Random Process of Metastasis and Generation of Heterogeneity in a Mouse Sarcoma Line

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The process of metastasis was analyzed in 505-05-01 cells, an established line of a methylcholanthrene-induced mouse sarcoma, by tagging the cells with the pSV2neo plasmid. A dominant clone was identified which, upon transplantation together with other clones, overgrew tumors in the kidney capsule. However, when this clone was transplanted in a mouse collaterally with recessive clones, both clones grew at the same rate and metastasized to the lung at an equal frequency. This suggests that the process of metastasis in this particular sarcoma line is stochastic, the dominant population having a better chance to colonize to the lung. The dominant neomycin-resistant clone was transfected with another marker plasmid, pY3, which confers resistance to hygromycin. Results of mixed inoculation of 9 independently isolated clones revealed the hierarchy of dominance among clones. This indicated the existence of heterogeneity within the parental clone. Upon mixed inoculation with hygromycin-resistant clones, the parental clone overgrew in the tumors. This indicated that some clone had changed its phenotype to become less aggressive. Thus, the direction of phenotypic drift *in vitro* seems to be random in terms of behavior *in vivo*.

Key words: Gene tagging — Stochastic metastasis — Phenotypic instability — Random drift

The phenotype of malignant tumor cells is unstable. Two mechanisms are thought to be responsible for the phenotypic instability of tumor cells. The first involves instability of gene expression at the transcription level.¹⁾ The second is due to instability of DNA sequences in tumor cells. The genetic instability of tumor cells arises at least from two sources, intrinsic and extrinsic mechanisms. The intrinsic instability manifests as a mutator phenotype leading to constant generation of gene and chromosomal mutations in tumor cells. The mutator phenotype can be brought about by functional abrogation of critical genes and sequences involved in stabilization of genomes. Recently, mutation at the p53 locus was reported to be associated with aneuploidy of gastric tumors.²⁾ Fusion with normal human fibroblasts was shown to suppress gene amplification in tumorigenic cells.³⁾ These results indicate that tumor suppressor genes may be involved in maintaining the genomic stability of the cells. Telomeric sequences are known to protect chromosome ends and shortening of telomeres was implicated in chromosome instability of human tumors.⁴⁾ An extrinsic source of genetic instability is oxygen radicals attacking tumor cell DNA. One product of these attacks, 8-hydroxydeoxyguanosine, was shown to be highly mutagenic.⁵⁾ A recent study indicated production of radicals not only by inflammatory cells, but also by tumor cells themselves.⁶⁾ The presence of damaged bases was demonstrated in DNA of human breast tumor cells.⁷⁾

Instability of tumor cells leads to generation of phenotypically heterogeneous subpopulations which are subject

to selection in the host environment. This selection favors the growth of more malignant clones which eventually dominate the tumors *in situ* and metastasize to other organs.⁸⁾ Metastasis is the final outcome of the process of tumor progression and involves genes such as those specifying adhesion molecules, proteases and cytoskeleton components which regulate cell-to-cell and cell-to-matrix interactions.⁹⁾ Thus, phenotypic instability and resulting tumor heterogeneity is the driving force for the progression of tumor cells, which originate from a single cell.^{10,11)}

We have shown previously that genetic tagging can effectively be used for the analysis of the clonal evolution of a mouse fibrosarcoma line, 505-05-01.¹²⁾ We report here the results of further analysis and demonstrate that the process of metastasis of this line is stochastic; 505-05-01 cells metastasize by chance and a subclone with a larger population size therefore colonizes to the lung more frequently. Our data also indicate that the direction of clonal variations in the capacity to dominate tumors *in vivo* is random.

MATERIALS AND METHODS

Animals and cells Male and female C57BL/Ka×C3H/He F1 mice (BCF1) were bred in our animal facility and used for the transplantation of 505-05-01 cells.

Mouse fibrosarcoma cells, 505-05-01, are an established cell line from a tumor induced by methylcholanthrene in BCF1 mice.¹³⁾ The cells used in the present

study are 22 randomly selected clones transfected with pSV2neo, which were described in a previous report.¹²⁾ The cells were kept in culture in alpha-modified minimum essential medium supplemented with 10% fetal bovine serum, 100 μ g/ml streptomycin and 100 U/ml of penicillin.

These clones were inoculated subcutaneously in syngeneic mice. About 4 to 6 weeks after the inoculation, tumors and/or lung metastases were excised and processed for further analyses.

DNA transfection Calcium phosphate-mediated transfection was performed as described previously.¹⁴⁾ pY3 plasmid, which confers resistance to hygromycin B,¹⁵⁾ was used for transfection of the dominant clone, neo 11. Transfected cells were selected in a medium containing 200 μ g/ml of hygromycin B (Sigma Chem. Co.). Colonies were randomly cloned and propagated for further analyses.

