c-Myc Suppresses the Tumorigenicity of Lung Cancer Cells and Down-Regulates Vascular Endothelial Growth Factor Expression

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ABSTRACT

The c-myc oncogene is frequently amplified in cells grown from lung tumors and has been linked to the malignancy of these cancers. In support of this, c-myc transfection enhances the in vivo proliferation and soft agar cloning of human small cell lung cancer (SCLC) cells. In this study, we surprisingly found that c-myc expression suppressed the formation of tumors by SCLC cells in athymic nude mice, c-myc expression down-regulated the protein and transcript for vascular endothelial growth factor (VEGF) in these SCLC cells, as well as VEGF transcript in rat fibroblasts manipulated for c-myc expression and in liver cells of c-myc-transgenic mice. Finally, bivariate and multivariate analyses demonstrated that the probability of tumor formation from lung cancer cell lines was negatively correlated with the relative expression of c-Myc, positively correlated with the relative expression of VEGF, and that the latent time to tumor formation was increased by the expression of c-Myc and decreased by the expression of VEGF. We hypothesize that, for lung cancer cells, c-Myc suppresses the formation of tumors in vivo by down-regulating VEGF, and that the amplification of c-myc seen in cells grown from lung tumors with a poor prognosis is an artifact of selection for growth in vitro.

INTRODUCTION

Lung cancer is the most common cause of tumor death in the United States. Both SCLC and NSCLC cell lines and tumors are frequently amplified for the c-myc gene (1–3). Because the dysregulated expression of c-myc contributes to the tumorigenicity of a variety of cell types (including lymphoma, cervical carcinoma, breast cancer, and colon cancer; Ref. 4), there has been much interest in analyzing its contribution to the prognosis of lung cancer. Johnson and colleagues (1) correlated c-myc amplification with prognosis for SCLC tumors. In their most recent analysis, they found c-myc amplification in 7 of 67 SCLC specimens from patients treated with chemotherapeutic agents and no c-myc amplification in specimens from 40 untreated patients (1). However, they had a matching tumor specimen for only one of these c-myc-amplified cell lines, and in this case, the c-myc amplification was not seen in the tumor. When tumors were examined, c-myc amplification was much less common and was seen in only 1 of 142 tumor foci of SCLC from 47 patients (3). Furthermore, the overexpression of c-Myc protein in tumor specimens did not correlate with the prognosis for SCLC (5) or NSCLC (5, 6).

To more directly examine the effect of c-myc overexpression on lung cancer tumorigenicity, we studied the consequences of c-myc transfection into a SCLC cell line that was not amplified for this oncogene, NCI H209 (H209 cells). We and others discovered previously that c-myc expression enhanced the cell proliferation and soft agar cloning of this cell line (7, 8). However, in the current studies, we were surprised to find that c-myc suppressed the ability of these cells to form tumors in athymic nude mice, and the few tumors that did form were poorly vascularized. We observed that c-myc expression in the H209 cells was associated with a decreased expression of the VEGF protein and transcript. That this relationship is of general biological importance is supported by the discovery of a similar inverse association between c-Myc and VEGF in unrelated cell systems, including fibroblast lines manipulated for c-myc gene expression, and in the livers of c-myc-transgenic mice. We therefore evaluated the relationships between c-Myc and VEGF protein expression and the tumorigenic outcome of six lung cancer cell lines, five of which were amplified for c-myc. Using bivariate and multivariate analyses to relate these factors to tumor outcomes in 81 xenotransplanted mice, we found that the probability of tumor formation was negatively correlated with the relative expression of c-Myc and positively correlated with the relative expression of VEGF, and that the latent time to tumor formation was increased by the expression of c-Myc and decreased by the expression of VEGF.

We suggest that the overexpression of c-myc is a negative factor for lung cancer tumorigenicity secondary to its down-regulation of VEGF protein expression. We hypothesize that the amplification of c-myc seen in cell lines derived from the tumors of patients with the worse prognosis is an artifact of selection for growth in tissue culture and does not reflect the status of the lung tumors.

