Increased Invasion and Spontaneous Metastasis of BL6 Melanoma with Inhibition of the Desmoplastic Response in C57 BL/6 Mice

Sanford H. Barsky2 and Rayudu Gopalakrishna
Department of Pathology, University of California at Los Angeles, Los Angeles, California 90024

ABSTRACT
BL6 melanoma cells injected s.c. in 18-month C57 BL/6 mice elicit a markedly fibrotic response similar in myofibroblast and collagen composition to that characterizing the desmoplastic response of human breast carcinoma. This host response can be quantitated by measuring hydroxyproline (total collagen) and incorporation of i.p.-injected [14C]proline into collagenase-sensitive protein (new collagen synthesis). Inhibition (70%) of the desmoplastic response can be achieved by daily injections of L-3,4-dehydroproline. Inhibiting the response in this manner promotes local invasion of tumor and increases the incidence of spontaneous pulmonary metastasis. 104 BL6 melanoma cells produce tumor nodules with a mean diameter of 1.5 ± 0.5 cm and mean collagen content of 36 ± 15 mg/g wet tissue at 4 weeks and 10% incidence of pulmonary metastasis at 7 weeks. L-3,4-dehydroproline produces nodules with a mean diameter of 2.3 ± 0.5 cm and mean collagen content of 12 ± 2 mg/g with a 40% incidence of metastasis. L-3,4-dehydroproline exerts a selective effect on myofibroblast collagen synthesis in vitro and no effect on [3H]thymidine uptake, doubling time, and viability of BL6 cells and myofibroblasts. Furthermore, this drug exerts no effect on BL6 invasion and metastasis in 6-week C57 BL/6 mice, hosts which exhibit a negligible desmoplastic response.

INTRODUCTION
The desmoplastic response to tumor invasion is a poorly understood host collagenous response mediated, in large part, by host myofibroblasts (1-3) and responsible for the “hard lump” appearance of many human cancers (4). Controversy has existed regarding the pathogenesis of the response. Some studies have argued that the response is a host response to tissue injury analogous to an organizing wound (5, 6) and other studies have argued that the response is elicited by tumor-derived growth factors which stimulate the recruitment and division of host fibroblasts (7, 8). Whatever the pathogenesis, the effects which the desmoplastic response exert on the growing tumor are not known. It can be argued that the response might benefit the host by “walling off” the invading tumor; alternately, it can be argued that the response might benefit the tumor by reducing access of host lymphocytes, macrophages, and other immune-regulator cells. The effects of the desmoplastic response on tumor invasion and metastasis have not been studied because they involve complex tumor cell-host interactions which are not easily reduced to simple variables. Recently our laboratory has developed an experimental model for studying the desmoplastic response to tumor invasion using BL6 melanoma cells in 18-month C57 BL/6 mice (9). It had been noted by others (10, 11) that aged C57 BL/6 mice form a fibrous capsule around BL6 melanoma. We investigated this fibrous response with the highly metastasizing variant, BL6 melanoma, and found that the myofibroblast and collagenous composition (relatively high amounts of Type V collagen) of this response were virtually identical to the composition of the desmoplastic response exhibited by human carcinomas. Furthermore, we were able to quantitate this fibrous response in terms of hydroxyproline content (total collagen) and incorporation of [14C]proline into collagenase-sensitive protein (new collagen synthesis) and selectively inhibit it with L-3,4-dehydroproline and thereby study the effects of this inhibition on tumor invasion and metastasis.

MATERIALS AND METHODS

In Vivo Experiments. 105 BL6 melanoma cells were injected into 4 groups (10 mice each) of 18-month female C57 BL/6 mice. Groups 1 and 3 received daily i.m. injections of L-3,4-dehydroproline (2.5 mg/100 g animal weight). L-3,4-dehydroproline is a commonly used inhibitor of collagen synthesis. This proline analogue blocks hydroxylation and assembly of the triple helix of the collagen molecule and hence inhibits extracellular deposition. The chains of the collagen molecule which are prevented from forming a triple helix are thought to undergo increased intracellular degradation (12). While many inhibitors of collagen synthesis exert toxic side effects on animals, in our hands and in those of others, L-3,4-dehydroproline exerted no untoward side effects on the mice. Groups 2 and 4 received saline injections and served as controls. At 4 weeks, tumor nodules from groups 1 and 2 were extirpated and subjected to measurements of hydroxyproline content (total collagen). Groups 3 and 4 received i.p. injections of [14C]proline, 5 μCi (specific activity, 275 mCi/mmol), 24-h prior to tumor extirpation after 4 weeks of growth. The animals were killed with ether anesthesia and the primary tumor nodules were excised and their degree of local invasion into adjacent host tissues was determined by measuring their mean diameters. The lungs were removed, inflated with Bouin’s fixative, and number of surface metastases (black colonies appearing on the pleural surfaces of both lungs) was determined with a dissecting microscope. In parallel sets of experiments, the primary tumor nodules were removed at 4 weeks but the animals were not killed until 7 weeks at which time pulmonary metastases were determined. As additional controls, the same set of experiments were performed on 6-week female C57 BL/6 mice. All experiments consisted of groups of 10 mice and were performed in triplicate.

