Early Growth of Experimental Lung Metastasis in Mouse

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Fibrosarcoma/Survival curves/Relative cell number/Doubling time.

A radiobiological method was developed to measure the initial growth of clonogenic tumor cells metastasizing to the lung. The thoraxes of mice were externally irradiated by γ rays after an intravenous transplantation of syngeneic fibrosarcoma cells. The lung colonies which developed 11 days after irradiation were counted and provided surviving fractions. Survival curves moved downward when the time interval between transplantation and radiation was delayed from 1 to 21 hr, but shifted upward at 48 hr or later. Survival ratios at given doses and the extrapolation number of survival curves fitted to multi-target model were calculated, and plotted against time after the intravenous transplantation. Doubling times of 13.3 and 13.1 hr were obtained by use of the survival ratio and of the extrapolation number, respectively. This method is useful to measure the growth dynamics of clonogenic tumor cells at the site of a metastasized organ.

INTRODUCTION

Metastasis formation consists of several steps including release of tumor cells from primary sites, circulation in the vessels, adherence to and passing through the endothelium of the target organ. Micrometastasis is experimentally detectable by LacZ gene-targetting1) and clinically detectable by methods including real-time reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemistry.2,3) Quantitative RT-PCR could detect 1 tumor cell out of 10 million lymphatic cells.4) However, initial growth of newly arrived cells is still poorly understood because of methodological limits. We here developed a novel method to study initial cell division. Tumor foci in lung could artificially be made by intravenously transplanting tumor cells into syngeneic mice. Irradiation to lung after tumor cells lodging kills tumor cells and results in reduction of the number of lung colony development. An assumption is that surviving fractions could be described by \( y = M^{\exp} (-D/Do) \) where \( M \) represents the product of extrapolation number of a tumor cell and cell number, and \( Do \) represents radiosensitivity of a tumor cell. As extrapolation number is constant irrespective of cell number, an increase of \( M \) value is equal to an increase in the number of clonogenic tumor cells at the time of irradiation. When time interval between an IV tumor cell injection and thoracic irradiation increases, resulting survival curves would move upward due to cell multiplication. Plotting \( M \) values against the time interval should have two phases; one is a leading plateau that reflects non-dividing single cells and second is an increasing phase that reflects cell multiplicity. Break point between the two phases corresponds to start of cell division while slope of the increasing phase represents cell division time.

MATERIALS AND METHODS

Animals

Specific pathogen-free male mice of the inbred strain C3H/HeMsNrsf were bred and kept in the National Institute of Radiological Sciences (NIRS). They were used for experiments at 8–12 weeks of age.

Tumors

The NFSa fibrosarcoma was kept in liquid nitrogen, and its 17th generation was inoculated into the lower legs of syngeneic mice before use. Single cell suspensions of tumor cells were prepared as described previously.5) Briefly, tumors were removed, minced with scissors, and then mixed with 20 ml of Dulbecco’s solution containing 0.2% trypsin (Difco 1:250), 0.02% pancreatin Grade VI (P-1750; Sigma Chemical Co., St Louis, MO, USA) and 5 mg DNAse I Grade II (Boehringer, Germany). The solution was stirred at 35°C for 5 min. The supernatant was discarded and fresh solution was added for another 20-min enzyme treatment. The second supernatant was filtered through stainless steel (#200) and centrifuged at 154g for 5 min. The pellet was resuspended
in Hanks’ balanced salt solution (HBSS) with 10% fetal calf serum (FCS). Viability exceeded 95% when determined with phase-contrast microscopy. All cell suspensions were mixed with $2 \times 10^6$ heavily-irradiated cells shortly before an intravenous transplantation into mice that were subcutaneously injected with 150 mg/kg of cyclophosphamide one day in advance. The use of heavily-irradiated cells and cyclophosphamide is to increase colony formation efficiency in the lung.

**Irradiation**

A Cs-137 $\gamma$-ray unit was used. Setting an interval time between an IV tumor cell transplantation and irradiation, mice under pentobarbital anesthesia (50 mg/kg) received a local radiation to thorax by shielding the rest of body with 5-cm thick lead blocks. Focus-skin-distance was 54 cm, and dose rate was 55cGy/min. A group of five mice were used for each radiation dose.

**Lung colony assay**

Eleven days after thoracic irradiation, lung was removed and fixed in Bouin’s solution. Number of tumor colonies on the surface of lung was counted macroscopically. Colony forming ability in unirradiated mice was ~10%. Surviving fractions at four radiation doses were obtained for each assay, and plotted against dose. Radiation dose to reduce surviving fraction by $1/e^1$, i.e., $D_0$ value, and extrapolation number at zero dose were obtained by fitting a multitarget model to survival curves.

**RESULTS AND DISCUSSION**

Survival curves were obtained for mice receiving thoracic irradiation 1 through 73 hr after the IV tumor cell transplantation (Fig. 1). Survival fractions linearly decreased with an increase of dose. A marginal downward move was observed for survival curves when the time interval between IV transplantation and radiation delayed from 1 hr to 21 hr (Fig. 1A). When the time interval further delayed from 48 to 73 hr, surviving fractions increased and survival curves shifted to upward (Fig. 1B).