MATERIALS AND METHODS

Cell Culture. For cells used for these studies, see Table 1. All lung cancer cells were grown in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) with either 9.5% bovine calf serum (for H209, 209 myc; Gemini Bio-Products, Calabasas, CA) or 9.5% fetal bovine serum (for all other cell lines, Sigma, St. Louis, MO) with 100 units/ml penicillin and 100 μg/ml streptomycin in 37°C incubators containing 5% CO₂. The medium for the 209 myc cells also contained 0.4 mg/ml G418 (Life Technologies, Inc., Grand Island, NY). Fibroblasts were grown in low glucose (1 g/l DMEM; Life Technologies, Inc.).

Cell lines were confirmed to be free of Mycoplasma infection (performed by the Johns Hopkins University Cell Biology Core Facility using the Gen-Probe Mycoplasma Rapid Detection System; Fisher, Pittsburgh, PA).

Soft Agar Cloning. Cells (1 × 10⁴) were triturated to single cells and then suspended in 150 μl of 0.4% agar in complete medium containing phenol red-free RPMI, layered above 150 μl of 0.8% agar, and overlaid with 150 μl in 24-well plates. Each experimental point was plated in three wells. Plates were grown in a 37°C incubator for 2–3 weeks (adequate for at least five cell doublings for each cell line). All of these cell lines cloned in soft agar, but with varying efficiency. Furthermore, the size of these colonies differed between cell lines, which may reflect differences in intrinsic cell doubling time or the propensity of each cell line for contact inhibition of growth. We therefore reasoned that the total cell number in each well at the end of a soft agar cloning experiment might be a better correlate of tumorigenicity than the colony count. We measured the viable cells in each well using a vital dye, alamarBlue (Accumed International, Westlake, OH; Ref. 13). The equivalent loading of viable cells at the start of each study was verified for each cell line by counting parallel wells using alamarBlue and measuring the 590-nm emission with 530 nm excitation after 18 h incubation on a CytoFluor 2300 system plate reader (Millipore, Marlborough, MA). The number of viable cells in each well at the
conclusion of the study was similarly assessed with alamarBlue dye. For each cell line, the alamarBlue emission in each well was normalized by dividing by the average emission of the H460 cells (a cell line that is highly efficient at soft agar cloning) for that experiment to give the relative soft agar cloning ability for each cell line for each experiment. The soft agar cloning measured in this way was found to be highly statistically correlated with soft agar cloning measured by colony counting (coefficient of correlation, 0.82).

**Growth Curves.** Growth measurements were done with the vital dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide using the MTT assay system (Sigma) exactly as described previously (7).

**Nude Mouse Tumorigenicity Studies.** Cells (4 × 10⁵) in log phase growth were washed and suspended in 100 µl sterile PBS on ice for s.c. injection into the left flank of 4–6-week-old unanesthetized male athymic nude mice (National Cancer Institute, Frederick, MD; Refs. 2 and 14). The total time from the start of cell preparation to injection never exceeded 40 min. Animals were maintained in a pathogen-free environment, and the size of tumors was measured every 4–6 days. The tumorigenicity of each cell line was examined over two to three separate experiments comprising a total of 12–21 animals/cell line. Each experiment ran for 6–12 weeks. The tumor volume was calculated as \( \frac{1}{2} \times a \times b \times 0.4 \), where \( a \) = short axis and \( b \) = long axis. The tumor volume doubling time was calculated by first plotting the log(tumor volume in mm³) by the time for each individual tumor. Then the range of the rapid phase of tumor growth was visually assessed from the graph of each individual tumor, and the doubling time of each tumor was calculated as the log⁡2 divided by the slope of the log⁡(volume):time over this range. The doubling times for tumors formed by each cell line are reported as the mean of all tumors ≥ SE. Finally, the latency time between inoculation and the first notation of tumor nodules was determined and is reported as the mean for all tumors formed by each cell line ≥ SE. In instances when tumors were first discovered at postmortem examination, the latency was recorded as the time to euthanasia. Animals were euthanized by CO₂ asphyxiation either at the end of the experiment or when the long axis of the tumor exceeded 2 cm. All animals were developed,

