Linomide Inhibits Angiogenesis, Growth, Metastasis, and Macrophage Infiltration within Rat Prostatic Cancers¹

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ABSTRACT

Linomide, a quinoline-3-carboxamide, has the ability to inhibit the growth of prostatic cancer in vivo but not in vitro (T. Ichikawa et al., Cancer Res., 52: 3022-3028, 1992). The reason for this discrepancy is that linomide inhibits tumor growth not directly but indirectly in vivo via its ability to inhibit the angiogenic response induced within the growing prostate cancer (J. Vukanovic, et al., Cancer Res., 53: 1833-1837, 1993). Tumor associated macrophages can stimulate angiogenesis via their ability to secrete various cytokines, particularly tumor necrosis factor α (TNF-α). Treatment of rats with linomide decreases significantly (P < 0.05), by more than 50%, the number of tumor associated macrophages from 60-70 to 15-37 macrophages/high power field). Monocytes/macrophages isolated from linomide treated rats had a decreased ability to secrete TNF-α when challenged in vitro with the bacterial endotoxin, lipopolysaccharide (i.e., 702 ± 76 (SEM) ng of TNF-α/10⁶ monocytes/macrophages from control versus 401 ± 2 ng of TNF-α/10⁶ monocytes/macrophages from linomide treated rats). In addition, when rats were treated with linomide and than challenged with lipopolysaccharide in vivo, the resulting elevation in serum TNF-α was inhibited by ~50% (i.e., 4.56 ± 1.8 ng/ml of TNF-α in control versus 2.9-2.2 ng/ml depending upon the dose of linomide). The ability of linomide to decrease monocyte/macrophage secretion of TNF-α is not immediate, however, since the secretion of TNF-α induced by lipopolysaccharide challenge of monocytes/macrophages isolated from untreated animals is not decreased by acute (i.e., <4 h) linomide treatment in vitro. These results demonstrate that the ability of linomide to inhibit the secretion of TNF-α by monocytes/macrophages isolated from untreated animals is not decreased by acute (i.e., <4 h) linomide treatment in vitro. These results demonstrate that the ability of linomide to inhibit the secretion of TNF-α by monocytes/macrophages is not immediate, however, since the secretion of TNF-α induced by lipopolysaccharide challenge of monocytes/macrophages isolated from untreated animals is not decreased by acute treatment (<4 h) of linomide in vitro.

INTRODUCTION

Linomide (Roquinimex), a quinoline-3-carboxamide (Fig. 1), has been demonstrated to have immunomodulatory and antitumor effects (1). Based on its immunomodulatory effects it has been used in clinical trials to accelerate engraftment following autologous bone marrow transplantation as part of the treatment for myeloid leukemias (2, 3). In these studies, linomide was given every 3–4 days at a very low dose (i.e., 0.05–0.2 mg/kg). At higher daily doses (i.e., >5 mg/kg/day) linomide has a reproducible ability to inhibit the growth rate of a series of androgen dependent and independent primary rat prostatic cancers and their metastases in vivo, but not in vitro, demonstrating that the antitumor effects of linomide are host mediated (1). These studies demonstrated that for maximal efficacy, animals should be treated with linomide at doses ≥5 mg/kg at least every other day. Studies using immunosuppressed animals demonstrated that neither B nor T cell function is effected or required for antiprostastic cancer effects of linomide (1, 4). One host mechanism which is effected by linomide is inhibition of angiogenesis. We have demonstrated that linomide treatment with daily doses of >10 mg/kg can inhibit the tumor angiogenic response (4) and thus reduce tumor blood flow (5). The demonstration that such linomide treatment has an antiangiogenic activity raises the question of the mechanism for this therapeutic effect. We have reported previously that linomide has the acute ability to inhibit the mobility and invasiveness of the endothelial cells in vitro in a dose dependent manner (5). These acute in vitro effects are paradoxical to the observation that 3–4 days of the linomide treatment are required before antitumor effects are observed in vivo (1). This slower kinetics of the in vivo antitumor response raises the issue of whether there are additional mechanisms, besides direct effects on endothelial cells, involved in the antiangiogenic activity of linomide.

