cells may contribute to the overall changes in cytokine expression, we examined by FACS analysis the time-dependent changes in the various cellular tumor components after PDT (5 mg/kg Photofrin, 100 J/cm²). Fig. 3A illustrates the changes in overall tumor composition occurring between the time immediately after PDT exposure (control tumors showed virtually identical results) and 24 h after PDT. The most obvious changes are the relative expansions of macrophages and, especially, granulocytes, at the expense of tumor cells, with the very small percentage of lymphocytes (mostly CD4+ T cells) remaining largely unchanged. The flow cytometric analysis in Fig. 3B illustrates for a representative sample the marked accumulation of granulocytes (Mac1+, Gr1+), rising from ~3% to over 50% of recovered cells, and the approximate tripling in the percentage of macrophages (Mac1+, Gr1−) over a 24-h period following PDT. No changes were observed in the composition of spleen cell populations after PDT to the shoulder region.

PDT Induces Expression of IL-10 mRNA and Protein in Skin. Skin samples examined after PDT with 630-nm light exposure also showed transient induction of IL-6 and IL-10. Although the IL-6 induction kinetics were similar to the tumor, IL-10 induction appeared delayed, with IL-10 mRNA becoming detectable between 24 and 48 h (data not shown). Because of the known involvement of IL-10 in the immunosuppressive effects of UV irradiation (16) and the observed suppression by PDT of the skin CHS responses (5, 17), IL-10 expression was also tested after a PDT protocol (10 mg/kg Photofrin, blue light, 1.3 J/cm²), which has been shown to induce CHS suppression in mice (17). IL-10 mRNA in control skin samples was absent but was strongly induced at 72 and 96 h after light exposure (Fig. 4A). IL-10 protein also was detectable at 72 h and progressively increased up to at least 120 h after treatment (Fig. 4B).

Discussion

This study demonstrates for the first time in an experimental in vivo system that PDT can significantly affect the expression of the cytokines IL-6 and IL-10. We confirm the enhancement of IL-6 in cells after PDT in vitro described earlier by Kick et al. (18) and show that IL-6 mRNA levels are strongly enhanced in tumor and normal tissues after PDT in vivo. This is accompanied, at least in tumor (other tissues were not tested), by a prolonged increase in IL-6 protein. As suggested by Kick et al. (18), TNF-α does not seem to play a role in the IL-6 induction by PDT because the changes in that cytokine are neither preceded nor accompanied by similar changes in TNF-α. TNF-α has been found induced by PDT in murine peritoneal macrophages in vitro (19), and a recent study by Anderson et al. (20) has demonstrated up-regulation of TNF-α in keratinocytes in vitro by PDT using a phthalocyanine-derived photosensitizer. The absence of TNF-α changes in our study might be related to the mouse strain used as the regulatory region of the TNF-α gene has been shown to have allelic differences (21). The TNF-α allele expressed in BALB/c mice is resistant to up-regulation by UV radiation and may also be resistant to alterations induced by PDT.

It remains to be determined whether the enhanced generation of IL-6 plays a role in the PDT tumor response. Intratumoral injection of IL-6 or transduction of the IL-6 gene into tumor cells can enhance tumor immunogenicity and inhibit tumor growth in experimental...
Fig. 3. Changes in the cell composition of EMT6 tumors determined by harvesting the tissue after PDT. Tumors were treated with PDT as described in Fig. 1 and analyzed by FACS analysis. In A, mean values from three independent experiments of the percentages of the different cell phenotypes comprising the tumor isolate were plotted. Tumor cells were identified as $>$ diploid, CD45$^-$, macrophages as Mac1$^+$, Gr-1$^+$, granulocytes as Mac1$^-$, Gr-1$^-$, and lymphocytes as Mac1$^+$, CD4$^+$, or CD8$^-$. Diploid, CD45$^-$ cells represent fibroblasts and endothelial cells. In B, scattergrams of representative tumor samples obtained before or at 5 min, 5 h, or 24 h after PDT as described in Fig. 1, showing PE-Mac1 versus FITC-Gr-1 staining (upper panels) and the appropriate isotype controls (lower panels), are shown.

Noteworthy is the induction of IL-6 mRNA with exposure to Photofrin in the absence of light activation in spleen (Fig. 1, B and C). Earlier, Luna et al. (24) have described in murine RIF cells in vitro the induction by Photofrin of the early-response genes c-fos and c-jun, which are related to IL-6 transcription via the AP-1 transcription factor (14, 18, 25). Although some of this effect was artifactual due to medium changes during the in vitro experiments, there clearly appeared to be additional gene induction due to Photofrin incubation in the dark. Kick et al. (18, 25) have not found dark-induction of either IL-6 or c-fos and c-jun in their in vitro human cell system. Our experiments support the observation of a Photofrin dark-effect. This may be of some significance for the hematopoiesis-stimulating effects of Photofrin observed by others (26) and us, because IL-6 can act as a cofactor with other cytokines in the growth of early hematopoietic stem cells (14).

