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REVIEW

MONOCLONAL GROWTH OF CANCER CELLS: EXPERIMENTAL EVIDENCE

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INTRODUCTION

"A cancer originates from a single cell." This proposition is now acceptable on the basis of various lines of experimental and clinical evidence. The monoclonal origin of tumors has been recognized in human lymphoproliferative diseases, in which only one type of immunoglobulin was found in contrast to various types present in humans without this disease, and further in various types of tumors which exhibited only one type of G6PD in patients with G6PD-mosaic somatic cells.[1] Recent developments in studies on oncogenes and restriction fragment length polymorphism (RFLP) have further supported the monoclonal nature of tumors in humans and experimental animals. Historically, experimental design to study the monoclonal origin of tumors followed human studies. Review articles on this subject have been presented previously.[1-5]

A significant indication of the monoclonal origin of tumors is that a tumor is considered to be like a clone of a mutated cell; however, the frequency of tumor formation is so low, compared with the expected frequency of mutation, that the existence of a mechanism to suppress tumor formation should be considered. The processes which allow a single transformed cell to develop to cancer necessarily involve the whole body system, and this will be an important theme in future studies.

This review first describes methods to examine the clonal origin of tumors in experimental animals, and then evidence for the monoclonal origin of a variety of experimental tumors. As will be shown, even some apparently multiclonal tumors could have originated from a clone of a single cell, a fused cell. Therefore, truly multiclonal tumors seem very rare.

METHODOLOGY

Principle of Application of X-Chromosome Inactivation Mosaicism to Study of the Clonal Origin of Tumors

In somatic cells of female mammals, only one of the pair of X-chromosomes is active and the other is inactive.[6] The inactivation of the X-chromosome involves methylation of DNA bases.[7] The choice of whether the paternal or maternal X-chromosome is inactivated is random. If a pair of X-chromosomes are heterozygous at a certain allele, for example, if one has a normal genotype and the other has a corresponding mutant genotype, the somatic cells will theoretically be a mixture of equal numbers of cells with the normal phenotype and those with the mutant phenotype. Since X-inactivation occurs early in embryogenesis,[8] the cells that develop into each organ are each expected to produce a clone with a single

common X-inactivation pattern of either of the two types. However, in fact, tissues consist of a fine mixture of the two types of cells.[8, 9] Therefore, differentiating cells are supposed to migrate among each other during embryogenesis and organogenesis.

If a tumor produced in a mosaic population of somatic cells has both phenotypes, it can be concluded to have originated from multiple cells, whereas if it exhibits a single phenotype, it can be concluded to have originated from only one, or a few cells with the same phenotype. The latter case is less likely on hit-probability considerations; i.e., the probability that a single phenotype of a tumor is obtained from n origins with the same phenotype is $1/2^{n-1}$.

Recently, on the basis of observation of the PGK-1 phenotypes of clones cultured from individual tumor cells[10] and analysis of the late-replicating X-chromosome in individual tumor cells,[11] the possibility was raised that a tumor with two phenotypes might be a clone of a fused cell carrying both paternal and maternal X-chromosomes in the activated form.

X-Chromosome-linked Markers

Useful X-chromosome-linked enzyme markers that have been applied in studies on the clonal origin of tumors are G6PD, PGK-1 and OCT. Mosaic female mice heterozygous at the PGK locus with a pair of normal and mutant genes[12] (genotype: *Pgk-1*^{b}/*Pgk-1*^{a}) have been most commonly used. The gene products coded by the normal and mutant genes, i.e., PGK-1 isoenzymes, are distinguishable by differences in electrophoretic mobilities on starch gel or a cellulose acetate membrane.

Translocation of an autosome to an X-chromosome in mice provides an especially useful marker. For example, in the Cattanach translocation[13] a part of chromosome No. 7, involving the gene encoding the hair pigment, is translocated to the X-chromosome. This translocation does not affect X-inactivation,[14] and so a heterozygous cell with a pair of X-chromosomes, one normal and the other with the translocated gene, can express either one of the two X-chromosomes with equal probability. These heterozygous mice show a homogeneous fine mixture of pigmented and non-pigmented hair, and in each cell it is possible to distinguish whether the translocated or normal X-chromosome is in the inactivated (late replicating) state.

