Heterogeneity of Angiogenic Activity in a Human Liposarcoma: a Proposed Mechanism for “No Take” of Human Tumors in Mice

Eike-Gert Achilles, Antonio Fernandez, Elizabeth N. Allred, Oliver Kisker, Taturo Udagawa, Wolf-Dietrich Beecken, Evelyn Flynn, Judah Folkman

Background: Tumor cells are known to be heterogeneous with respect to their metastatic activity, proliferation rate, and activity of several enzymes. However, little is known about the heterogeneity of tumor angiogenic activity. We investigated whether heterogeneity of angiogenic activity could be responsible for the well-known observation of “no take” of human tumors transplanted into immunodeficient mice.

Methods: Severe combined immunodeficient (SCID) mice were xenotransplanted subcutaneously with tumor tissue (n = 55) or cell suspension of a human liposarcoma cell line (SW-872) or subclones (n = 28), with varying cell proliferation rates. Xenograft tumor growth was recorded for up to 6 months. Tumor tissues were then removed and analyzed for tumor cell apoptosis, microvessel density, and cell proliferation. All statistical tests were two-sided.

Results: Pieces of tumor derived from the parental cell line or its clones gave rise to three kinds of tumors: 1) highly angiogenic and fast-growing (aggressive) tumors, 2) weakly angiogenic and slow-growing tumors, and 3) nonangiogenic and stable tumors. Most tumors retained the original phenotype of their parental tumor. Tumor volume correlated positively with microvessel density (Spearman correlation coefficient $r = .89; P = .0001$) and inversely with tumor cell apoptosis (Spearman $r = -.68; P = .002$). Tumor volume was less strongly but positively correlated with tumor cell proliferation in vivo (Spearman $r = .55; P = .02$).

Conclusions: Human liposarcoma cells appear to be heterogeneous in their angiogenic activity. When tumor cells with little or no angiogenic activity are transplanted into SCID mice, a microscopic, dormant tumor results that may not grow further. Because such tiny tumors are neither grossly visible nor palpable, they have previously been called “no take.” The finding that an angiogenic tumor can contain subpopulations of tumor cells with little or no angiogenic activity may provide a novel mechanism for dormant micrometastases, late recurrence, and changes in rate of tumor progression. [J Natl Cancer Inst 2001;93:1075–81]

Tumor growth and metastasis are angiogenesis dependent (1). However, after tumors have switched to the angiogenic phenotype (1,2), it is not clear whether all of the cells in a given tumor are similar with respect to their angiogenic ability. Quantification of microvessel density in histologic sections of breast cancer (3,4) and in other tumors (5,6) reveals “hotspots” of localized angiogenic activity.

Furthermore, we have shown previously that, in tumors arising spontaneously in transgenic mice, where all of the beta cells in the pancreatic islets uniformly express the large T-antigen oncogene (7), the individual islets can be considered as a single tumor, but disseminated throughout the pancreas as subclones. This configuration makes it possible to visualize and to quantify the angiogenic switch. Only 1% of the tumors in that reported study became angiogenic at 6–7 weeks of age, and 4% of the tumors were angiogenic at 13 weeks of age when the mice were dying of tumor burden. Thus, approximately 95%–96% of tumors remained nonangiogenic and did not grow beyond a volume of about 0.6–0.8 mm$^3$. Taken together, these findings suggest that subpopulations of cells within a given tumor may be heterogeneous in their angiogenic activity. On the basis of these results, we asked whether an established human tumor could contain subpopulations of tumor cells that were not angiogenic or had varying degrees of angiogenic activity. If so, this might clarify certain unexplained observations, such as “no take” of human tumors transplanted into immunodeficient mice (8–11).

To address this question, we chose a human liposarcoma (SW-872) cell line that grows reproducibly in severe combined immunodeficient (SCID) mice. Tumor growth was recorded for up to one fourth the life span of the animal. (The life span of SCID mice is approximately 2 years.)