<table>
<thead>
<tr>
<th>Cell line Source*</th>
<th>c-myc status</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCI H209 (H209)</td>
<td>Human SCLC</td>
<td>Normal</td>
</tr>
<tr>
<td>209 myc</td>
<td>H209</td>
<td>c-myc transfected</td>
</tr>
<tr>
<td>NCI H60 (H60)</td>
<td>Human SCLC</td>
<td>Amplified</td>
</tr>
<tr>
<td>NCI H460 (H460)</td>
<td>Human SCLC/adenoCA</td>
<td>Amplified</td>
</tr>
<tr>
<td>NCI H157</td>
<td>Human SCLC</td>
<td>Amplified</td>
</tr>
<tr>
<td>NCI H82 (H82)</td>
<td>Human SCLC</td>
<td>Amplified</td>
</tr>
<tr>
<td>NCI H417 (H417)</td>
<td>Human SCLC</td>
<td>Amplified</td>
</tr>
<tr>
<td>Rat1a</td>
<td>Rat fibroblasts</td>
<td>Normal</td>
</tr>
<tr>
<td>RMS</td>
<td>Rat fibroblasts</td>
<td>c-myc transgenic</td>
</tr>
<tr>
<td>TGR</td>
<td>Rat fibroblasts</td>
<td>Two c-myc genes</td>
</tr>
<tr>
<td>HET15</td>
<td>TGR</td>
<td>One c-myc gene</td>
</tr>
<tr>
<td>H105</td>
<td>TGR</td>
<td>Null for c-myc gene</td>
</tr>
<tr>
<td>Transgenic livers</td>
<td>Mice</td>
<td>c-myc transgenic</td>
</tr>
</tbody>
</table>

* LCC, large cell lung cancer; adenoCA, pulmonary adenocarcinoma; SqCC, squamous cell lung cancer.

**Analysis of Transcript Expression.** Total RNA was isolated by the Trizol method (Life Technologies, Inc.), and 20 µg were separated by gel electrophoresis on 1.5% agarose-formaldehyde gels, transferred to nylon membranes (Zeta-Probe; Bio-Rad, Hercules, CA), and hybridized as reported previously (7). Probes were prepared as inserts of VEGF and human glyceraldehyde-3-phosphate dehydrogenase (American Type Culture Collection, Rockville, MD) and were labeled by random priming with [α-³²P]dATP using the Prime-It Random Primer Labeling kit (Stratagene) to a specific activity of ~10⁹ cpm/µg DNA (16). These blots were sequentially stripped and reprobed according to the manufacturer’s instructions (Bio-Rad), and images were quantitated with a PhosphorImager and ImageQuant analyses (Molecular Dynamics).

**Confirmation of Retained Exogenous c-myc Expression in Tumors.** The expression of exogenous c-myc was examined by PCR analysis of DNA from the tumors formed by 209 myc cells. Genomic DNA was isolated from material either freshly excised or flash frozen in liquid nitrogen in OCT (Sakura Finetek, Torrance, CA); digested in SDS-protease K overnight at 50°C, extracted in phenol:chloroform, and washed in 70% ethanol. Primers spanned from the 5’ region of the neomycin resistance gene (Escherichia coli transposon 5; Ref. 17) to the 3’ end of c-myc exon 3 (18): upstream primer, 5’-GGGAACCTTGTTGCGTAAGGA-3’; and downstream primer, 5’-GGTGTGCTCACTACGGCC-3’. After a hot start, 35 cycles of PCR were done with denaturation at 94°C for 30 s, annealing at 50°C for 2 min, extension at 72°C for 2 min, and a final elongation step at 72°C for 6 min. These conditions yielded specific priming of a 2-kb fragment only in the c-myc-transfected 209 myc cells and not in the parental H209 or the H60 cells. The identity of this product was confirmed by sequencing (Johns Hopkins Genetics Core facility).