DNA extraction and Southern blotting Tumors were excised and minced with scissors. Tissue fragments were transferred onto plastic dishes and cultured for 5 days with as small an amount of medium as possible to facilitate adhesion of the fragments. Tumor cell explants were trypsinized and transferred to new dishes. This procedure efficiently eliminated contaminating stromal cells and inflammatory cells.

DNA was extracted from the cultured tumor cells thus obtained and analyzed by Southern blotting hybridization according to the procedure described previously.¹⁶⁾

RESULTS

Dominance of neo 11 clone *in vivo* In our previous study, 22 randomly selected clones of 505-05-01 cells genetically tagged with the pSV2neo plasmid were analyzed and one of them, neo 11, was shown to overgrow the other 21 clones in tumors after subcutaneous inoculation.¹²⁾ The dominance of neo 11 clone was tested again at a different site. A mixture of 22 clones (an equal number of cells of each clone) was inoculated at 1×10^5 cells/site under the kidney capsule of syngeneic mice. Tumors were excised 4 weeks after the inoculation. Fig. 1 shows the result of Southern analysis of DNA from 3 tumors recovered from kidney; all of them had the pattern of pSV2neo integration identical to that of neo 11 clone. Thus, the dominance of neo 11 clone was observed in tumors grown under kidney capsules as well as subcutaneous sites.

Growth of dominant and recessive clones in collateral inoculation The dominance of neo 11 clone *in vivo* suggests that this clone has a growth advantage over other clones. It was shown previously that the growth rates *in vivo* of pSV2neo-transfected 505-05-01 sarcoma cells were the same for dominant and recessive clones when they were inoculated individually.¹²⁾

Fig. 1. Dominance of neo 11 clone in mixed inoculation under the kidney capsule. DNA was purified from tumors grown under the kidney capsule after mixed inoculation of 22 neo clones. DNA was digested with *Bam* H1 and Southern blotting was performed. Filters were hybridized with the 2.3 kb *Hind* III-*Bam* H1 fragment of the pSV2neo plasmid. Lane 1 is the neo 11 clone and lanes 2 to 4 are 3 independently isolated tumors.

Collateral inoculation was applied to determine whether or not the hierarchy of the clonal dominance was still observable at a distance in a mouse. We employed three clones, neo 1, neo 3 and neo 11, which had previously shown differences in dominance in a mixed inoculation experiment *in vitro*, neo 11 being the most dominant and neo 3 the most recessive. One of the three was inoculated on one side of the back of mice and another clone on the other side. In contrast to the dominance in the mixed inoculation experiment, the growth rates of the clones in collateral inoculation were the same among the three clones (Fig. 2). This suggests that the dominance of neo 11 clone does not operate at a distance and it functions only when the clones are in close contact with each other, as in the mixed inoculation.

Fig. 2. Growth of neo 1 (A), neo 3 (B) and neo 11 (C) clones at collateral sites in a mouse. Each of the clones were injected subcutaneously at 5×10^5 cells/site in collateral sites on the back of a mouse. For each combination, 8 mice were used and the average diameters of tumors in 8 mice were plotted.

Metastasis of dominant and recessive clones in collateral inoculation When the dominant and recessive clones were tested individually, the frequencies of spontaneous metastasis were found to be the same among them.¹²⁾ This was found to be true even when two clones were inoculated at independent sites of a mouse. Dominant neo 11 clone and recessive neo 1 clone were inoculated collaterally on both sides of mice. In Fig. 3, 23 lung nodules recovered from 4 mice were analyzed for the origin of metastasis. Although there were variations in the rates of metastasis among 4 mice tested, total numbers of metastases by both clones were almost the same, 11 nodules for neo 1 and 12 nodules for neo 11. Additional cases were analyzed and out of 36 nodules examined, 16 originated from neo 1 clone and 20 from neo 11, indicating similar rates of metastasis. As has been

shown in Fig. 2, the size of tumors for neo 1 and neo 11 on opposite sides of the mice was the same under these conditions.

Further analysis by secondary tagging with pY3 The possibility of heterogeneity within a clone was tested by secondary tagging with the hygromycin resistance gene. The dominant clone neo 11 was passed about 10 times in culture and then transfected with pY3. Nine clones, hyg 1 to hyg 9, were selected randomly (Fig. 4). These clones were then mixed and inoculated subcutaneously into 4 mice. The tumor of the first mice had an integration pattern of pY3 similar to that of hyg 2 clone. However, the pattern of three lung metastases indicated that the tumor was a mixture of hyg 2 and hyg 8 clones. Analysis of the three tumors indicated that hyg 8 clone overgrew the other clones.