A recent study has demonstrated that both bacterial endotoxin LPS³ and Staphylococcus aureus enterotoxin B induced septic shock is prevented by daily treatment of mice with 100 mg/kg of linomide (6). The same study demonstrated that this effect is due to an inhibition of TNF-α secretion. Although it was first identified as a factor inducing tumor necrosis in vivo, it is now clear that TNF-α has a broad spectrum of biological effects, including a highly dose dependent ability to modulate angiogenesis (7). Monocytes/macrophages are considered to be the major cellular source of TNF-α (8). TAMs are a major component of the tumor infiltrating cells (9). Although originally considered as mere scavenger cells, macrophages have been demonstrated to have both positive and negative effects with regard to tumor growth (9). One mechanism for the positive effect of macrophages is via their ability to stimulate tumor vascularization (10, 11). It has been demonstrated that in response to chemoattracants, TAMs accumulate at the site of implanted tumors even before new capillary formation occurs. Evans (12, 13) has shown that mice depleted of macrophages by total body X-irradiation or azathioprine administration before or after implantation of a syngeneic fibrosarcoma demonstrated a delay in the appearance of tumors, a suppression in the growth of established tumors, and a marked reduction in tumor vascularization. Mostafa et al. (14, 15) and Stenzinger et al. (16) demonstrated that vascularization of several human tumor cell lines grown on the chorioallantoic membrane of the chick embryo or s.c. in nude mice occurred coincidentally with TAM infiltration at the tumor site. Studies by Polverini and Leibovich (10) suggested that the mechanism for TAM induced angiogenesis is via the secretion of a factor or factors that stimulate capillary formation. These latter studies are highly relevant since it has been suggested that TNF-α is the major angiogenic molecule in macrophages (17).

Based upon these leads, the number of TAMs and other immune cells in linomide treated and untreated hosts was determined in both locally invasive and metastatic primary prostatic cancer. In addition,
the effect of linomide treatment on LPS induced elevation in serum TNF-α levels in vivo was tested as well as the direct effect of linomide treatment on LPS induced macrophage secretion of TNF-α.

MATERIALS AND METHODS

Reagents. Linomide (N-phenylmethyl-1,2-dihydro-4-hydroxy-1-methyl-2-oxoquinoline-3-carboximide) is the registered trademark for roquinimex (Kabi Pharmacia Therapeutics, Helsingborg, Sweden). Linomide was generously provided by Kabi Pharmacia Therapeutics.

Animals. The animals used in these studies were inbred male Copenhagen (Cop) rats weighing 175—200 g, obtained from Harlan Sprague-Dawley (Indianapolis, IN). Rats were housed in groups 3—5/individual microisolator stainless steel cage placed in well-ventilated rooms at an ambient temperature of 21—22°C and 50% relative humidity, lighted 12 h/day (lights at 7 a.m.). Animals had ad libitum access to tap water and food (i.e., 5053 Rat Chow; Purina Mills, Inc., Richmond, IN).

Prostatic Cancers. The nonmetastatic AT 2.1 and the highly metastatic Mat-Lu prostate cancer sublines utilized in this study are both androgen independent and anaplastic in histology and are members of the Dunning R-3327 system of serially transplantable rat prostatic cancers (18). For the in vivo studies, animals were inoculated s.c. with approximately 10-mg trocar pieces of tumor tissue as described previously (1). To test the effects of various treatments on metastatic ability of prostatic cancer cells, an ~10-mg trocar piece of Mat-Lu tumor tissue was inoculated in the leg of rats as described previously (19). Following the development of palpable tumors, individual tumor dimensions were serially measured with calibrated microloupe at various times following tumor inoculation. Tumor volumes in cm³ were calculated as

\[(l \times w \times h) \times 0.5236\]

and used to determine tumor volume doubling times as described previously (18). In the animals bearing Mat-Lu cancer in the leg, the tumor was removed en bloc at the indicated tumor size as described previously (19).