Microvessel density, tumor cell apoptosis, and tumor cell proliferation rates were quantitated on histology sections. We determined whether a single human tumor type contained subpopulations of tumor cells that were heterogeneous in angiogenic activity. We define the variable—angiogenic activity—as the total output of positive and negative regulators of angiogenesis by the tumor cells. We measured the angiogenic activity indirectly by quantifying the microvessel density in the areas of intensive neovascularization (hot spots) and by analysis of apoptosis and proliferation throughout the tumor. Other factors or sources contributing to the angiogenic heterogeneity, such as tumor site (12,13) or genetic background (14), were unchanged in this study.

We attempted to elucidate whether angiogenic activity correlated with tumor growth rate, tumor cell apoptosis, and tumor cell proliferation. We hypothesized that the growth rate (increased tumor vol-

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ume) is controlled mainly by the angiogenic activity because tumor cell apoptosis rate rises in direct proportion to inhibition of angiogenesis (15).

**MATERIALS AND METHODS**

**Cell Growth In Vitro**

The human liposarcoma cell line SW-872 (passage 13) was purchased from the American Type Culture Collection (Manassas, VA). The cells were cultured in Dulbecco’s modified essential medium (DMEM) (JRH Co., Lenexa, KS) supplemented with 5% heat-inactivated fetal calf serum (Intergen, Purchase, NY) in 75-cm² Falcon tissue-culture flasks (Becton Dickinson, Franklin Lakes, NJ) and a humidified atmosphere containing 10% CO₂ at 37 °C. Subclones were established and selected according to high, intermediate, or low proliferation rates in vitro. These subclones were prepared as follows: Monolayers of cells from the parental line were trypsinized, appropriately washed, and suspended at one cell/mL concentration. The cells were then plated (200 μL/well) in 96-well plates (Corning Costar Corp., Cambridge, MA). After the cells became confluent (approximately 1 week), they were transferred to 24-well plates and then to T-75 flasks. After the cells were propagated for 6 weeks in DMEM, six of 11 clones (of either slow, intermediate, or fast proliferation rate in vitro) were randomly chosen for investigations in vivo.

The proliferation rate was determined in vitro by plating 2500 cells/well in 24-well plates. Cell counts in each well were performed by use of a hemacytometer (Hauser Scientific, Horsham, PA) every 24 hours during the following 7-day period.

For xenotransplantation, confluent tumor cells were rinsed in phosphate-buffered saline (Sigma Chemical Co., St. Louis, MO), briefly trypsinized, and suspended in DMEM, and cell suspensions were adjusted to a density of 25 × 10⁶ cells/mL. The cells were centrifuged at 300g for 1 minute at room temperature (20 °C) and finally suspended at a density of 25 × 10⁶ cells/mL in DMEM without serum. From this suspension, 200 μL was immediately injected subcutaneously with a 30-gauge needle into the anterior flank or into the dorsum at the midline of male 6-week-old SCID mice.

**Animal Studies**

In this study, the following terms are used to distinguish different growth rates of tumors. The term “dormant” is used to describe a microscopic tumor that is not expanding, “stable disease” describes a macroscopic tumor that is not visibly expanding, “slowly growing” describes a tumor that is expanding slowly over months, and “fast growing or aggressive” describes a tumor that is expanding over a few weeks. The growth rates (95% confidence intervals) in log₁₀ mm²/day for different tumors were as follows: dormant tumors, -0.029 (-0.033 to -0.026); stable tumors, -0.009 (-0.014 to -0.004); slow-growing tumors, -0.003 (-0.009 to 0.003); and aggressive tumors, 0.028 (0.022–0.035).

The negative values observed for the dormant, stable, and slow-growing tumors were due to the initial decrease in volume following wound edema immediately after transplantation. In initial experiments, 6- to 8-week-old male SCID mice (obtained from Massachusetts General Hospital, Boston, MA) kept in microisolator cages (four mice/cage) were inoculated subcutaneously in their anterior flanks with SW-872 tumor cells (5 × 10⁶ cells in 200 μL injection).

In the next set of experiments, tumor-bearing mice (mean tumor volume, 855 mm³; range, 12–3302 mm³) were killed with methoxyflurane, and the lesions were removed. Tumor tissues were rinsed in DMEM, and macroscopically viable areas were cut with a scalpel into small pieces of similar size in each experiment (mean size, 17 mm²; range, 0.5–66 mm²). The reason for varying the size of the transplanted tumor tissue was to demonstrate or exclude any relation of the resulting tumor phenotype and the tissue size transplanted initially.