**Statistical Analysis.** The variables were expressed as proportions or means, as appropriate. Three different outcome measures of tumorigenicity were developed, i.e., appearance of a tumor at the injection site at any time during the study or at a postmortem examination, latency time from inoculation to first appearance of tumor, and volume doubling time of tumor growth. For bivariate analysis of tumor formation, mean values of predictor variables were compared between tumor formers and nonformers, with statistical significance determined using the t test. Bivariate analysis of factors relating to tumor latency was conducted using simple linear regression, and a similar approach was used to examine the relationship of factors to tumor doubling time. Statistical significance is reported for Ps of <0.05. Factors that were statistically significant in bivariate analysis, or that were considered by the investigators to be scientifically important, were used to develop multivariate models. Logistic regression was used for multivariate analysis of tumor formation, and linear regression was used for tumor latency and tumor volume doubling time. 

**RESULTS**

**c-myc Transfection Suppresses Tumorigenicity in Athymic Nude Mice.** The NCI H209 (H209) cells grow slowly in medium, exhibit poor cloning efficiency, and express little to no detectable c-Myc protein (8). The transfection and increased expression of c-myc in H209 SCLC cells produced cells (209 myc cells) with one-half of the doubling time and three-quarters of the soft agar cloning ability of the H209 parental cells (7, 9, 19). Tumorigenicity was assessed by s.c. inoculation of each of these cells into athymic nude mice. Tumorigenic outcome was measured in three ways: (a) the appearance of a tumor at the injection site at any time during the study or at postmort-
H209 cells highly express VEGF protein and transcript. c-myc (data not shown). If c-myc was retained in two and absent by PCR analysis in one (data not shown). The tumors formed by the 209 myc cells exhibited less vascularity than those formed by the H209 cells (Fig. 1). This observation suggests that myc may alter the expression of VEGF, an angiogenic factor secreted by tumor cells (20–22). Hence, we examined the effect that c-myc transfection has on VEGF expression in the H209 cells. H209 cells highly express VEGF protein and transcript. c-myc transfection was associated with a 4-fold reduction in VEGF protein (Fig. 2, a and b) and transcript (Fig. 2, c and d). Because VEGF expression is necessary for tumor establishment, we postulate that the decrease in VEGF expression in the 209 myc cells contributes to their reduced tumorigenicity.

**c-Myc Down-Regulates VEGF Transcript.** We examined VEGF expression in other cell systems in which the expression of c-myc was manipulated. We found that the transfection of c-myc into the Rat1a fibroblast cell line (creating RM8 cells) was associated with a 10-fold reduction in VEGF transcript expression (Fig. 3a). Fibroblast cell lines homozygous for c-myc deletion (H015 cells) show twice the VEGF protein expressed by the heterozygotes (HET15 cells) or the normal controls (TGR cells; Fig. 3b). In addition, hepatocytes isolated from mice transgenic for c-myc expression demonstrate one-half of the VEGF transcript expression of those from normal controls (Fig. 3c). Thus, the inverse relationship between c-myc and VEGF transcript expression is of widespread biological importance.

**Inverse Correlation between c-myc and VEGF Expression for Pulmonary Tumorigenesis in Vivo.** c-myc amplification in cell lines established from lung cancer tumors has been associated with a worsened prognosis (1). In contrast, we observed here that c-myc transfection suppressed the tumorigenicity of a SCLC cell line, and furthermore, that c-myc expression was associated with a decrease in VEGF. Thus, our findings would lead to the prediction that c-myc amplification would have the opposite effect on prognosis. To investigate this discrepancy, we studied the in vitro and in vivo characteristics of a series of c-myc-amplified lung cancer cell lines. We statis-