Gene tagging of neo 11 clone by pY3 revealed the existence of heterogeneity within the clone. This heterogeneity was supposed to be generated during *in vitro* cultivation of the clone. The direction of the phenotypic drift under tissue culture conditions may be towards either more malignant or less malignant for *in vivo* growth. This can be tested by examining the dominance of hyg clones and the parental neo 11 clone in mixed inoculation. Fig. 5 shows the result of such analysis; all the hyg clones were lost during growth of tumors when they were mixed with the parental neo 11 clone. Thus, hyg clones with limited heterogeneity were overwhelmed by the parental neo 11 clone which should have contained more variations than its daughter clones.

DISCUSSION

Genetic tagging is a potent tool for analyzing the process of tumor metastasis.^{17,18)} We have shown previously that the neo 11 clone was recessive in tissue culture when mixed with other clones which were overgrown by the neo 11 clone in the *in vivo* subcutaneous environment.¹²⁾ In our present experiment, neo 11 again dominated tumors grown under the kidney capsule. Although other sites were not tested, it seems quite likely that the dominance is a general trait of neo 11 clone grown *in vivo*. Cell-to-cell contact seems to be required for this dominance to operate, because neo 11 and other recessive clones grew at the same rate when inoculated collaterally in the same mouse. The molecular nature of the cell-to-cell interaction and the mechanism for the suppression of recessive clones *in vivo* by neo 11 require further investigation.

Generally, metastasis is thought to be a selective process in which tumor cell clones equipped with molecules conferring invasive properties implant other organs more readily.⁹⁾ Our previous study indicated that the dominant neo 11 clone is the one that metastasizes to the lung.¹²⁾

Fig. 3. Metastasis of two clones injected at collateral sites. Lung metastatic nodules were recovered from four mice in which neo 1 and neo 11 clones were injected at collateral sites. The first two lanes are the patterns of neo 1 and neo 11 clones, respectively.

Fig. 4. Secondary tagging by the pY3 plasmid. The dominant clone, neo 11, was transfected with the hygromycin resistance marker gene. The mixture of 9 clones was injected subcutaneously into mice. Tumors and metastatic nodules were recovered and the Southern blot was probed with the 1 kb *Kpn* I-*Sac* I fragment of the pY3 plasmid. Lanes 1 to 9 are DNA of hyg 1 to hyg 9 clones. T1 indicates DNA from the tumor in mouse number 1 and m1 to m3 are from lung metastatic nodules of this mouse. T2 to T3 are tumors grown in other mice.

However, it was reported that tumor subpopulations already selected for their metastatic ability metastasize in a random fashion.¹⁹⁾ This was confirmed in our present experiment using the already invasive 505-05-01 tumor line. When dominant neo 11 and recessive neo 1 were

tested by collateral inoculation in the same mouse, the frequency of metastasis was the same for the two. This indicates that the dominance in metastasis of neo 11 clone following mixed inoculation is simply a reflection of the population size of the clone in tumors grown

Fig. 5. Mixed inoculation of hyg clones with parental neo 11 clone. Nine hygromycin-resistant clones, each 1×10^6 cells, were mixed with 9×10^6 cells of neo 11 clone. The mixture was injected subcutaneously on the back of mice at 5×10^5 cells/site. DNA was purified from the tumors and analyzed by Southern blotting. The left panel was probed for the hygromycin resistance gene and the right for the neomycin resistance gene. Lane 1 is hyg 9 clone and lanes 2 to 6 are DNA from 5 independently isolated tumors.

subcutaneously. Therefore, although growth of clones in tumors *in situ* is selective, the process of spontaneous metastasis in 505-05-01 tumor cells is stochastic: clones with a larger population size have a better chance of metastasis. The random nature of metastasis observed here holds only for 505-05-01 cells which have already gone through a long process of progression. These cells have been selected for their ability to grow in the micro-environment of the lung.

The results of secondary tagging with the hygromycin resistance gene indicate the presence of heterogeneity

within the dominant population of neo 11 clone. It was reported that DNA transfection may generate heterogeneity in tumor cells.²⁰⁾ However, molecular analysis at monomorphic and polymorphic minisatellite loci has indicated the constant generation of genetic heterogeneity in untransfected 505-05-01 cells.²¹⁾ These results suggest that 505-05-01 cells are genetically unstable. Genetic tagging of neo 11 clone with the hygromycin resistance gene revealed that the clone had already undergone changes which lead to differences in capacity for competitive *in vivo* growth. In addition, mixed inoculation indicated that the hygromycin-resistant subclones were overgrown by the parental neo 11 clone. This suggests that the phenotypic drift due to genetic instability is not directional in 505-05-01 cells.

Genetic instability, once acquired by tumor cells, becomes a driving force of tumor progression and constantly generates heterogenous subpopulations. This automatically leads to appearance of more malignant clones through selection in host environments. An understanding of the molecular mechanism of genetic instability of malignant tumor cells would be of great importance in tumor biology.

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