In Vitro Experiments. Explants of BL6 melanoma grown in mice under the conditions enumerated for the in vivo experiments were established in organ culture at 37°C, 95% O2, 5% CO2, for 5 h in proline-glutamine-free Dulbecco-Vogt medium supplemented with 5% dialyzed fetal calf serum, ascorbate (100 μg/ml), β-aminopropionitrile (50 μg/ml) with added [14C]proline (5 μCi/ml) to assess collagen synthesis in vitro. Separate cell cultures of BL6 melanoma cells and myofibroblasts derived from explants of desmoplastic BL6 melanoma according to previously established methods (1) were grown under similar conditions to demonstrate the selective effect of L-3,4-dehydroproline on myofibroblast collagen synthesis. The possible effects of this drug on BL6 and myofibroblast viability, doubling time, and [3H]thymidine uptake were also investigated. Cell cultures were grown in either the absence or the presence of L-3,4-dehydroproline (25 μM/ml) under conditions appropriate for the parameter being studied.

Measurement of Hydroxyproline (Total Collagen). The tumor nodules (100-500 mg) were homogenized in 5 ml of 10% trichloroacetic acid at 4°C and centrifuged at 4000 x g for 10 min. The pellet was washed twice successively with 10% trichloroacetic acid, ethanol:ether (3:1), and ether. The washed pellet was dried. Collagen was measured after acid hydrolysis (6 N HCl for 18 h) as hydroxyproline (13). Melanin pigment from the melanoma cells present in the extracts initially
interfered with the colorimetric measurement of hydroxyproline. Hence after the acid was removed by vacuum drying, the protein hydrolysate was applied to a Dowex 50W × 8 (200–400 mesh H+ form) column (0.8 × 2 cm) and the amino acid fraction containing hydroxyproline was eluted and dried under vacuum. Hydroxyproline measurements were determined to obtain a reliable quantitation of total collagen accumulation. Collagen (Type I obtained from calf skin) was used as a reference standard.

Measurement of Incorporation of [14C]Proline into Collagenase-sensitive Protein (New Collagen Synthesis). The tumor nodules and tumoral explants were similarly homogenized in 10% trichloroacetic acid and centrifuged at 4000 × g, and the pellet was washed successively with 10% trichloroacetic acid, ethanol:ether, and ether. Total protein synthesis was determined as the amount of [14C]proline incorporation into this trichloroacetic acid-insoluble pellet. The pellet, following resuspension and neutralization was then digested with protease-free bacterial collagenase (*Clstridium histolyticum*). The radioactivity released into the supernate was taken as a measurement of collagenase-sensitive protein (new collagen synthesis). Collagen (Type I) was labeled with [3H]acetic anhydride (14). Collagen (acetylated-H]) of about 5000 cpm was added to the tissue pellet in which [14C]collagen had been solubilized by collagenase treatment to ensure complete digestion of the substrate and to correct for the recovery of the substrate, if appropriate. Both [14C] and [3H] radioactivity released after collagenase digestion were measured in a β-scintillation counter. The ratio of collagenase-sensitive protein synthesized to total protein synthesized was calculated. The conditioned media of both cultured BL6 cells and myofibroblasts grown in the absence and presence of L-3,4-dehydroproline was dialyzed against 0.5 M acetic acid containing EDTA (15 mm) and N-ethylmaleimide (3.8 mM) to wash free [14C]proline. 10% NaCl was added to precipitate all collagens. The precipitated collagens were resolubilized with 0.5 M acetic acid and lyophilized. Lyophilized samples were resuspended and neutralized and then incubated with purified bacterial collagenase and counts liberated in the supernate were taken as a measurement of collagenase-sensitive protein.