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Relative c-Myc protein</th>
<th>Relative VEGF protein</th>
<th>In vitro DT&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Relative soft agar cloning</th>
<th>Relative ODC protein</th>
<th>Predictors</th>
<th>Outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(days)</td>
<td></td>
<td></td>
<td>No. of animals receiving injections</td>
<td>No. of tumors formed</td>
</tr>
<tr>
<td>H209</td>
<td>0</td>
<td>3.96</td>
<td>5.32 ± 1.29</td>
<td>0.16 ± 0.05</td>
<td>1</td>
<td>21</td>
<td>19</td>
</tr>
<tr>
<td>209 myc</td>
<td>1</td>
<td>1</td>
<td>2.90 ± 0.55</td>
<td>0.62 ± 0.08</td>
<td>1.81</td>
<td>21</td>
<td>4</td>
</tr>
<tr>
<td>H60</td>
<td>2.25 ± 0.84</td>
<td>2.44</td>
<td>4.24 ± 0.37</td>
<td>0.34 ± 0.18</td>
<td>0.96</td>
<td>12</td>
<td>5</td>
</tr>
<tr>
<td>H82</td>
<td>2.69 ± 1.40</td>
<td>3.2</td>
<td>2.56 ± 0.28</td>
<td>0.49 ± 0.15</td>
<td>4.09</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td>H460</td>
<td>2.51 ± 0.81</td>
<td>2.68</td>
<td>1.45 ± 0.10</td>
<td>1</td>
<td>1.38</td>
<td>12</td>
<td>9</td>
</tr>
<tr>
<td>H417</td>
<td>3.76 ± 0.98</td>
<td>8.02</td>
<td>2.60 ± 0.38</td>
<td>0.64 ± 0.07</td>
<td>0.70</td>
<td>12</td>
<td>9</td>
</tr>
<tr>
<td>H157</td>
<td>0.59 ± 0.32</td>
<td>2.3</td>
<td>1.93 ± 0.47</td>
<td>0.93 ± 0.01</td>
<td>0.16</td>
<td>12</td>
<td>8</td>
</tr>
</tbody>
</table>

<sup>a</sup> DT, doubling time.
bivariate analysis. Neither the in vitro P (50%; in VEGF protein increased the odds of tumor formation by probability of tumor formation (multivariate analysis, they both were significant predictors of tumor levels of c-Myc and VEGF protein were then incorporated into a agar cloning was close to significant by bivariate analyses. When the P exhibited a trend to positive predictability, with a P the relative level of c-Myc was a significant negative predictor with a P the other biological reason to support their importance were then examined by they were significant by bivariate analysis or because there was correlation with the other parameters measured for these cell lines (data not shown).

We next examined the relationships among the four possible predictors and the three possible outcomes by bivariate analysis of the 81 xenotransplantations. Predictors that looked promising either because they were significant by bivariate analysis or because there was biological reason to support their importance were then examined by multivariate analysis (Table 3).

For the outcome of tumor formation, bivariate analysis showed that the relative level of c-Myc was a significant negative predictor with a P of 0.05 (Table 3). In contrast, the relative level of VEGF expression exhibited a trend to positive predictability, with a P of 0.13 by bivariate analysis. Neither the in vitro cell doubling time nor the soft agar cloning was close to significant by bivariate analyses. When the levels of c-Myc and VEGF protein were then incorporated into a multivariate analysis, they both were significant predictors of tumor formation; a unit increase of c-Myc protein approximately halved the probability of tumor formation (P = 0.005), whereas this same change in VEGF protein increased the odds of tumor formation by ~50% (P = 0.008; Table 3).

For the outcome of tumor latency, bivariate analyses of the 51 animals that formed tumors again showed the relative expression of c-Myc to be a negative predictor of tumorigenicity and correlate with an increase latency (coefficient of 4.43; P = 0.05; Table 3). Again, the relative expression of VEGF had the opposite effect, with a trend to a decreased latency (coefficient of −2.32; P = 0.17). Multivariate analysis of c-Myc and VEGF showed both to be significant predictors of latency, and the coefficient for c-Myc was 7.31 (P = 0.004), whereas for VEGF, the coefficient was −4.68 (P = 0.01; Table 3).

For the outcome of tumor volume doubling time, bivariate analyses of the 51 animals that formed tumors suggested that the relative expression of c-Myc protein was inversely related to doubling time of the tumor, but not by much (coefficient of −1.08; P = 0.005; Table 3). Furthermore, the doubling time of the cells in medium was directly correlated with the volume doubling time of the tumors in vivo (coefficient, 0.774; P = 0.034). Finally, soft agar cloning efficiency inversely associated with tumor doubling time by bivariate analysis (coefficient, −3.04; P = 0.08), as did VEGF (coefficient, −0.436; P = 0.143), although neither significantly (Table 3). Multivariate analyses differed from these bivariate findings in that none of these factors proved to be significant predictors of tumor volume doubling time (Table 3).