To transplant these tumor implants, we made a 5-mm-long horizontal incision in the midline dorsum 1 cm from the base of the tail of SCID mice after they were anesthetized by administering Avertin (40 μg/kg) intraperitoneally. Through the incision, a 1-cm subcutaneous tunnel was made cephalad (toward the head of the animal) by use of fine forceps. The tumor implant was inserted into this tunnel so that it was positioned 1 cm from the skin incision. The wounds were closed with fine sutures (PDS 4.0; Ethicon, Somerville, NJ). All procedures were done under aseptic conditions. In further studies, the parental cell line, or clones derived from cell culture of the parental line, were injected (5 × 10⁶ cells in 200 μL/injection) or transplanted as tissue pieces. Tumors were measured every 3–4 days, and volumes (in millimeters cubed) were calculated by use of the formula (width [mm]² × length [mm] × 0.52). All of the experiments were carried out in accordance with the Animal Research Regulations at Children’s Hospital, Boston, MA. The animals were observed daily, and those bearing big tumors were monitored carefully for any signs of discomfort.

**Histology and Immunohistochemistry**

Mice were killed at intervals during a period of 2–6 months after transplantation. All of the animals underwent a thorough autopsy to search for hidden secondary tumors, which could possibly suppress the subcutaneous tumor. Such a tumor was found in only one animal, thus eliminating the complication of a hidden primary tumor in the other animals (13,16). Representative tumor tissues were harvested and fixed in 10% neutral buffered formalin at 4 °C for 24 hours. All tissues were paraffin embedded. Sections (10 μm thick) were first stained with hematoxylin–eosin to evaluate tissue viability and quality. The microvessel density was determined after immunocytochemical staining by use of the Vectastain avidin–biotin detection system (Vector Laboratories, Inc., Burlingame, CA) with anti-CD 31 monoclonal antibody (dilution: 1:250; Pharmingen, San Diego, CA) according to the manufacturer’s protocol (CD 31 is also known as PECAM [platelet/endothelial cell adhesion molecule] located on the cell surface). With the use of the method of Weidner et al. (3), the regions of highest vessel density (“hot-spot” regions) were scanned at low magnification (×400 to ×1000) and then counted at ×200 magnification (0.738-mm² field) by an observer who was blinded to the code for the tumor source. At least five fields were counted in a representative tumor section, and the highest count was taken as described previously (3).

**Statistical Methods**

Because the measurements of tumor volume, cell apoptosis, microvessel density, and proliferation rate were not normally distributed, we evaluated relationships among them with nonparametric regression methods (17). This evaluation was achieved by employing standard least-squares regression methods on the ranks of the data values (Stata, version 6; Stata Corp., College Station, TX). We also examined some relationships with least-squares regression after log₁₀ transformation of the data. All statistical tests were two-sided.

**RESULTS**

**Animal Studies**

All mice were healthy and gained weight normally throughout the experiments. Xenotransplantations were performed by injections of tissue culture-derived tumor cell suspensions (series 1) (Fig. 1, A) or were performed via tumor tissue transplants (series 2–6) derived from a carrier mouse with a known phenotype after a follow-up of 1–6 months (Fig. 1, B). The results are summarized in Table 1. We numbered the series to clarify separate experiments with different tumor volumes and different tumor phenotypes.

In experimental series 1, we used a total of 28 animals. Six different clones of cell line SW-872 and the parental cell line itself were injected subcutaneously (5 × 10⁶ cells suspended in 200 μL volume/mouse, four animals/group), and the mice were followed 2–6 months after transplantation (Fig. 1, A). Among all of the xenotransplanted animals, the parental cell line and one clone (clone 9) showed a highly aggressive phenotype (Fig. 1, A—group a: tumor growth curves with open squares). The others (Fig. 1, A—group b: tumor growth curves with closed circles) showed either in general a stable (clones 8 and 17) or consistently a stable (clone 1) or a dormant phenotype (clones 4 and 5). Dormant tumors (often of approximately 200 μm in largest diameter)
phenotype with aggressive growth (parental cell line and clone 9), and group b clones 1, 8, and 17 and an aggressive tumor of clone 9. And one of five (clone 17, stable phenotype) showed slow growth.

