Short Communication

LUNG COLONY FORMATION: A SELECTIVE CLONING PROCESS FOR LUNG-COLONY-FORMING ABILITY

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Received 21 June 1978 Accepted 2 November 1978

Metastasis is a phenomenon consisting of many steps, including release of cells from the tumour, circulation of released cells and their lodging at distant sites, and growth where these new sites are favourable. Both tumour-cell and host factors could be involved and interact in all these steps (Zeidman, 1957; Rubin & Green, 1968; Thompson, 1976). Metastatic foci are formed at low frequency in spite of the release of many malignant cells into the circulation (Engel, 1959; Roberts et al., 1961) or despite i.v. injection of many living cells (Iwasaki, 1915; Takahashi, 1915; Warren & Davis, 1934). Our working hypothesis to help explain this inefficiency of metastasis has been as follows: tumours vary with respect to their karyotype or malignant characteristic (Makino, 1957; Foulds, 1969; Nowell, 1976) so that cells within a tumour may be heterogeneous in various characteristics including malignant properties. Metastasis may require properties other than local growth ability, and variants with different potentials for metastatic growth may evolve during tumour development and progression. For metastatic growth, variants must meet the requirements for both dissemination and growth in the new tissue environment. Thus, metastasis could involve selective cloning. We have isolated clones from a mouse fibrosarcoma and characterized some of their cell characteristics, including their malignant potential. Plating efficiency (Suzuki & Withers, 1978a) DNA content (Suzuki et al., 1977b) cell volume and lung-colony-forming efficiency (LCFE) (Suzuki & Withers, 1978a; Suzuki et al., 1978b) were heterogeneous among clones. These results are consistent with our working hypothesis. In the experiments reported here, the LCFE of cells cultured from secondary lung nodules was compared with that of cells cultured from subcutaneous tumours or with that of the original cells (FSA 1233) in culture.

FSA 1233 is one of the clones isolated from a mouse fibrosarcoma (Suzuki & Withers, 1978a) and, after 5 subcultures, has been kept in liquid N₂. The cells were cultured in McCoy’s 5A medium as modified by Hsu, containing 20% foetal calf serum. Cell suspensions from secondary lung nodules or subcutaneous tumours were made 25 days after injection, using trypsin and DNase I (Suzuki & Withers, 1978a). These cells were grown in culture for 10–20 days (3 subcultures) prior to i.v. injection into the tail veins of mice. No significant normal-cell contamination was observed by flow microfluorometry after this period of culture.

LCFE is affected by several factors: cell-cycle stage (Suzuki et al., 1977a) the in vitro growth conditions prior to i.v. inoculation (Bosmann & Lione, 1974) and

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TABLE.—Mean lung colonies/mouse ± s.e. (based on 10–20 mice) when inoculated with cells cultured from lung nodules or tumours

| Cells injected/mouse† | 1:1 × 10⁴ | 3:3 × 10⁴ | 5 × 10⁴ | 10⁵ |
|-----------------------|-----------|-----------|---------|------|
| Exp. 1                |           |           |         |      |
| The original cells (FSA 1233) | 6.8 ± 1.4 | 0.4 ± 0.2 | 0.9 ± 0.3 | 12.0 ± 3.0 |
| A tumour (T1)         | 9.1 ± 0.7 |            |         |      |
| A lung nodule (LC1)   | 8.5 ± 1.8 |            |         |      |
| Exp. 2                |           |           |         |      |
| A tumour (T2)         | 11.6 ± 1.3| 4.8 ± 1.9 | 18.3 ± 5.2| 207 ± 19 |
| A lung nodule (LC1)   | 9.3 ± 1.0 | 11.2 ± 2.3 | 62.7 ± 12.2| 182 ± 18 |
| Exp. 3                |           |           |         |      |
| The original cells (FSA 1233) | 51.9 ± 6.2 |            |         |      |
| Mixed lung nodules (LM1) | 28.4 ± 2.6 |            |         |      |