Possible Effects of L-3,4-Dehydroproline on Cell Growth and Viability. Growth of BL6 cells and myofibroblasts in cell culture was determined by studies of doubling time and cell proliferation according to established methods (15). Each cell type was grown in the presence and absence of L-3,4-dehydroproline (25 µg/ml). The cells were plated at low densities and counted with a hemocytometer at regular intervals to determine the time needed for doubling. Cell proliferation was assessed by [3H]thymidine uptake studies. Cells grown in the presence and absence of L-3,4-dehydroproline were seeded into individual wells of a multiwell tissue culture plate. 1.0 µCi of [3H]thymidine (specific activity, 1.9 Ci/mmol) in 25 µl was added to each assay well. Four hours later, medium was removed and the cells harvested onto filters and counted in a β-scintillation counter. Cell viability was determined by exclusion of 0.2% trypan blue.

Measurement of Protein. Cell and tissue protein were determined by taking the trichloroacetic acid-insoluble protein fraction from tissue homogenates and/or trypsinized cells, and solubilizing in 1 N NaOH at 100°C for 10 min and measuring protein by the procedure of Lowry et al. (16), using bovine serum albumin as the standard.

Statistical Analyses. Differences between the experimental groups were evaluated using both the Student’s t test and the Mann-Whitney test.

**RESULTS**

Examination of the primary tumor nodule after 4 weeks of growth revealed striking differences in appearance (Fig. 1), histology (Fig. 2), and collagen content (Table 1) in the untreated group compared to the L-3,4-dehydroproline-treated group. 70% decrease in collagen content was achieved with L-3,4-dehydroproline (P < 0.02). This decrease in collagen content was due to a comparable decrease in collagen synthesis which could be demonstrated both in the tumoral nodules in vivo (P < 0.02) and in the tumoral explants in vitro (P < 0.05)
Further examination of the untreated primary nodule revealed prominent interlacing bands of collagen containing numerous host myofibroblasts (Fig. 2, top) which strongly resembled the desmoplastic response of many human carcinomas (1-3). These interlacing bands of collagen and influx of myofibroblasts could be visibly abolished with l-3,4-dehydroproline. The interface between tumor and host tissue in treated animals showed melanoma cells invading adjacent skeletal muscle and s.c. tissues with no appreciable intervening collagenous or myofibroblast response (Fig. 2, bottom). Six-week C57 BL/6 mice demonstrated no visibly apparent fibrotic bands or myofibroblast response and total collagen was very low (<2 mg/g) (Table 1). The decrease in collagen synthesis within the tumor nodules from l-3,4-dehydroproline was apparently due to a selective effect of the drug on myofibroblast collagen synthesis (P < 0.05) (Table 2). Since BL6 cells synthesize very small amounts of collagen in cell culture, the inhibitory effect of this drug on collagen synthesis of these cells was insignificant (P > 0.25).

There was a striking inverse correlation noted between collagen content and mean diameter of the primary nodules in 18-month C57 BL/6 mice (P < 0.05) (Fig. 3). Since l-3,4-dehydroproline exerted no effect on viability, doubling time, or [3H] thymidine uptake of either BL6 cells or myofibroblasts in cell culture (P > 0.25) (Table 2) but a significant and direct inhibitory effect on collagen synthesis of host myofibroblasts in cell culture and collagen synthesis of the BL6 tumoral explants in organ culture, it was concluded that the action of l-3,4-dehydroproline on the enhancement of mean diameter of the primary tumor nodule was due to this inhibition of host collagen synthesis. Decreased host collagen synthesis allowed then for increased expansion of the primary tumor into host tissue or increased tumor invasion. Although the tumor cells had been initially injected s.c., the tumor nodules which were excised showed extensions of tumor cells into adjacent skeletal muscle, underlying bone, and overlying skin (Fig. 2, bottom). The mean diameter of the tumor nodules strongly correlated with the degree of invasion into these adjacent host tissues.

1-3,4-dehydroproline increased the incidence of spontaneous pulmonary metastasis from 10 to 40% (P < 0.05). This trend was first noted at 4 weeks but became even more noticeable at 7 weeks (Table 3). Not only was the incidence of metastasis increased in the l-3,4-dehydroproline treated mice, but the number of colonies/positive lung was also increased (P < 0.05) (Fig. 4). The drug exerted no effect on tumor invasion or metastasis in 6-week C57 BL/6 mice, hosts which exhibited a negligible desmoplastic response (P > 0.25).

