Modulation of clonal progression in B16F1 melanoma cells

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(Received 31 July 1990; accepted 5 November 1990)

We have examined the effects of the microenvironment on the frequency and
generation of metastatic variant cells in both parental B16F1 melanoma cells and
nascent clones. The metastatic abilities of cultured B16F1 cells were tested after a
period of growth in the presence or absence of a second cell population separated
from each other by a transwell membrane (0.45 µm pore size). The first
population is defined as the 'responder' cells and the second as the 'stimulator'
cells. We found that the presence of $10^5$ B16F1 stimulator cells during the growth
of responder B16F1 cells from $\sim 10^4$ to $\sim 10^6$ cells resulted in cells with an
increased metastatic phenotype (>8-fold increase in median number of lung
tumors relative to untreated B16F1 parental cells). The presence of stimulator
cells also increased the metastatic phenotype of nascent clones, which were grown
to a population size of $<10^6$ cells, suggesting that the rate of generation of
metastatic variants of the responder B16F1 clones was affected by the stimulator
cells. Other cell lines, including highly metastatic B16F10 and BL6 melanoma
cells, and KHT35-L1 fibrosarcoma cells, were effective stimulator cells when as
few as $10^4$ cells were added to transwells. In addition, normal immortalized NIH
3T3 cells were effective stimulator cells only at $10^5$ cells/transwell. The cell
density at which untreated parental B16F1 cells were harvested ($3 \times 10^5$–
$3 \times 10^6$ cells/cm$^2$) did not affect the median number of lung tumors significantly.
These results suggest that factors released from both tumor and immortalized
normal cells can modulate epigenetic changes in the metastatic phenotype of
B16F1 melanoma cells.

Introduction

The generation of variant cells and the action of selective forces on these tumor
cells results in a phenotypically heterogeneous tumor cell population, which is
believed to contribute to tumor progression [12, 17, 20, 21, 23, 32]. We have
previously examined the dynamics of generation and loss of metastatic variant cells
('dynamic heterogeneity') in clonal tumor cell populations grown to small, defined
population sizes. We have used this strategy to study the generation and loss of B16
melanoma [13] and KHT fibrosarcoma [8, 9] cells able to form experimental lung
metastases, and embryonal carcinoma cells [10] able to form experimental liver
metastases. Other laboratories have taken this approach to examine clonal isolates
of mouse fibrosarcoma [16], rat adenocarcinoma [27] and Chinese hamster ovary
cells [5].

While the rates of generation of metastatic variant cells have been measured in
several tumor models, the mechanisms underlying the generation of the observed
heterogeneity in metastatic phenotype are not understood. Two explanations for
the origin of the metastatic variant cells are: (a) genetic mechanisms, such as mutation
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(or amplification) of genes that contribute to metastasis; and (b) epigenetic mechanisms, including extracellular signals [23], that lead to quasi-stable, heritable changes in the metastatic phenotype. To date, the genetic hypothesis for the origin of metastatic variant cells has been tested, using mutant cells with increased rates of gene amplification (i.e. 'amplificator' phenotype [5]) and correlations of metastatic ability with karyotypic abnormalities [15]. The results of the 'amplification' study indicated that while metastatic variant cells were indeed generated stochastically, there were no differences in effective rate of generation of metastatic variant cells between mutant cells with the amplificator phenotype and wild-type cells [5]. Although there are many other 'mutator' phenotypes that could be tested in this context, the epigenetic origin of the metastatic variant cells also needs to be considered.

We have initiated an examination of an epigenetic hypothesis for the generation of metastatic variant cells, by modifying the microenvironment in which clones of B16F1 cells are grown. We tested the metastatic ability of clones and parental B16F1 cells expanded to small, defined population sizes ('responder' cells) in the presence and absence of various 'stimulator' cells. The responder cells were grown in the bottom compartment of transwell dishes, with stimulator cells in the upper chamber of the dishes. This arrangement permits passage of factors able to pass through the 0.45 μm pore size of the transwell, while prohibiting direct contact between the responder cells and the stimulator cells. We found that the presence of stimulator cells during the expansion of B16F1 cells in some cases resulted in responder B16F1 cells with increased metastatic ability, relative to controls expanded in the absence of stimulator cells. This modulation was dependent on the type and number of stimulator cells. These results suggest that the microenvironment can influence the development of metastatic cells during clonal expansion.

Materials and methods

Cells and cell culture

The following murine cell lines were used in this study: B16F1 [6, 7], B16F10 [6, 7] and B16BL6 [11] melanoma, KHT35-L1 fibrosarcoma [4, 9] and NIH 3T3 fibroblast cells. The cell lines were cultured routinely in alpha minimal essential medium containing nucleosides (Gibco/BRL Canada, Burlington, Ont.) and 10 per cent fetal calf serum (Gibco/BRL) from selected lots giving high plating efficiency with these cell lines, as described [6, 7]. The cells were routinely grown to approximately 80 per cent confluency (~5 × 10⁴ cells/cm² for KHT35L1; ~8 × 10⁴ cells/cm² for the B16 lines) in tissue culture flasks before harvesting for experimental use. Cells were tested routinely for mycoplasma infection [28] and were negative.

Experimental metastasis assay

The experimental metastatic ability of B16F1 cells and the derived clones was determined as described previously [13] by the intravenous injection (lateral tail vein) of 5 × 10⁴ cells, in a volume 0.2 ml of medium, into syngeneic C57BL/6 mice (Harlan Sprague Dawley Inc., Indianapolis, IN). The mice were killed 21–25 days later, the lungs fixed in Bouin’s solution, and the number of lung tumors counted. All animal experimentation was performed according to standards of the Canadian Council for Animal Care, under a protocol approved by the University of Western Ontario. The Mann–Whitney U-test (P. C. Pitman for IBM-PC) was used to...