In series 2, five mice received tissue transplants of an approximate volume of 0.5 mm³ from an aggressive tumor derived from the parental cell line. Three of the five mice had early-onset aggressive tumor growth, and two showed a slowly growing phenotype.

In series 3, five mice received tissue transplants of an approximate volume of 4 mm³ taken from a stable tumor produced by the parental cell line. Only one tumor showed aggressive growth, whereas three tumors remained stable and one showed very slow growth.

In series 4, 10 mice received tissue transplants (approximate volume, 15 mm³) derived from a stable tumor produced by the parental cell line. The majority (seven of 10 animals) of the tumors showed long-term stable or very slow growth, whereas aggressive growth was observed less frequently (three of 10 animals). Only one tumor of series 2–4 underwent spontaneous regression. This means that one tumor in a total of 20 mice of series 2–4 underwent spontaneous regression.

In series 5, each mouse (total, 10 animals) received a tissue transplant of an approximate volume of 62 mm³ taken from an aggressively growing tumor derived from the parental cell line. The majority (eight of 10 animals) of tumors grew aggressively but at slightly different growth rates, whereas only two (two of 10 animals) showed a phenotype with stable growth.

In series 6, pieces (approximate volume, 0.5–14 mm³) of tumors with a known phenotype were taken from the carrier mice. The tumors were derived from the parental cell line as well as from the clones (clones 1, 8, 9, and 17), which produced macroscopically measurable stable or aggressive tumors. The pieces from tumors of each phenotype were retransplanted subcutaneously in five animals for a given phenotype, and tumor growth was followed up to a period of 6 months.

All xenotransplants of tissue pieces from five experiments (series 2–6) by use of a total of 55 animals generally resulted in tumors (34 of 55 animals) (Fig. 1, B), with a phenotype similar to the one from which they were derived (45 of 55 tumors) (Table 1, B). In addition, they retained the same phenotype as those primarily derived from cell suspension. Slowly growing tumors when subsequently transplanted tended to maintain the same stable phenotype as their parental tumor (clones 1, 8, and 17; Fig. 1, B—group b). Similarly, those tumors (parental cell line and clone 9) with an original aggressively growing phenotype generally retained the same phenotype as their parent after further transplantation (Fig. 1, A and B—group a). Several of the long-term stable tumors eventually began to grow near the end of a follow-up period of up to 6 months. The median time (range) in days when tumors were measured was 95 (29–173). The median times (range) when types of tumors were measured were as follows: parental cell line, 61 (50–173); clone 1, 96 (74–154); clone 4, 95 (95–95); clone 5, 117 (102–140); clone 8, 96 (74–133); clone 9, 78 (29–102); and clone 17, 96 (74–133). The median (range) of the tumor volume (in mm³) derived from parental cells and the different clones were as follows: parental, 641 (8–5608); clone 1, 7 (1–31); clone 4, 1 (1–1); clone 5, 1 (1–948); clone 8, 1 (1–1315); clone 9, 2812 (895–5680); and clone 17, 22 (1–205).

In mice bearing small or microscopic tumors, no lung or other organ metastases could be detected macroscopically. In some animals with highly aggressive lesions, a few (<10) small lung metastases could be observed.

**Histology and Immunohistochemistry**

Of a total of 83 tumors in the different experiments, 18 specimens (with stable
Table 1. Phenotypic results of 83 subcutaneous xenotransplantations of the parental liposarcoma cell line SW-872 and derived clones in severe combined immunedeficient mice performed in six different experimental series