Elimination of NK Activity in Vivo. Lyophilized rabbit antiserum raised against purified glycoprophilinoid asialo-GM₁ (Wako Chemicals, Inc., Richmond, VA) was diluted in sterile saline. One hundred ml of the reconstituted antiserum were injected i.p. every 5 days starting 4 days before tumor inoculation since this dose regimen was demonstrated previously to deplete 90% of the NK cell activity in rats (1).

Quantitation of Tumor Infiltrating Host Cells. Tumors (n = 5) from control and linomide treated animals were observed 2 weeks after tumor inoculation and fixed in formalin for immunocytochemical staining. Formalin fixed, paraffin embedded tumor sections (5 mm thick) were serially deparaffinized with xylene and then rehydrated with ethanol. After quenching endogenous peroxidase activity in 3% H₂O₂ in methanol, sections were incubated in a 1:20 dilution of either normal mouse or goat serum (Vector Laboratories, Burlingame, CA) for 20 min. Sections were then incubated with the appropriate mouse monoclonal antibody demonstrated a granular, patchy cytoplasmic distribution of the antigen in monocytes and in most macrophages.

Effect of Linomide Treatment upon Tumor Infiltrating Host Cells in Rat Prostatic Cancers. Previous studies demonstrated that linomide has the ability to inhibit tumor induced angiogenesis [i.e., the number of blood vessels in both locally invasive AT 2.1 and highly metastatic Mat-Lu rat prostate cancers growing in the syngeneic hosts treated daily with linomide is reduced by 38% and 35% respectively compared to tumors growing in untreated host (4)]. To test whether this decrease in tumor angiogenesis is correlated with changes in the number of tumor infiltrating host cells, the number of such host infiltrating cells was determined in tumors from linomide treated versus untreated rats. To do this, Copenhagen rats were inoculated with AT 2.1 tumor tissue and treatment for one-half of the animals was begun with daily i.p. injections of 100 mg/kg of linomide. To obtain size matched tumors (i.e., n = 5/group) by 2 weeks postinoculation, twice as much tumor tissue was inoculated into rats given linomide as control rats since tumor growth is reduced by ~50% in the linomide treated animals (1, 4). After 2 weeks, animals were sacrificed, and tumors were removed, weighed, and processed for immunohisctochemical staining.

The macrophage populations within the rat tumors were studied using 2 monoclonal antibodies [i.e., ED₁ and ED₂ (22)] directed against rat macrophage specific epitopes. Staining with the ED₁ monoclonal antibody demonstrated a granular, patchy cytoplasmic distribution of the antigen in monocytes and in most macrophages.
The monoclonal ED\textsubscript{2} antibody recognized a membrane antigen on tissue macrophages and showed a more diffuse membrane staining. AT 2.1 cells grown in \textit{in vitro} cell culture are not positively stained by either the ED\textsubscript{1} or ED\textsubscript{2} monoclonal antibodies. ED\textsubscript{1} and ED\textsubscript{2} mAbs stained the monocytes and macrophages, respectively, inside clumps of tumor cells and in the inflammatory cells at the periphery in the capsule of tumor. The number of ED\textsubscript{1}, or ED\textsubscript{2} positive TAMs per high power field (×400) was reduced (P < 0.05) by 2–4-fold, respectively in linomide treated versus untreated hosts (Table 1).

The majority of the host cells infiltrating the transplanted tumors are macrophages. Only minor numbers of the tumor infiltrating host cells are composed of T cell subpopulations (i.e., <5 cells/high power field) and NK cells (<1 cell/high power field). T cells were almost exclusively located in the capsule surrounding the tumors. Rare T and NK cells were found inside the tumor itself in direct contact with the tumor cells. No difference in tumor cell density or location between control and linomide treated animals was observed for either T or NK cells. B lymphocytes were detectable in the lumen of the tumor microvessels. No difference in the low B cell number was observed for AT 2.1 tumors obtained from linomide treated versus control untreated animals.