Chimeric mice have also been used.[5] A large patch size which is unsuitable for testing the clonal nature of tumors has been suggested for chimeric mice. However, Bodenstein and Sidman recently reported the fineness of the patch size in chimeric mice.[15]

Basic Assumptions in Application of X-Inactivation Mosaicism

The following assumptions should be valid in application of X-inactivation mosaicism to studies on the clonal nature of tumors.

1. Cellular mosaicism should be homogeneous in a normal tissue. This is a safe assumption, as described earlier in the discussion on the patch size. Imbalance of inactivation between paternal and maternal X-chromosomes is often seen, depending on the combination of mouse strains. However, it does not affect the applicability of this method.

2. X-Inactivation should not be disturbed during treatment with a carcinogen, cellular carcinogenesis, and further growth. If X-inactivation is controlled by DNA methylation, carcinogens such as alkylating agents may disturb the X-inactivation. However, this has not yet been observed.[8]

3. No selection of either type of cells should occur during tumor growth. For examination of possible selection, equal numbers of tumor cells of two types, e.g., those with PGK-1A and PGK-1B, were transplanted subcutaneously. The resulting tumors were found to contain the two types of tumor cells,[16] and the two types were found to grow at equal rates.[17] Therefore, these three assumptions are currently accepted as valid.

Abbreviations used: AAF, 2-acetylaminofluorene; BHBN, N-butyl-N-(4-hydroxybutyl) nitrosamine; DEN, diethylnitrosamine; DMBA, 7,12-dimethylbenz[*a*]anthracene; DMH, 1,2-dimethylhydrazine; G6PD, glucose-6-phosphate dehydrogenase; MCA, 3-methylcholanthrene; HPRT, hypoxanthine phosphoribosyl transferase; NDEA, N-nitrosodiethanolamine; OCT, ornithine carbamoyl transferase; PGK, phosphoglycerate kinase; RFLP, restriction fragment length polymorphism.

Other Genetic Markers: Oncogene Rearrangement or Integration Sites, Immunoglobulin Gene Rearrangement Site, and RFLP

The Philadelphia chromosome,[18] human chromosome No. 9 with the c-*abl* oncogene translocated into the middle of the *bcr* gene on chromosome 22, is found in more than 85% of chronic myelocytic leukemias (see review 1) and 13% of acute lymphocytic leukemias,[19] and its junction site is distinguished in the two types of leukemia at the nucleotide level.[20] Although the same translocation is found in all leukemic cells in the same patient, and it could be an indicator of the monoclonal origin of these leukemic cells, this translocation is not only a genetic marker but also the cause of the disease. Therefore some limitations exist in judging the clonal nature of such disease.

In human lymphoproliferative diseases, the type of immunoglobulin[1] or rearranged immunoglobulin gene[21] is a very useful indicator of clonal origin. The advantage of their use is that only one type of immunoglobulin or gene rearrangement is observed in the case of monoclonal disease among numerous possibilities. This method could be applied to animal experiments. However, immunoglobulin mosaicism can be used as a marker only in lymphoproliferative diseases.

The site of integration of an oncogenic virus (e.g., adult T-cell leukemia in humans[22]) or oncogene into a host genome, or the site of the host oncogene rearrangement can be a useful marker to test the clonal nature of tumors. However, again, if the site of integration or rearrangement on the host genome is limited to specific sites and is the cause of the tumor, the applicability of this method is limited.

RFLP in DNA of X-chromosomes is also a useful marker.[23,24] The RFLP test involves alteration of methylation in DNA bases associated with X-chromosome inactivation, and distinguishes paternal from maternal DNA of X-chromosomes in the activated or inactivated state by restriction enzyme recognition. RFLP at the HPRT locus has been used to prove the monoclonal nature of human tumors.[23,24] These markers should be useful for studies on the clonal origin of tumors with experimental animals.

Experimental Evidence for Monoclonal Origin of Tumors

MCA-induced Fibrosarcomas

Reddy and Fialkow first used PGK mosaic cell mice in studies on the clonal nature of experimental tumors.[25] They obtained fibrosarcomas by subcutaneous injection of a high dose (0.2 and 2 mg per mouse) of MCA. All the tumors, like normal tissues, had both PGK-1 phenotypes. Since clones derived from isolated tumor cells had only one PGK-1 type, the tumors appeared to be mixtures of multiple clones. Thus, Reddy and Fialkow concluded that these MCA-induced fibrosarcomas were of multiple cellular origin.