$D_0$ values initially fluctuated with time after IV transplantation, and reached a constant level at 33 hr after transplantation (Fig. 2). The negative slope of regression was so small ($-0.006; r = 0.433$) that did not significantly ($p = 0.094$) differ from zero. The slope was close to zero when data of 21 hr or later alone were used. These results indicate that cell multiplicity would not change the slope of cell survival curves. In vitro cultured small colonies also show the same $D_0$ values as single cells, a phenomenon known as survival independence. $^6$

$M$ values also initially fluctuate with time after IV transplantation, and reached minimum 21 hr after transplantation (Fig. 3). A negative regression ($r = -0.5669$) was observed for data of 1 through 21 hr, but statistically not significant ($p = 0.087$) from slope zero. After 21 hr, $M$ values linearly increased to reach maximum at 73 hr. The positive regression ($r = 0.9686$) was statistically significant ($p = 0.00033$). Doubling time calculated from the positive regression line was 13.1 hr (12.6–13.6 hr; 95% confidence limits). As $M$ values are too much sensitive to a small change of $D_0$ value, we introduced an interpolation method to measure cell proliferation. The interpolation method is based on an assumption that cell multiplicity should be reflected to any surviving fractions. Process for the interpolation consists of the following two steps: first, surviving fractions at given doses (we selected 4, 6, 8 and 10 Gy) were compared between a reference time of 1 hr and a time of interest; second, ratios thus obtained were averaged and designated as “relative cell
number” at the time of interest. Relation between relative cell number and time after IV transplantation again showed two phases (Fig. 3). The initial phase between 1 and 21 hr after transplantation showed a negative regression with statistical significance \( p = 0.0053 \) in this analysis. Slope of the regression line possessed a half-life of 12.3 hr \((11.4–13.1 \text{ hr}; 95\% \text{ confidence limits})\). The second phase between 21 and 73 hr showed a positive regression \( p = 0.000068 \) with a doubling time of 13.3 \((13.1–13.6; 95\% \text{ confidence limits}) \text{ hr}.\)

The present study showed that the \( D_0 \) and \( M \) values of tumor cells metastasizing in lung were not constant and fluctuated during 21 hr of IV transplantation (Fig. 2 and 3). The smallest and largest \( D_0 \) values were 1.55 Gy and 2.39 Gy, respectively (Fig. 2), indicating a factor of 1.5 as a maximum variation of \( D_0 \) values. \( M \) values showed a large variation factor of 4.9, i.e., 5.61 vs. 1.15 (Fig. 3). This fluctuation may relate to initial metastatic processes such as adhesion and invasion of tumor cells into lung tissue. We have observed that tumor cells adhered to cultured vascular endothelial cells within 30 min. after plating, and started to invade into the endothelial cells by 1 hr (Hori, unpublished data). As radiosensitivity of some cells depends on cell-to-cell contact,\(^7\) it is possible that radiosensitivity of tumor cells changes depending on type of cells with which tumor cells are interacting at the time of irradiation.

A decrease of relative cell number at initial phase (Fig. 3) is puzzling. A majority of tumor cells detaches from lung shortly after arriving, and fails to develop metastasis.\(^8\) Radiolabelled NFSa tumor cells also detach from lung shortly after IV transplantation.\(^9\) The present radiation method indicated that clonogenic cells also detached from lung shortly after arrival. As the negative regression pointed 0.9995 cells on vertical scale at time zero, exponential detachment of clonogenic cells started shortly after cells arriving to lung. It is not clear whether the cell detachment is related to cell division, even though the cell detachment half time of 12.3 hr was similar to the cell doubling time of 13.3 hrs. It is possible that radiation not only kills cells but also affects clonogenicity of survived cells. Taking account that clonogenic tumor cells should attach to and not detach from lung prior to proliferation, we examined whether pre-irradiation of lung would counteract with the initial cell detachment. Thorax was pre-irradiated with 5 Gy of \( \gamma \)-ray one day before IV transplantation, and then received test radiation doses either 1 or 20 hr after IV transplantation. The survival curve of 20 hr was downward displaced and separated from that of 1 hr, indicating that pre-irradiation did not prevent initial cell detachment (data not shown). Therefore, it is unlikely that radiation inhibited the cell detachment.

This method demonstrated that murine NFSa fibrosarcoma cells started to proliferate in lung 21 hr after IV transplantation with a delay time of 21 hr. Using a method similar to the present study, McCullough and Till measure proliferation of hemopoietic stem cells \textit{in vivo}.\(^{10} \) Cell doubling time in the lung was 13 h (Fig. 3), irrespective of extrapolating (\( M \) values) or interpolating (relative cell number) methods. This figure is similar to 15 hr, which is the doubling time of \textit{in vitro} NFSa-Y83, a cloned NFSa cell line.\(^{11} \) The microenvironment for proliferating metastatic cells in lung may be similar to that of \textit{in vitro} culture.

As conclusions, clonogenic NFSa tumor cells started to proliferate in lung 21 hr after IV transplantation with a doubling time of 13 hr.

**ACKNOWLEDGEMENTS**

The animals involved in these studies were procured, maintained and used in accordance with the Recommendations for Handling of Laboratory Animals for Biomedical Research, compiled by the Committee on the Safety and Handling Regulations for Laboratory Animal Experiments, NIRS. This work was supported in part by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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Received on March 1, 2005
1st Revision received on April 26, 2005
Accepted on May 2, 2005