Finally, we wondered whether the negative impact of c-Myc protein on tumorigenicity for these lung cancer cells might be attributable to
a dissociation of c-Myc protein and c-Myc action. We therefore measured the expression of a known transactivation target of c-Myc, ODC transcript (25) and correlated this with the tumor outcomes as above. ODC tended to correlate with c-Myc expression for these six cell lines. However, bivariate analyses of ODC and the three tumor outcomes gave significant results only for tumor latency, where ODC had a coefficient of 5.84 and a \( P = 0.004 \). Bivariate analyses did not show ODC as a significant predictor of either tumor formation or tumor volume doubling time.

**DISCUSSION**

In these studies, we made a series of observations about the tumorigenic outcome of c-myc expression that differed from the current paradigm. We first found that the transfection of c-myc into a SCLC cell line suppressed tumor formation, despite its action to enhance in vitro cell growth and soft agar cloning. Because the few small tumors that formed were not as highly vascularized as the tumors formed by the parent cell lines, we examined the expression of VEGF. We discovered that c-myc decreased the expression of both VEGF protein and transcript in this SCLC cell line. Because VEGF has been shown previously as essential for the formation of other tumors, this implied that the reduction in this factor might be contributing to the suppressed tumorigenicity of the c-myc-expressing SCLC cells. We found that this inverse relationship between c-myc and VEGF transcript was recurrent across diverse cell systems manipulated for c-myc expression. The amplification of c-myc in cell lines established from lung cancers has been correlated previously with a worsened prognosis. To further examine this apparent contradiction between our observations and these previous findings, we examined the relationship between c-Myc and VEGF protein expression and the formation of tumors by cell lines amplified for c-myc. We found that the relative expression of c-Myc was a significant negative predictor of tumor take and tumor latency, and that the expression of VEGF was a significant positive predictor of these outcomes, whereas the expression of other in vitro
factors did not contribute to tumorigenic outcomes when assessed by multivariate analyses.

How can we reconcile the suppressive effect of c-myc amplification on tumor formation and tumor latency in nude mouse xenotransplants with its negative impact on patient survival? One possibility is that the c-myc amplification assessed by other lung cancer researchers may have been an artifact of the cell culture technique rather than a prominent characteristic of the primary tumor. Such a phenomenon has been observed for the expression of c-myc in a variety of tumors passaged in cell culture (26) and for a breast carcinoma tumor passaged in athymic nude mice (27). Consistent with this concern, Yokota et al. (28) detected c-myc amplification in 3 of 12 squamous cell carcinomas but in only two of 17 SCLCs examined, both of which were only in the cell lines but not in the original tumors, and Brennan et al. (1) similarly found c-myc amplification in a cell line but not in its original tumor. The c-myc-amplified cells may have been selected for in culture because of the positive in vitro proliferative effects of c-myc expression. The effect of c-myc transfection to decrease the doubling time of the H209 SCLC cell line supports this hypothesis. However, because the level of C-Myc expression did not significantly correlate with the doubling time of the five c-myc-amplified cell lines, we cannot rule out that some other effect of c-myc expression may offer a selective advantage to the growth of cells in culture.

The observations of others imply that c-Myc expression may be important for processes occurring after the establishment of the tumor. For example, analysis of lung cancer tumors has shown that the dysregulated expression of c-myc may be seen in metastases more often than the primary tumor (3, 29). In addition, c-myc amplification has been associated with the invasion of breast carcinoma (30). Furthermore, c-myc amplification was found to only occur in cell lines derived from patients treated previously with chemotherapeutic agents (generally in association with a cyclophosphamide-based regimen; Ref. 1), and c-myc overexpression has been related to the enhanced resistance of SCLC to chemotherapy (31, 32). All of these outcomes would be expected to alter patient prognosis but were not examined in our investigation. c-myc amplification may also be a surrogate marker of genomic instability and hence may correlate with clinical outcome but not with the tumorigenicity of the resultant cell lines.