Tanooka and Tanaka[16] used a low dose of MCA (0.05 mg per mouse) to induce fibrosarcomas in PGK-mosaic cell mice. To confirm the PGK-1 type of tumor, they transplanted pieces of the tumor into a pair of mice, one with PGK-1A only and the other with PGK-1B only. This was a useful method for distinguishing PGK-1 of the contaminating background of blood and supporting tissue from that of the tumor. They found that 7 out of 8 of the resulting tumors had only one PGK-1 phenotype.

Very similar results were obtained by Woodruff *et al.*,[10] and Deamant and Iannaccone[26] on MCA-induced tumors in PGK mosaic mice. These workers interpreted the formation of monoclonal tumors as representing selection of one of multiple clones. However, an alternative interpretation is that only one of many dormant cancer cells started to grow out.

It was furher found that the formation of single-phenotypic tumors in the PGK mosaic mice depended on the dose of carcinogen; i.e., the tumor incidence reached 100% with MCA doses of more than 0.25 mg, and tumors with a single PGK-1 type decreased to 57% of the total with a dose of 2 mg MCA per mouse.[27] Monoclonal tumors were produced predominantly with low doses of carcinogen, while multiclonal tumors were produced more frequently with increase in the dose. Most human tumors are thought to be produced by relatively low doses of environmental carcinogens, and so from this

dose-response relation, it can be understood why most human tumors are monoclonal.[1]

It is expected from a consideration of cell-transformation frequency that many transformed cells exist in the carcinogen-treated host tissue, but only one of these cells seems to develop into a tumor. Possibly some tumor-suppressing mechanism may act on the cell with a potential for tumor formation, so that the majority of transformed cells may be suppressed to the dormant state. The presence of dormant tumor cells or monoclonal clones of precancerous cells, as shown in the following sections, suggests the existence of such a suppressive mechanism in the host.

Skin Tumors

Reddy and Fialkow reported that most papillomas produced in PGK-mosaic mice painted with DMBA followed with TPA had a single PGK-1 phenotype, while about half the papillomas produced by repeated DMBA painting alone had both PGK phenotypes.[28] Later, Taguchi *et al.* showed with 1/4 the dose of DMBA used by Reddy and Fialkow that almost all papillomas induced by either DMBA plus TPA or DMBA alone had a single PGK-1 type.[29] Again, the induction of a tumor with a single phenotype depended on the dose of carcinogen. It is noteworthy that Taguchi *et al.* demonstrated further that most of these benign papillomas developed into malignant squamous cell carcinomas, maintaining the same PGK-1 pattern as that of the original papilloma.[29] They concluded that the papillomas were in a transitional state that later changed into malignancy.

Kim-Burnham *et al.* showed that when PGK-mosaic C3H/HeN mice were irradiated with UV 5 times a week, the skin tumors produced (spindle cell carcinomas) all had a single PGK type.[30]

Table I. A List of Various Experimental Tumors Shown to be Monoclonal with Mosaic Cell Mice

Target organ	Tumor	Carcinogen	Tumors with single phenotype (%)	Reference
Skin	Fibrosarcoma	MCA (0.2 and 2 mg)	0	25)
	〃	〃 (0.005–2 mg)	57–93	16, 27)
	〃	〃 (0.1 and 0.5 mg)	95–97	10)
	〃	〃 (1.25 and 2.5 mg)	90	26)
	Papilloma	DMBA	54	28)
	〃	〃	94	29)
	〃	DMBA + TPA	92	28)
	〃	〃	90	29)
	Squamous cell carcinoma	DMBA	96	29)
	〃	DMBA + TPA	90	29)
	Spindle cell carcinoma	UV	100	30)
Bladder	Adenocarcinoma	BHBN	100	31)
Liver	Preneoplastic nodule	AAF	98	34)
	Hepatocellular carcinoma*	Phenobarbitone alone or with NDEA	95	35, 36)
Stomach	Papilloma	DEN	90	32)
Colon	Adenocarcinoma	DMH	90	33)
Blood-forming organ	AKR leukemia	Spontaneous (AKR virus)	100	37)
	Rauscher leukemia	Rauscher leukemia virus	100	38)
	Thymic lymphoma	X-radiation	100	39)
	Myeloid leukemia	〃	100	40)
Mammary gland	Adenocarcinoma	Spontaneous (MTV)	86	46, 49)

All tumors were tested with mosaic cell mice with PGK-1 isozyme marker, except for one case with OCT marker (*).