<table>
<thead>
<tr>
<th>Experimental series*</th>
<th>Original cell type (phenotype)†</th>
<th>No. of mice with tumor/total No.‡</th>
<th>No. of tumors with similar phenotype/total No. (%)§</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Parental (aggressive)</td>
<td>4/4</td>
<td>4/4 (100)</td>
</tr>
<tr>
<td>1</td>
<td>Clone 1 (stable)</td>
<td>4/4</td>
<td>4/4 (100)</td>
</tr>
<tr>
<td>1</td>
<td>Clone 4 (dormant)</td>
<td>4/4</td>
<td>4/4 (100)</td>
</tr>
<tr>
<td>1</td>
<td>Clone 5 (dormant)</td>
<td>4/4</td>
<td>3/4 (75)</td>
</tr>
<tr>
<td>1</td>
<td>Clone 8 (stable)</td>
<td>4/4</td>
<td>3/4 (75)</td>
</tr>
<tr>
<td>1</td>
<td>Clone 9 (aggressive)</td>
<td>4/4</td>
<td>4/4 (100)</td>
</tr>
<tr>
<td>1</td>
<td>Clone 17 (stable)</td>
<td>4/4</td>
<td>3/4 (75)</td>
</tr>
<tr>
<td>Total aggressive</td>
<td></td>
<td>8/8</td>
<td>8/8 (100)</td>
</tr>
<tr>
<td>Total stable or dormant</td>
<td></td>
<td>20/20</td>
<td>17/20 (85)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>28/28</td>
<td>25/28 (89)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experimental series*</th>
<th>Original cell type (phenotype)†</th>
<th>No. of mice with tumor/total No.‡</th>
<th>No. of tumors with similar phenotype/total No. (%)§</th>
</tr>
</thead>
<tbody>
<tr>
<td>2, 5, 6</td>
<td>Parental (aggressive)</td>
<td>20/20</td>
<td>16/20 (80)</td>
</tr>
<tr>
<td>3, 4</td>
<td>Parental (stable)</td>
<td>14/15</td>
<td>10/15 (66)</td>
</tr>
<tr>
<td>6</td>
<td>Clone 1 (stable)</td>
<td>5/5</td>
<td>5/5 (100)</td>
</tr>
<tr>
<td>6</td>
<td>Clone 8 (stable)</td>
<td>5/5</td>
<td>5/5 (100)</td>
</tr>
<tr>
<td>6</td>
<td>Clone 9 (aggressive)</td>
<td>5/5</td>
<td>5/5 (100)</td>
</tr>
<tr>
<td>6</td>
<td>Clone 17 (stable)</td>
<td>5/5</td>
<td>4/5 (80)</td>
</tr>
<tr>
<td>Total aggressive</td>
<td></td>
<td>25/25</td>
<td>21/25 (84)</td>
</tr>
<tr>
<td>Total stable</td>
<td></td>
<td>29/30</td>
<td>24/30 (80)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>54/55</td>
<td>45/55 (81)</td>
</tr>
</tbody>
</table>

*Experimental series as described in the “Results” section.
†Types with different tumor growth patterns (see “Results” section for definition).
‡Number of mice developing microscopic or macroscopic tumors of the total number of mice used in that experiment.
§Number of mice with tumor phenotype similar to that displayed by tumor cells/tissues that they were derived from, of the total number of mice in that experiment. Percentages are shown in the parentheses.

[n = 3], slowly progressive [n = 6], and aggressive [n = 9] phenotypes) suitable for immunocytochemical investigation were selected randomly. Statistical analysis was conducted by comparing the tumor group whose tissues were studied by immunohistochemistry with the other group whose tissues were not studied by immunohistochemistry. The median values (and interquartile range, i.e., the 25th to 75th percentile values) of the post-transplantation day when the tumor tissue was suitable for analysis and the tumor volumes on that same day in both groups showed no statistically significant differences (median value of 95 days for mice whose tumors were not studied by immunohistochemistry versus 102 days for mice whose tumors were studied by immunohistochemistry; P = .35; Wilcoxon rank sum test). Although, the median tumor volume was slightly higher in those tumors selected for immunohistochemical analysis, this difference did not reach statistical significance (the median tumor volume [range] in mm³ for those selected for immunohistochemical analysis was 229 [35–870] versus 41 [0–2153] for those not selected; P = .23; Wilcoxon rank sum test).