**Effect of Linomide on LPS Induced Secretion of TNF-α.** LPS induced up-regulation of TNF-α has been demonstrated to be decreased by daily treatment of mice with 100 mg/kg of linomide (6). To determine if this is the case in rats, animals (5/group) were untreated or treated with linomide either via p.o. feeding of 25 mg/kg/day or via i.p. injection of 100 mg/kg/day for 7 days before i.p. injection with 0.5 mg/kg of LPS. The drug is well absorbed after p.o. administration and our previous data demonstrated no difference in antitumor effects if linomide is applied either i.p. or s.c. Serum was obtained 90 min after LPS injection (when TNF-α serum levels are maximal (25)) and assayed for TNF-α. These studies demonstrate that linomide treatment alone, when given either p.o. or i.p. injection, has no ability to increase serum TNF-α level but it decreases the elevation in serum TNF-α levels induced by LPS treatment (Table 2).

Since monocytes/macrophages are the major source of TNF-α, monocytes/macrophages were tested \textit{in vitro} for their direct response to the inhibition by linomide of TNF-α secretion. To do this peripheral blood monocytes/macrophages were isolated from both untreated and linomide treated animals (i.e., 100 mg/kg/day p.o. for 1 week) and then cells were exposed \textit{in vitro} in culture to linomide alone, LPS alone, or LPS after pretreatment with or without linomide. To do this, monocytes/macrophages were pretreated with media containing 0–100 μg/ml of linomide [i.e., levels achievable in the serum of rats treated \textit{in vivo} with therapeutically effective doses of linomide (i.e., ≤100 mg/kg/day)] (5). After 4 h of pretreatment, media were replaced with media containing 100 μg/ml of linomide alone or 1 μg/ml of LPS and the pretreatment concentration of linomide. After 16 h, the media were assayed for TNF-α levels (Table 3). These results demonstrated that when monocytes/macrophages from linomide untreated hosts are exposed to linomide alone, there is no effect on TNF-α secretion. When these cells were exposed to LPS, there is a massive secretion of TNF-α into the tissue culture media. When monocytes/macrophages from untreated hosts are pretreated for 4 h with linomide and then continued in the presence of linomide during the 16-h LPS challenge, there was no effect on TNF-α secretion (Table 2). Thus, linomide does not have an acute ability to inhibit TNF-α secretion by monocytes/macrophages \textit{in vitro}. In contrast, when monocytes/macrophages were obtained from animals treated \textit{in vivo} with linomide for 1 week p.o. at a dose of 100 mg/kg/day \textit{in vivo}, their response to the 16-h LPS challenge \textit{in vitro} is decreased by more than 40% (P < 0.05). Thus, the ability of linomide to decrease monocytes/macrophages secretion of TNF-α \textit{in vivo} requires host factors not present during \textit{in vitro} cell culture.