IL-10 was not expressed constitutively in EMT6 cells, nor could it be induced by PDT in vitro. It was, however, present in most, albeit not all, untreated tumors. When it was present, PDT caused a reduction in IL-10 mRNA over time after treatment. Differences in IL-10 expression among EMT6 tumors have been described by Kurt et al. (9), who have demonstrated higher IL-10 levels in regressing than progressing tumors. The inconsistency of mRNA levels in our tumors may reflect differences in their immune status. In most tumors, the mRNA expression of IL-6 and IL-10 appeared inversely related, showing a marked decrease at the same time point (1 h after PDT) at murine tumor systems (15, 22, 23). Thus, PDT may enhance local antitumor immunity by up-regulating IL-6. The mechanisms by which this is achieved are not yet clear. Dougherty et al. (23) have suggested that IL-6 may further the recruitment of tumoricidal macrophages into the tumor bed. FACS analysis of PDT-treated tumors in the present study does demonstrate an approximate 2-3-fold increase in the percentage of macrophages over a 24-h period. On the other hand, Mullé et al. (15) have shown that IL-6-mediated tumor regression could be abrogated by in vivo depletion of either CD4$^+$ or CD8$^+$ T-cell subsets. Although this study did not detect any T-cell accumulations in PDT-treated tumors, changes in T-cell function might be present, which are presently being analyzed.

IL-10 was expressed constitutively in EMT6 cells, nor could it be induced by PDT in vitro. It was, however, present in most, albeit not all, untreated tumors. When it was present, PDT caused a reduction in IL-10 mRNA over time after treatment. Differences in IL-10 expression among EMT6 tumors have been described by Kurt et al. (9), who have demonstrated higher IL-10 levels in regressing than progressing tumors. The inconsistency of mRNA levels in our tumors may reflect differences in their immune status. In most tumors, the mRNA expression of IL-6 and IL-10 appeared inversely related, showing a marked decrease at the same time point (1 h after PDT) at
which IL-6 was markedly up-regulated. IL-10 is known to inhibit the IL-6 mRNA and protein expression by macrophages by down-regulating the transcripational activity of the IL-6 gene through inhibition of AP-1 binding activity (27, 28).

The cells responsible for altered overall cytokine mRNA and protein expression in tumors after PDT are not yet identified. Although IL-6 and/or TNF-α could have derived from EMT6 tumor cells, macrophages, lymphocytes, fibroblasts, and endothelial cells, the lack of IL-10 expression in EMT6 cells in vitro indicates that tumor IL-10 must have been generated by host cells such as macrophages and lymphocytes. The significance of the pronounced granulocyte accumulation in tumors after PDT is also not yet clear. Such accumulation has been observed by others, and experiments have shown that depletion of granulocytes in animals in vivo may retard the PDT tumor response (4, 29).

In contrast to tumor, both IL-6 and IL-10 were induced simultaneously in the spleen. Induction was independent of the presence of tumor and appears to be related to the penetration of some therapeutic light to the spleen. This hypothesis is supported by the results from animals treated with subcurative light doses (34 J/cm²) in which cytokine alterations in the tumor were similar to those seen with higher light doses but where little or no change was seen in cytokine levels in spleen (data not shown). This observation demonstrates that care must be exercised both in the delivery of PDT to small animals and in the interpretation of the results.

The IL-10 induction in the PDT-treated skin may be of particular biological significance because it may explain, at least in part, the transient suppression of the CHS reaction after PDT exposure of extended areas of skin in vivo. Keratinocyte-derived IL-10 has been found responsible for the UV-induced systemic suppression of CHS and delayed-type hypersensitivity reactions (30). The kinetics of IL-10 induction in the skin after PDT, with IL-10 becoming prominent at 72 h after exposure, coincide with the development of suppression of CHS after Photofrin PDT (17), suggesting a mechanistic role in the observed immunosuppressive effects. This is further supported by the fact that IL-10 is known to down-regulate MHC class II and costimulatory molecule expression, resulting in suppression of Langerhans cell antigen-presenting function (31). Such down-regulation has been observed recently by Obochi et al. (32) after low-dose PDT of skin allografts.

Acknowledgments

We are grateful to Dr. Carleton C. Stewart, Flow Cytometry Facility, Roswell Park Cancer Institute, for assistance and advice, to Dr. Allan Oseroff for support and suggestions, and to Dr. Thomas B. Tomasi, Molecular Medicine, Roswell Park Cancer Institute, for his continued support and helpful suggestions.

References


28. Dokter, W. H. A., Koopmans, S. B., and Vellenga, E. Effects of IL-10 and IL-4 on LPS-induced transcription factors (AP-1, NF-IL6 and NF-kB) which are involved in IL-6 regulation. Leukemia (Baltimore), 10: 1308—1316, 1996.


Altered Expression of Interleukin 6 and Interleukin 10 As a Result of Photodynamic Therapy *in Vivo*

Sandra O. Gollnick, Xiaonan Liu, Barbara Owczarczak, et al.

*Cancer Res* 1997;57:3904-3909.

Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/57/18/3904

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, contact the AACR Publications Department at permissions@aacr.org.

Downloaded from cancerres.aacrjournals.org on April 13, 2017. © 1997 American Association for Cancer Research.