### Table 1 Effect of l-3,4-dehydroproline treatment on total collagen and new collagen synthesis within the desmoplastic response

<table>
<thead>
<tr>
<th>Group</th>
<th>In vivo</th>
<th>New collagen synthesis a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total collagen (mg/g)</td>
<td>In vitro</td>
</tr>
<tr>
<td></td>
<td>CSP</td>
<td>TP</td>
</tr>
<tr>
<td>BL6, 18 month, untreated</td>
<td>36 ± 15</td>
<td>29,250 ± 3,500</td>
</tr>
<tr>
<td>BL6, 18 month, treated</td>
<td>12 ± 2</td>
<td>15,560 ± 1,850</td>
</tr>
<tr>
<td>BL6, 6 week, untreated</td>
<td>1.8 ± 0.4</td>
<td>NT</td>
</tr>
<tr>
<td>BL6, 6 week, treated</td>
<td>1.6 ± 0.5</td>
<td>NT</td>
</tr>
</tbody>
</table>

a Collagen is expressed as mg/g wet tissue. Results are expressed as mean ± SD of groups of 10 tumors for all data shown.

b Approximately 107 cpm of [14C]proline was added to each assay as enumerated in "Materials and Methods." Measurements included collagenase-sensitive protein synthesized (CSP), total protein synthesized (TP), and the ratio of these two parameters.

* NT, not tested. The amount of total collagen present in tumors of 6-week mice was so low that collagen synthesis studies were not performed in these groups.

### Table 2 Selective effect of l-3,4-dehydroproline treatment on collagen synthesis of myofibroblasts in vitro

<table>
<thead>
<tr>
<th>Group</th>
<th>Viability a</th>
<th>Doubling time (days)</th>
<th>[3H]Thymidine uptake</th>
<th>Collagenase-sensitive [14C]protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myofibroblasts</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>&gt;90%</td>
<td>2.7 ± 0.3 b</td>
<td>580 ± 125 c</td>
<td>15,860 ± 2,580 d</td>
</tr>
<tr>
<td>Treated</td>
<td>&gt;90%</td>
<td>2.5 ± 0.2</td>
<td>603 ± 110</td>
<td>5,560 ± 1,050</td>
</tr>
<tr>
<td>BL6 cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>&gt;90%</td>
<td>0.75 ± 0.2</td>
<td>1,850 ± 250</td>
<td>310 ± 150</td>
</tr>
<tr>
<td>Treated</td>
<td>&gt;90%</td>
<td>0.82 ± 0.3</td>
<td>1,790 ± 225</td>
<td>290 ± 180</td>
</tr>
</tbody>
</table>

a Cell viability was determined by exclusion of 0.2% trypan blue.
b Short-term cultures of myofibroblasts were obtained from explants of desmoplastic BL6 melanoma in an identical manner from which they had been obtained previously from explants of desmoplastic human breast carcinoma (1).
c Each assay was performed in triplicate. Values are expressed as mean ± SD.
d [3H]Thymidine uptake and collagenase-sensitive [14C]protein are expressed as cpm/mg cellular protein.

### Table 3 Effect of l-3,4-dehydroproline on tumor invasion and metastasis of BL6 melanoma

<table>
<thead>
<tr>
<th>Group</th>
<th>Invasion of primary nodule (mean diameter, 4 weeks)</th>
<th>Spontaneous pulmonary metastasis a</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL6, untreated, C57 BL/6, 18 month</td>
<td>0/30 (0)</td>
<td>3/30 (10)</td>
</tr>
<tr>
<td>B6, treated, C57 BL/6, 18 month</td>
<td>2.3 ± 0.5</td>
<td>6/30 (20)</td>
</tr>
<tr>
<td>BL6, untreated, C57 BL/6, 6 week</td>
<td>2.8 ± 0.4</td>
<td>12/30 (40)</td>
</tr>
<tr>
<td>B6, treated, C57 BL/6, 6 week</td>
<td>2.7 ± 0.5</td>
<td>13/30 (43)</td>
</tr>
</tbody>
</table>

a All metastasis experiments consisted of groups of 10 mice and were performed in triplicate. Results shown represent the cumulative data of all experiments. Data depict incidence of spontaneous metastasis. Numbers in parentheses, percentage.