The lack of effect of c-Myc on tumor volume doubling time when assessed by multivariate analysis may be a result of competing actions of c-Myc on a cell population: (a) c-Myc enhances cell proliferation (33); (b) it increases apoptosis in nutrient deprivation conditions (34, 35); and (c) it alters the expression of enzymes in the glycolytic pathway to favor growth in oxygen-deprived, but not glucose-deprived, conditions (36). Thus, the result of dysregulated c-myc expression on the tumor cell population doubling time will be a function of the relationship between the kinetics of these actions. These actions explain the observations of Rygaard et al. (37), who correlated the expression of c-myc mRNA with the proliferative index measured by flow cytometry but not with the population doubling time of lung cancer cell lines, and of Bepler et al. (38). As an illustration of the competition between tumor cell proliferative capacity and tumor cell death, all of the tumors of >4 mm³ in our study demonstrated a peripheral zone of viable tumor cells interspersed with connective tissue stroma containing blood vessels and a central necrotic region. The kinetics of tumor growth may ultimately relate to the rapidity of cell growth compared with the nutrient supply and the susceptibility of each cell type to death in nutrient deprivation conditions. Thus, the H460 cell line, which does not decrease in cell number despite nutrient deprivation, has a tumorigenic advantage over the 209 myc cells, which rapidly decline in cell number after plateauing (Fig. 4c). Furthermore, the longer doubling time of the H209 cells would allow adequate VEGF-stimulated vascularization prior to the cell population overgrowing available nutrients and their ensuing cell death by apoptosis or necrosis.

We need to reconcile the apparent differences in regard to c-Myc action on tumorigenicity of lung cancer cells and its effect on the tumorigenicity of other types of cells. For example, although c-myc transfection of the Rat1a fibroblasts depressed VEGF expression (Fig. 3), it increased the growth of tumors formed by transplantation into athymic nude mice. Similarly, c-myc-transgenic mice develop liver tumors only in mice older than 1 year of age (12), in contrast to the appearance of tumors of the mammary epithelium and the lymphocytes in much younger mice (39–43). These differences between cell types may reflect specific characteristics of each cell type that complement tumorigenicity. For example, if the enhancement of cell proliferation induced by c-Myc is checked by a down-regulation of VEGF, some cells may express other factors that can substitute for the angiogenic effect of VEGF, such as fibroblast growth factor (44), or cell types may vary in their propensity to undergo DNA damage and achieve the second “hit” necessary to establish tumors. In addition, it is possible that the decrease in VEGF must reach a threshold prior to affecting tumor growth. Indeed, this threshold may not be absolute but may relate to other tumor growth kinetic characteristics as discussed previously.

c-Myc Decreases VEGF Expression. We hypothesize that tumor take and tumor latency time reflect the process of tumor initiation, whereas the tumor volume doubling time is a measure of tumor maintenance. Therefore, the differential effect of c-Myc on these different outcomes is consistent with its action to down-regulate VEGF. This latter molecule has been shown previously to be important for tumor initiation but not for tumor maintenance (21).

Why does c-Myc down-regulate VEGF? As discussed, the cell proliferation promoted by c-Myc expression is limited by the increased apoptosis of Myc-overexpressing cells when nutrient deprived. The suppression of VEGF expression, resulting directly or indirectly from c-Myc expression, would contribute to this process by bridling nutrient supply. These barriers would theoretically allow limited cell division to replace what was there previously but prohibit excess proliferation, because this would require increasing the nutrient supply beyond that which is already present. This would serve for tissue modeling and would also function as an additional barrier against tumor formation every time Myc expression is deregulated in a cell. This biology has functional consequences, because VEGF expression is a significant independent prognostic factor for the survival of patients with SCLC (6, 45) and colon cancer (46).

REFERENCES


B. Lewis, personal communication.
myc

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Cancer Res 2000;60:143-149.

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