**Role of NK Cells in Antitumor, Antimetastatic, and Antimacrophage Effects of Linomide.** Our previous studies have demonstrated that the antitumor effect of linomide (i.e., 2-fold inhibition of tumor growth) occurs in nude as well as in immunocompetent rats bearing the AT 2.1 rat prostate cancer (1). Similar antitumor effects of linomide (i.e., 2-fold inhibition of tumor growth) also occur in SCID mice bearing the AT 2.1 rat prostate cancer (1). Since SCID mice lack both B and T cell function, these data demonstrate that B and T cell function is not required for the activities of linomide \textit{in vivo}. SCID mice do have normal NK cell functional activity (28). In addition, NK cells have been demonstrated to affect macrophages (29). Previous studies have demonstrated that when tumor bearing mice are treated daily with linomide at doses >10 mg/kg, there is an inhibition of tumor growth and an increase in the number of NK cells suggesting an important role of NK cells in the antitumor activity of daily linomide treatment in mice (30). In addition, linomide treatment inhibits the secretion of TNF-α into the serum of LPS treated mice (6). These data suggest that NK cells might be a critical host factor in mediating the \textit{in vivo} abilities of linomide to decrease TAMs and inhibit tumor growth in rats. To test this possibility, male Cop rats were inoculated s.c. with highly metastatic MAT-Lu cancer cells and immediately randomized into 4 groups (i.e., 6–8 rats/group). One group (i.e., control) received no further treatment. One group received daily i.p. injections of 100 mg/kg of linomide, one group received i.p. injections of 100 μl of polyclonal aAMG\textsubscript{1} antisera every 5th day [a protocol that we have previously demonstrated depletes NK activity in rats by >90% (1)], and the final group received both linomide and aAMG\textsubscript{1} antisera treatment. As demonstrated in Fig. 2, growth of the primary tumor was inhibited by about 67% (P < 0.05) by day 25 post-tumor inoculation in linomide treated hosts. This effect is due to ~50% decrease in the growth rate of the tumors in linomide treated hosts [i.e., reflected by increased tumor volume doubling time (Table 4)]. Treatment i.p. with anti-asialo GM\textsubscript{1} antisera alone had no effect upon the growth rate of the tumor and in combination did not decrease the ability of linomide to inhibit the growth of the primary MAT-Lu tumors (Table 4). At ~7 cm\textsuperscript{2} size (range, 6–8 cm\textsuperscript{2}) primary tumors were surgically removed and all groups of rats were allowed to go untreated for the additional 3 weeks. At the end of the experiment animals were killed and the

**Table 1**: Effect of linomide treatment on the number of TAMs in AT 2.1 tumors

<table>
<thead>
<tr>
<th>Treatment (N = 5 tumors/group)</th>
<th>ED\textsubscript{1} positive TAMs/high power field</th>
<th>ED\textsubscript{2} positive TAMs/high power field</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24 ± 3\textsuperscript{a}</td>
<td>41 ± 1\textsuperscript{a}</td>
</tr>
<tr>
<td>Linomide (100 mg/kg/day) for 2 wk</td>
<td>10 ± 4\textsuperscript{a} (-58)\textsuperscript{b}</td>
<td>10 ± 4\textsuperscript{a} (-76)\textsuperscript{b}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Mean ± SEM.

\textsuperscript{b} P < 0.05 compared to control group.

\textsuperscript{c} Numbers in parentheses, percentage decrease compared to control group.

**Table 2**: Ability of linomide to decrease the LPS induced elevation in serum TNF-α \textit{in vivo}

<table>
<thead>
<tr>
<th>Treatment (N = 5/group)</th>
<th>Serum TNF-α (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Linomide (100 mg/kg/day)</td>
<td>4.56 ± 1.81\textsuperscript{c}\textsuperscript{e}</td>
</tr>
<tr>
<td>LPS alone\textsuperscript{d}</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LPSalone + linomide (25 mg/kg/day p.o.)\textsuperscript{f}</td>
<td>2.47 ± 1.52\textsuperscript{c}\textsuperscript{e} (-39)\textsuperscript{f}</td>
</tr>
<tr>
<td>LPSalone + linomide 100 mg/kg/day i.p.\textsuperscript{g}</td>
<td>2.16 ± 1.29\textsuperscript{c}\textsuperscript{e} (-53)\textsuperscript{g}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} 0.5 mg/kg LPS injected IP 90 min before harvesting of blood

\textsuperscript{b} Mean ± SEM.

\textsuperscript{c} P < 0.05 compared to control.

\textsuperscript{d} Linomide started 7 days before LPS injection.

\textsuperscript{e} Linomide started 7 days before LPS injection.