Bladder Tumors

Kakizoe *et al.* showed that four carcinomas produced in the bladder of PGK mosaic mice by oral administration of BHBN had a single PGK-1 type and concluded that these tumors were of monoclonal origin.[31] In this experiment, only one tumor developed per bladder. Further studies are required on the development of bladder tumors analogous to human bladder carcinomas that form multiple foci, and on the clonal origin of these multiple foci.

Stomach Papillomas and Colon Cancers

Fukushima *et al.* found that 90% of the stomach papillomas that they induced in PGK-mosaic mice by oral administration of DEN had a single PGK-1 phenotype.[32] Furthermore, Inoue *et al.* showed that most colon cancers that they induced in mosaic mice by treatment with DMH also had a single PGK-1 phenotype.[33] These papillomas and colon cancers formed multiple foci. The single PGK-1 phenotypes of these multiple foci indicated that each focus was an independent clone of a single tumor cell origin.

Liver Tumors and Preneoplastic Nodules

Rabes *et al.* obtained preneoplastic nodules in the liver of PGK-mosaic mice by oral administration of AAF. The site of the preneoplastic nodules was judged by the reduced activity of ATPase, and this area of the liver showed only one type of PGK-1, while other parts of the liver contained multiple PGK-1 types.[34] It is interesting that these precancerous nodules were already monoclonal before they became malignant. Williams *et al.* obtained histochemical evidence for the monoclonal origin of liver tumors induced by oral administration of phenobarbitone alone or in combination with injection of NDEA in OCT-mosaic mice by demonstrating that they had only one enzyme phenotype.[35, 36]

Leukemias

Since leukemic cells in the PGK mosaic host include a relatively few normal blood cells with both PGK-1 types, they provide the clearest results on PGK-1 analysis. Leukemic cells of spontaneous AKR leukemias and leukemias induced by Rauscher leukemia virus in PGK mosaic mice were shown to have a single PGK-1 type by Collins and Fialkow[37] and Reddy and Fialkow,[38] respectively. Bessho *et al.* showed that all thymic lymphomas induced in PGK mosaic mice by repeated whole-body X-irradiation had a single PGK-1 phenotype.[39] Bessho and Hirashima later produced myeloid leukemias in PGK mosaic mice and again found a single PGK-1 type in all these leukemias.[40] They also observed one case in which the PGK-1 type of red blood cells coincided with that of the leukemic cells. This finding provides evidence that some myeloid leukemias originate from a progenitor cell that differentiates into both erythroid and myeloid cells.

Uniformity of the immunoglobulin heavy chain gene rearrangement pattern has been found in lymphoid tumors produced in transgenic mice, in which the *myc* oncogene was introduced.[41–43] It should be noted here that sarcomas induced by direct injection of v-*src* into chicken also exhibited uniformity of the integration site.[44] These cases represent examples of only one choice out of many varieties, as seen also in the uniformity of immunoglobulin in human lymphoproliferative diseases[1] and the uniformity of the ATLV-integration site in DNA of leukemic cells of ATL patients,[22] which provide a strong indication of the monoclonal origin of tumors. The question again arises as to why only one cell can grow into a tumor among many infected cells.

Spontaneous Mammary Tumors

Nagasawa *et al.* developed a mouse strain, SHN, that has a high incidence of spontaneous mammary tumors.[45] These mammary tumors are viral and dependent on the hormone prolactin. Multiple tumors can be produced when pre-formed tumors are resected. To obtain PGK-mosaic mice with a high incidence of mammary tumors, Tanaka *et al.*[46] mated female SHN mice (*Pgk-1*^{b}) with male C3H/He mice (*Pgk-1*^{a}). All the female F_1 mosaic mice developed mammary tumors, and 86% of these tumors exhibited a single PGK-1 type, indicating that most of them were monoclonal.