Seventeen of the remaining 65 tumors were used for retransplantation. Nineteen of the dormant or stable tumors could not be fully investigated by immunocytochemistry because of the limited amount of the tissue. All tumors showed variable areas of viable neoplastic tissue consistent with a pleomorphic or undifferentiated sarcoma in slides stained by hematoxylin–eosin. Statistical analysis revealed that the tumor volume was positively associated with microvessel density and inversely associated with tumor cell apoptosis (Spearman correlation coefficient |r| = .89 [P<.001] and r = –.68 [P = .002], respectively). Therefore, the microvessel density was highest in the largest tumors with an aggressive phenotype in vivo (Fig. 2, A), whereas the apoptotic rate was highest in the smallest tumors with a slowly progressive or stable phenotype in vivo (Fig. 2, B). The proliferation rate of tumor cells was less strongly, but positively, correlated with tumor volume (Spearman r = .55; P = .02) (Fig. 2, C). Representative specimens stained for the different antigens are shown in Fig. 3.

The microvessel density in aggressive tumors was statistically significantly higher than in the stable or slowly progressive tumors. The medians (ranges) were 245 (161–414) and 49 (14–176) (P = .0005, respectively).

The median of tumor cell proliferation (percent of proliferating cells/high-power field) in aggressive tumors was 48 (range, 29–66), which is slightly higher than in the stable or slowly progressive tumors (median, 35; range, 18–47) (P = .02). The median of tumor cell apoptosis (percent apoptotic cells/high-power field) was lower in aggressive tumors (median, 0.69; range, 0.57–0.95) compared with 1.65 (range, 0.56–3.69) in stable or slowly progressive tumors (P = .007).

**DISCUSSION**

The experiments described in this report show that a single type of human tumor contains subpopulations of tumor cells with different angiogenic activities. Tumor volume appears to correlate directly with microvessel density. Because of compelling evidence that tumor growth is angiogenesis dependent (6), it is likely that the intensity of angiogenic activity in each subpopulation is rate limiting for tumor growth.

These results also show that tumor cell apoptosis is inversely proportional to angiogenic activity. This suggests that the angiogenic regulation of tumor growth operates mainly by its effect on tumor cell apoptosis but to a lesser extent on tumor cell proliferation. The observation that the microvessel density is inversely proportional to tumor cell apoptosis but generally independent or, to a lesser extent, propor-
The essential role of angiogenesis as the early-onset event in neoplastic progression has also been demonstrated in human preneoplastic breast lesions that showed a range of high and low angiogenic activities in the rabbit cornea, despite the fact that there was no difference in their morphology (28).

There are several clinical implications of this work. Our studies provide a possible model for the clinical observation that some tumors, such as breast cancer, may recur as metastases of the original tumor, as late as 10–20 years after removal of the primary tumor. The long-term dormant or stable tumor implants in our study that were nonangiogenic and that generally did not expand their tumor volume during at least 140 days would be equivalent to human tumors that had remained dormant for up to 13.4 years (based on the equivalence of 1 mouse-day to 35 human days).

Our findings also propose a mechanism for the common observation that certain kinds of human tumors transplanted into immunodeficient mice have a poor rate of “take” (8–11). This has been ascribed until now to immune differences, to the transplantation of necrotic pieces, or to the observation that human tumors may be heavily infiltrated by fibroblast and collagen deposits. However, we show that tumors that may have formerly been labeled as “no take” in fact are viable, dormant, or stable and of small size due to lack of angiogenic activity. Furthermore, our study shows that a subpopulation of these dormant or stable tiny tumors eventually become angiogenic and grow rapidly into large visible tumors. The fact that this angiogenic switch can take as long as 40–160 days may explain why tumors that were previously “no take” were thought to be dead.

The importance of understanding and evaluating angiogenesis in the early phase of tumor growth is underscored by numerous reports of the high incidence of occult dormant microscopic tumors in the breast (29), the thyroid (30), and the prostate (31). The lack of suitable models to fully understand the problem of tumor dormancy has recently been emphasized (32).
Our results may also explain why, in some patients with retroperitoneal liposarcoma, the tumor remains stable or "indolent" for decades while, in other patients, the same tumor type follows a highly aggressive course over a single year (33).

REFERENCES


NOTES

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