\textsuperscript{f} Numbers in parentheses, percentage decrease compared to LPS alone.
lungs were analyzed for the number of metastases (Table 4). In animals given the aaGM1 antiserum alone, there is a more than 2-fold ($P < 0.05$) increase in the number of lung metastases compared to control animals. Linomide treatment alone resulted in an 87% ($P < 0.05$) reduction in the number of lung metastases established per animal. In contrast, depletion of NK cells when combined with daily linomide treatment neither enhanced nor decreased the ability of linomide to inhibit lung metastases in rats (Table 4). Thus, unlike the situation in mice, NK cells are not critically required in rats for the ability of linomide to inhibit either primary tumor growth or establishment of metastases.

To test whether such depletion of NK cell activity affects the ability of linomide to reduce the number of TAMs, size matched primary Mat-Lu tumors were harvested from five animals in each group. Morphometric and image analysis were performed to quantify the numbers of TAMs within the primary MAT-Lu rat prostate carcinomas. These studies demonstrated a significant decrease ($P < 0.05$) in the number of ED1 and ED2 positive TAMs present in the linomide treated tumors using either type of quantitation (Table 5). Depletion of the NK cells with aaGM1 antiserum alone did not significantly reduce the number of TAMs nor did combination of the aaGM1 antiserum with linomide prevent the ability of linomide to significantly lower TAMs. Thus, the effects of linomide in rats are NK cell independent.

**DISCUSSION**

The growth and formation of capillary blood vessels (i.e., tumor angiogenesis) and essential components of solid tumor growth. In addition to being necessary for the growth of primary tumors beyond a size of 2–3 mm³, tumor angiogenesis is also critical at the beginning and the end of the metastatic cascade (31). Thus, tumor angiogenesis permits the shedding of cells from the primary tumor and decreased angiogenesis is associated with a decreased rate of metastasis (31–33). Products derived from both tumor cells and a variety of non-neoplastic mediator systems, including macrophages, have been implicated in this angiogenic response (11–13). TAMs are a major component of the tumor infiltrating cells [up to 80% in human breast cancers (34)].

**TAMs play a complex role in the regulation of primary tumor growth and metastasis.** Originally considered as mere scavenger cells, macrophages have been demonstrated to have both positive and negative abilities with regard to tumor growth. Polverini (11) demonstrated that macrophages isolated from solid tumors are potent inducers of angiogenesis *in vivo* and endothelial cell proliferation *in vitro*. His studies suggest that the mechanism for TAM induced angiogenesis is via the secretion of factors, including TNF-α (35), that stimulate capillary formation. Therefore, approaches which either prevent the initial

**Table 3** *In vitro* ability of monocytes/macrophages to secrete TNF-α when exposed to LPS alone or in combination with varying doses of linomide

<table>
<thead>
<tr>
<th>In vivo treatment</th>
<th>4-h in vitro pretreatment</th>
<th>16-h in vitro treatment</th>
<th>TNF-α (ng/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>None (negative control)</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>LPS [1 μg/ml (positive control)]</td>
<td>702 ± 766</td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>Linomide (100 μg/ml)</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>None</td>
<td>Linomide (100 μg/ml)</td>
<td>LPS (1 μg/ml) + linomide</td>
<td>792 ± 116</td>
</tr>
<tr>
<td>None</td>
<td>0.1 μg/ml</td>
<td>1 μg/ml</td>
<td>714 ± 157</td>
</tr>
<tr>
<td>None</td>
<td>1 μg/ml</td>
<td>10 μg/ml</td>
<td>812 ± 81</td>
</tr>
<tr>
<td>None</td>
<td>10 μg/ml</td>
<td>100 μg/ml</td>
<td>834 ± 92</td>
</tr>
<tr>
<td>Linomide (100 mg/kg/day p.o. for 7 days)</td>
<td>None</td>
<td>LPS (1 μg/ml)</td>
<td>401 ± 25 (−43)²</td>
</tr>
<tr>
<td>Linomide (100 mg/kg/day p.o. for 7 days)</td>
<td>Linomide (100 μg/ml)</td>
<td>None</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

| *Mean ± SEM.*  | *P < 0.05* compared to LPS positive control group.  | *Numbers in parentheses, percentage decrease compared to LPS positive control group. |