Monoclonal Metastasis

By implantation of tumor cells with cellular and chromosomal markers, Fidler *et al.* have

obtained evidence that metastasis is caused by expansion of a single variant tumor cell.[47,48] The occurrence of monoclonal metastasis was shown more directly by studies on metastases of monoclonal autochthonous tumors. Ootsuyama *et al.* obtained frequent metastases of spontaneous mammary tumors to the lung of female (SHN×C3H/He) F_1 mice.[49] Resection of successively appearing mammary tumors resulted in survival of the mice for a maximum of 8 mammary tumors. Comparison of the PGK-1 types of the primary mammary tumors with those of the metastatic colonies, together with histological analysis of tumor types, indicated the monoclonal nature of the metastatic colonies. In mice with multiple primary mammary tumors and multiple metastases, not only each individual metastasis but also all the metastases in the same lung had the same pattern of PGK-1 isozymes and the same histologic type, indicating that the metastatic colonies were derived from only one of many primary tumors. Thus it seems that a tumor with a high metastatic potential predominates over tumors with low potentials. As proposed by Fidler and Hart,[50] it seems that a tumor acquires the metastatic potential during monoclonal growth as a consequence of development of biological diversity (heterogeneity).

Test of Recurrence of Monoclonal Tumors

Experimental therapy of autochthonous tumors often results in a response that is unexpected from results on transplanted tumors: cure cannot be obtained, though it would be expected from the results on transplanted tumors.[51] It is essential to prove whether apparent recurrence is due to true recurrence or to formation of a new tumor at the same site. Fibrosarcomas with a single phenotype produced by MCA in PGK mosaic mice were used to distinguish between these two possibilities. Tanooka and Tanaka showed that the PGK-1 type of recurrent tumors after radiation therapy of MCA-induced fibrosarcomas in PGK mosaic mice coincided with that of the primary tumors and thus concluded that the tumors that reappeared were indeed true recurrent tumors.[52]

Possible Origin of Tumors from Fused Cells

The multi-phenotypes of fibrosarcomas formed in mosaic mice treated with a high dose of carcinogen have attracted attention. Woodruff *et al.* found by cloning single cells from tumors with double phenotypes that one clone consisted of cells which themselves were of double phenotypes.[10] This finding suggested the possible simultaneous activations of both X-chromosomes in a single tumor cell. Consequently, Takagi *et al.* examined X-inactivation in individual cells of fibrosarcomas induced by MCA in female heterozygous mice with one normal X-chromosome and one with Cattanach translocation, and found polyploidal tumor cells in which both the paternal and maternal X-chromosome were activated.[11] This finding indicates that the tumors may have originated from fused cells of the two different types. Such fusion is very rare, but some tumors with apparently multiple phenotypes are considered to be clones of fused cells. This problem should be considered further in relation to the mechanism of metastasis, since acquisition of a metastatic potential may involve fusion of cells.[53]

Future Perspectives

It now appears that cancers originate from single cells and acquire heterogeneity during growth. Rare cases of multiclonal tumors are thus of interest. Some apparently multiclonal tumors appeared to originate from clones of polyploidal (probably fused) cells. This problem should be further pursued in new experimental systems in the future. The finding that precancerous nodules are already monoclonal before progress to the malignant state is very intriguing. The frequency of monoclonal tumor formation is low relative to the mutation or transformation frequency of somatic cells. This low frequency must be due to the existence of a tumor-suppressing mechanism acting *in situ*, which should be clarified in the future.

Forty years after exposure of humans to atomic bomb radiation, the carcinogenic effect of the exposure is still maintained in survivors and is now being expressed. An

increasing incidence of skin cancers in survivors has recently been recognized by Sadamori *et al.*[54] More importantly, Honda *et al.* found that cultures of biopsy specimens taken from the skin of humans exposed to the radiation showed monoclonal expansion of cells with a common chromosomal translocation, although it is unknown whether these cells are established tumor cells.[55] This finding indicates that cells with chromosomal damage are maintained in skin tissue in the latent form and start to grow *in vitro* when host-suppression is released. These latent cells may be present even in healthy hosts. Host-suppression and latency of tumors must be related to the rare event of monoclonal growth of tumors and should be experimentally investigated.

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