### DISCUSSION

The origins and significance of the desmoplastic response to tumor invasion have been controversial and not well studied.
For many years, the tumor cells were thought to secrete the collagenous desmoplastic matrix (17). Most recent studies have demonstrated that the origins of the desmoplastic response are host stromal cells recruited in response to the invading tumor (2, 3, 7). Both the mechanism of the desmoplastic response and its effect on tumor invasion and metastasis have been difficult to study because both tumoral and host factors are involved. The observation that the response is prominent in aged mice but absent in young mice (10) demonstrates the importance of host factors in the genesis of the response. The observation that the response is lacking in some human tumors arising in the same sites as desmoplastic tumors (for example, medullary breast carcinoma versus scirrhous breast carcinoma) demonstrates the equal importance of tumoral factors in the genesis of the response (2). Some observers have noted that tumors eliciting the highest desmoplastic reaction behave more aggressively than those eliciting a less pronounced response. This has been noted in scirrhous breast carcinoma compared to medullary breast carcinoma and in certain pulmonary “scar” carcinomas (18). Although these observations may indicate that those tumors that elicit the desmoplastic response are inherently more biologically malignant than those that do not, no conclusions can be made concerning the effect of the response itself on invasion and metastasis. Other observers have noted an inverse correlation between the amount of desmoplasia and biological aggressiveness in certain animal tumors. For example, in studies of bile duct guinea pig carcinomas (lines 1 and 10), line 1 was noted to be more desmoplastic but less malignant than line 10 (19, 20). Since these observations may reflect two separate properties which are unrelated, still no conclusions can be made regarding the effect of the desmoplastic response on tumor invasion and metastasis from these sorts of studies.

Before one can meaningfully evaluate the effect which the desmoplastic response exerts on the tumor, one must selectively inhibit the response experimentally. L-3,4-dehydroproline exerted a selective inhibition of myofibroblast collagen synthesis (Table 2) which accounted for both decreased collagen synthesis and total collagen noted within the desmoplastic tumor nodules (Table 1). In the animals treated with L-3,4-dehydroproline, there was a dramatic decrease in numbers of myofibroblasts noted within the response. Since this drug exerted no effects on viability or growth of myofibroblasts in vitro, it is likely that the effects noted in vivo were mediated through the inhibition of collagen synthesis. The deposition of collagen within the desmoplastic response most likely stimulated the additional recruitment of myofibroblasts via some sort of positive feedback mechanism. L-3,4-dehydroproline similarly exerted no effects on viability or growth of BL6 cells in vitro. Therefore any effects of this drug on tumor size (mean diameter) most likely occurred through its inhibition of host collagen synthesis in vivo. Indeed, inhibition of the desmoplastic response in such manner increased mean diameter size (Table 3). Although the mean diameter may have been reflective of other tumor parameters such as cell number and growth rate, the mean diameter of the tumor nodules certainly reflected the degree of local invasion of adjacent host tissues (Fig. 2, bottom). Inhibition of the desmoplastic response also increased the incidence of spontaneous pulmonary metastasis (Table 3) (Fig. 4). These observations argued that the desmoplastic response has some “protective value” to the host. The lack of effect of L-3,4-dehydroproline on tumor invasion and metastasis in hosts which exerted a negligible desmoplastic response (6-week C57 BL/6 mice) (Tables 1, 3) argued against other possible mechanisms accounting for the effect observed in 18-month mice. For example, the increase in mean diameter size and incidence in metastasis noted in 18-month mice with L-3,4-dehydroproline could have been a consequence of a metabolite of that drug which stimulated tumor cell growth independent of the drug’s inhibition of the desmoplastic response. Although it is still possible that a metabolite of L-3,4-dehydroproline could be stimulating tumor cell growth in vivo, this metabolite would have to be produced selectively in 18-month-old mice and this possibility is considered much less likely. The lack of effect of L-3,4-dehydroproline on tumor invasion and metastasis in 6-week C57 BL/6 mice, hosts which exerted a negligible desmoplastic response, further demonstrated that the effects on tumor invasion and metastasis which this drug produced most likely were mediated through its selective inhibition of host collagen synthesis within the desmoplastic response. This study does not address the mechanism by which inhibition of the desmoplastic response enhances tumor invasion and metastasis or conversely the mechanism by which the normal desmoplastic response inhibits tumor invasion and metastasis. Since recent studies had implicated tumor-derived metalloproteinases (Type IV and Type 1 collagenases) in tumor invasion and metastasis, and since the observations in the present study indicated that the desmoplastic response inhibited invasion, the existence of metalloproteinase inhibitors within the desmoplastic response is being investigated. Preliminary studies have revealed that the desmoplastic response in human breast carcinoma contains a high concentration of Type IV and Type 1 collagenase inhibitors (21). The presence of similar inhibitors within the myofibroblast-mediated host collagenous response to BL6 melanoma could be one mechanism by which the response directly inhibits tumor invasion and metastasis. A second more indirect mechanism by which metalloproteinase inhibitors might limit tumor invasion is via inhibition of angiogenesis, a process dependent, in part, on the release of metalloproteinases by migrating endothelial cells (22). The study also does not address the issue of why the desmoplastic response is only partially successful in limiting tumor invasion and metastasis. The mechanisms by which tumor cells can overcome the inhibition of the desmoplastic response and still metastasize are probably the most important unanswered questions of all.
REFERENCES


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