**Table 4** Effects of linomide and aaGM1 treatment alone and in combination on Mat-Lu tumor metastases

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor bearing rats/group</th>
<th>Tumor vol doubling time at the time of removal of primary size matched tumors</th>
<th>Tumor vol (cm³)</th>
<th>Av. no. of lung metastases*</th>
<th>Range of lung metastases*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>7</td>
<td>4.2 ± 0.1⁶</td>
<td>6.7 ± 0.9</td>
<td>45 ± 12 (100)²</td>
<td>12–81</td>
</tr>
<tr>
<td>aaGM1 (100 ml/5 days)</td>
<td>6</td>
<td>4.0 ± 0.15</td>
<td>8.3 ± 1.9</td>
<td>100 ± 33 (222)</td>
<td>18–193</td>
</tr>
<tr>
<td>Linomide (100 mg/kg)</td>
<td>8</td>
<td>7.5 ± 1.0</td>
<td>6.2 ± 0.3</td>
<td>6 ± 1 (13)</td>
<td>0–19</td>
</tr>
<tr>
<td>Linomide (100 mg/kg)</td>
<td>8</td>
<td>7.4 ± 1.0</td>
<td>6.4 ± 0.9</td>
<td>6 ± 2 (13)</td>
<td>2–9</td>
</tr>
</tbody>
</table>

* Rats were analyzed for lung metastases 3 weeks post-leg amputation, i.e., 7 weeks for control and aaGM1 and 9 weeks for linomide and linomide plus aaGM1 treated groups.

* *Mean ± SEM.*

* Numbers in parentheses, percentage metastatic ability normalized to the control group.

* *P < 0.05* compared to control group.
infiltration of TAMs into tumors or inhibit TAMs within the tumor from releasing growth factors should have therapeutic usefulness. For example, IL-10 is known to deactivate macrophage migration and suppress production of cytokines, including TNF-α (36). When tumorigenic CHO cells are transsected with an IL-10 expression vector and the resulting IL-10 expressing CHO cells are injected into appropriate animals, there is a virtual absence of infiltrating TAMs within the tumors. Coincident with this TAM suppression is a major suppression in the tumorigenicity of these IL-10 expressing CHO transfectants (37).

Results of the present study demonstrate that TAMs constitute the major type of tumor infiltrating host cells within serially transplantable rat prostatic cancers. Daily treatment with 100 mg of linomide/kg decreased by ≥50% the number of TAMs in both nonmetastatic AT 2.1 and metastatic Mat-Lu rat prostatic cancers grown in vivo. Likewise, such daily linomide treatment decreased by 35–40% the blood vessel number and area within both of these tumors (4). Such decrease in blood supply resulted in ~50% decrease in both the growth rate of primary AT 2.1 and MAT-Lu tumors and a >85% reduction in the number of MAT-Lu lung metastases which were established (1, 4). At least one of the mechanisms for this antiinflammatory/antimetastatic effect is due to the inhibition by linomide of tumor angiogenesis (4, 5) which may be via the presently demonstrated inhibition in the number of TAMs infiltrating these cancers in vivo.

A variety of angiogenic factors (e.g., basic fibroblastic growth factor, vascular endothelial growth factor, TNF-α, etc.) have been shown to be produced by macrophages (7, 11, 17, 35). It has been suggested that TNF-α is the major angiogenic molecule in macrophages (11). Although it was first identified as a factor inducing tumor necrosis in vivo, it is now clear that this pro-inflammatory cytokine has a broad spectrum of biological effects. For example, TNF-α has a highly dose dependent effect on angiogenesis. At very low levels TNF-α can inhibit angiogenesis induced by other angiogenic factors within the tumor. At slightly higher levels, TNF-α stimulates angiogenesis; and at even higher levels, TNF-α produces a destructive effect on capillaries (7). Leibovich et al. (35) demonstrated that TNF-α from TAM is angiogenic in the chicken chorioallantoic membrane assay. Likewise, Passaniti et al. (38) demonstrated that TNF-α is angiogenic in vivo using the Matrigel assay.