*Mean ± s.e. of 3–8 tubes. Plating efficiency was determined in screw-capped culture tubes by soft-agar cloning (Suzuki & Okada, 1976).
†Single-cell suspensions of 10⁴–10⁵ cells in 0.5 ml of medium were injected into tail veins of unirradiated mice without addition of microspheres or heavily irradiated cells. Mice were killed 19 days after injection and lungs were removed and fixed in Bouin’s solution. Colonies were counted macroscopically (Suzuki et al., 1977a; Suzuki et al., 1978b).

whether the cells were prepared from tumours or in vitro cultures (Suzuki & Withers, 1978a). In these experiments, cells in the late log-phase of growth in the third subculture were used for both LCFE and host survival-time experiments. Late-log-phase cultures were trypsinized, washed by centrifugation, and resuspended in medium. Cells were counted with a Coulter counter (model ZBI) and a multi-channel analyser (Channelyzer II) (Suzuki et al., 1977a; Suzuki & Withers, 1978a). The suspensions were routinely checked for clumps and viability using a phase-contrast microscope. One of the features of the fibrosarcoma used is that it is easy to make single-cell suspensions without clumps (Suzuki & Withers, 1978a). C3Hf/Bu male mice, 8–10 weeks old, were obtained from our specific-pathogen-free breeding colony.

The Table describes details of the experiments and the results, and the Figure summarizes these results. Experiment 1 (Table) shows that cells from a single nodule in a lung (LC1) had a higher LCFE than the original FSA 1233 cells, whereas cells from a subcutaneous tumour (T1) had an LCFE similar to that of the original FSA 1233 cells. Experiment 2 was a repetition of Experiment 1. Experiment 3 shows that cells from a mixed culture of all lung colonies from one mouse (LM1) had a higher LCFE than the original FSA 1233 cells. The in vitro plating efficiencies of these cells are also shown in the Table, but differences in PE's do not explain the difference of LCFE among cells from nodules, tumours or the original FSA 1233 culture.

These results were interpreted as follows. The higher LCFE of cells from lung nodules was not related solely to growth in vivo, since growth as subcutaneous tumours did not yield cells of higher LCFE. The fact that cells from a single nodule or from a mixture of metastatic nodules showed similar high levels of LCFE suggests that metastasis could be a process in which variants from the initial primary tumour showing increased metastatic growth ability are selectively cloned for a particular metastatic site.

When 10 mice per group were inoculated s.c. with 10⁶ cells per locus at 4 loci, the days for 50% survival of the hosts were 33 and 38 days for FSA 1233, and 33 and 37 days for a mixture of cells from several lung nodules (LM1). The results of 2 separate experiments showed no difference between the survival time of mice inoculated s.c. with either LM1 or the
parental FSA 1233, despite the 10-fold greater artificial lung-colony-forming ability of the LM1 cells. This indicates that some factors responsible for lung-colony formation are different from those involved in local subcutaneous growth.

The present experimental results agree with various clinical and experimental facts: the inefficiency of metastasis formation despite the presence of many malignant cells in the blood stream (Engel, 1959; Roberts et al., 1961) tumour-specific distribution of metastatic foci, the "seed soil theory" of Paget (1889), and the development concept of malignancy which suggests heterogeneity of karyotype or malignant characteristics among cells in a
tumour (Makino, 1957; Foulds, 1969; Nowell, 1976). Fidler (1973) has already isolated highly metastatic variants of B16 melanoma cells by 5 to 10 passages of the cells as lung metastases. B16 melanoma is a long-established line, whereas the clone used in these experiments was freshly isolated from a mouse fibrosarcoma. Our experiment showed that the selection of increased metastatic efficiency was specific to the organ in which the metastases were passaged, because s.c. passage did not enhance lung-colony-forming efficiency.

In summary, lung-colony formation was a selective cloning process for factors involved in lung-colony formation.

We thank Ms Marcia W. Kehler for excellent technical assistance with the experiments, and for the preparation of this manuscript. We are also grateful to Larry Wilborn and his staff for the supply and care of the animals used in these experiments.

This investigation was supported in part by research grants CA-06294 and CA-11138, awarded by the National Cancer Institute, DHEW.

Animals used in this study were maintained in facilities approved by the American Association for Accreditation of Laboratory Animal Care, and in accordance with current United States Department of Agriculture and Department of Health, Education, and Welfare, National Institutes of Health regulations and standards.

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