In vivo septic shock induced in mice by the bacterial endotoxin lipopolysaccharide results in the stimulation of monocytes/macrophages to produce TNF-α (6). A recent study has demonstrated that such septic shock is prevented by daily treatment of mice with 100 mg/kg of linomide and that this effect is due to an inhibition of TNF-α secretion. Our rat studies agree with these previous observations in mice since elevation in TNF-α serum levels are decreased in linomide treated rats exposed to LPS. This effect of linomide is not an acute (i.e., within 4 h) effect upon monocytes/macrophages, however, since acute in vitro exposure to linomide does not inhibit the LPS induced increase in TNF-α secretion by these rat cells. Preliminary data demonstrated that IL-10 secretion by isolated monocytes/macrophages is enhanced in vitro by linomide treatment and that this enhancement requires a chronic exposure to linomide for more than 10 h. Since IL-10 is known to both inhibit migration of macrophages and suppress TNF-α secretion (36), such enhanced IL-10 secretion could provide an explanation for inhibitory effects of linomide upon TAM infiltration, angiogenesis, and prostatic cancer growth in vivo.4

We have demonstrated previously that linomide inhibits the migration of endothelial cells in a dose dependent manner and that it is cytostatic but not cytotoxic to endothelial cells (5). Endothelial cell proliferation occurs predominantly at the tumor periphery (39) with the endothelial cells migrating into the center of the tumor to form new blood vessels (39). Such migration does not require endothelial cell proliferation (40). Thus, proliferation, migration, and invasion are essential requirements for the formation of tumor blood vessels. The rate of tumor endothelial cell proliferation is rate limiting for tumor growth (31). Part of the difficulty in the treatment of the human prostate cancer is that the rate of proliferation of prostatic cancer cells, even in metastatic sites, is remarkably low [i.e., <3% of cancer cells proliferating per day (41)] thus limiting the usefulness of standard chemotherapies which are targeted at proliferating cells. Since the rate of tumor endothelial cell proliferation is usually slightly lower than that of the cancer cells they are supporting (39) this suggests that endothelial cells within prostatic cancer lesions will have an extremely low rate of proliferation (39). As demonstrated in the present and previous studies (5), the major effects of linomide appear to involve the recruitment, migration, and invasion of macrophages and endothelial cells. Since these effects are proliferation independent, the efficacy of linomide is not limited by a low rate of endothelial cell proliferation. Tumor angiogenesis is required for the maintenance and the growth of the cancer cells (31, 33). Thus, tumor angiogenesis is a major target for effective therapy even for slowly proliferating cancers like that of the prostate. To be effective, angiogenesis inhibitors must be given chronically which raises critical quality of life and toxicity issues. In addition, an angiogenesis inhibitor could affect wound healing. There are significant differences, however, between the neovascularization associated with wound healing and tumor angiogenesis (17, 41, 42). For example, while macrophages and TNF-α are involved in tumor angiogenesis they are not the predominant cell type or growth factor involved during wound healing (41, 42). Therefore, the inhibitory effects of linomide on macrophages should allow normal wound healing to occur. This may explain why chronic linomide treatment neither blocks the healing of the original inoculation site of tumor implants5 nor induces major host toxicities (1–5). An additional advantage is that linomide is active p.o. (1, 4, 5). Thus, long term use of linomide may prove to be beneficial in prostate cancer patients by...
suppressing the progression of the disease to its lethal conclusion via its antiangiogenic effects.

REFERENCES


Linomide Inhibits Angiogenesis, Growth, Metastasis, and Macrophage Infiltration within Rat Prostatic Cancers

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