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MALIGNANT TRANSFORMATION OF HUMAN FIBROBLASTS *IN VITRO*

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Although carcinogens cause human tumors, human cells in culture have not been successfully transformed to malignancy by exposure to carcinogens. It is now recognized that malignant transformation involves multiple changes within a cell and, therefore, successive clonal selection of cells containing such changes must occur. One explanation for the failure to induce in vitro malignant transformation of human cells could be inability to recognize cells that have undergone intermediate changes so as to expand the population, expose the cells a second time, cause further changes, etc. Therefore, we transfected finite life span diploid human fibroblasts with oncogenes known to be active in cells derived from human fibrosarcomas or effective in transforming animal fibroblasts to determine the phenotypes they produced. Transfection of a sis gene, or an H-, or N-ras oncogene caused the cells to acquire many characteristics of malignant cells, but not to acquire an infinite life span or become malignant. We recently succeeded in developing an infinite life span human fibroblasts cell strain, designated MSU-1.1, which has a stable, near-diploid karyotype, composed of 45 chromosomes including two marker chromosomes. We have shown that these cells can be transformed to malignancy by transfection of the H-, K-, or N-ras or the v-fes oncogene. All of the malignant H-, K-, or N-ras transfected derivatives examined have exhibited the stable karyotype of the parental MSU-1.1 cells. We have also found rare spontaneous clonal variants of MSU-1.1 that are malignantly transformed and have shown that malignant variants can also be induced by carcinogen treatment.

IN VITRO TRANSFORMATION OF FINITE LIFE SPAN HUMAN FIBROBLASTS BY TRANSFECTION OF ONCOGENES

Exposure to chemical carcinogens or radiation is considered to cause most human cancer, but human cells in culture have not been successfully transformed to malignancy by such agents. Malignant transformation is a multi-step process, and there is growing evidence that at least five changes are required and that these are clonally acquired. A normal cell that by chance acquires one of these changes must undergo clonal expansion so that among the progeny cells, a cell with the first change can acquire a second change, and so on until by sequential clonal expansions, a malignant cell arises. One explanation for the failure to induce such transformation of human cells in culture could be inability to recognize the phenotypes of cells that have undergone intermediate changes, so that these cells can be isolated, expanded, and exposed a second time to cause further changes, etc. To identify possible intermediates, we transfected diploid human fibroblasts with oncogenes known to be active in cell lines derived from fibrosarcomas or effective in transforming animal fibroblasts, such as H-ras, or N-ras, or a sis oncogene and determine the phenotypes produced. The sis oncogene codes for a protein structurally and immunologically related to the B chain of the platelet-derived growth factor (PDGF B) (Doolittle et al, 1983; Robbins et al, 1983). Oncogenes from DNA tumor viruses such as simian virus 40 (SV40) or the papilloma viruses were not utilized in these studies since they do not have a homolog in the DNA of human cells. The plasmids we constructed or used for these experiments also contained a gene coding for a selectable marker so the transfectants could be identified and selected by drug resistance and examined for one or the other characteristics of tumor-derived cells, such as morphological alteration, focus formation, ability to form colonies in soft agar, growth-factor independence, and tumorigenicity.

Using this approach, we and our colleagues (Fry et al, 1986) found that diploid human fibroblasts transfected with the v-sis oncogene grew

to 6- to 10-fold higher saturation densities than control cells transfected with the vector plasmid alone, formed large, well-defined foci, and exhibited growth factor independence, growing well in the absence of serum, and formed colonies in soft agar at a high frequency. But they retained their normal fibroblastic morphology, demonstrated a finite life span in culture, and were not tumorigenic.

Similar studies were carried out using the T24 H-*ras* oncogene derived from the human EJ bladder carcinoma cell line (Hurlin *et al*, 1987) or human N-*ras* oncogenes (Wilson *et al*, 1989) inserted into vectors designed to give various levels of expression of the oncogene. The *ras* oncogenes that were flanked by suitable enhancer and promoter sequences caused the cells to acquire many characteristics of malignant cells, i.e., morphological transformation, anchorage independence, focus-formation, etc., but they did not acquire an infinite life span and did not form tumors in athymic mice.

Since the human fibrosarcoma-derived cell line HT1080 expresses both a mutated N-*ras* gene (Brown *et al*, 1984) and the B chain of PDGF (Pantazis *et al*, 1985), we attempted to develop strains expressing both oncogenes. Our efforts to introduce the v-*sis* oncogenes into the *ras* transformed cell strains described above and a *ras* oncogene into the *sis*-transformed cells were thwarted by the finite life span of these human diploid fibroblasts. Even though the first oncogene transfection experiments were carried out with early-passage cells, the drug-resistant transfectant cell strains isolated and expanded to serve as recipients for the second oncogene represent individual clones. The progeny cells from such clones can be expanded through 20 to 24 additional population doublings, yielding from 1×10^6 to 16×10^6 cells, but the cells in the transfectant clones from the second transfection can only undergo a few population doublings before they senesce. This early senescence is not unexpected since Holliday *et al* (1977) showed that the life span of cells is reduced by 10 to 15 population doublings if they are cloned, a phenomenon that is sometimes referred to as a "bottleneck effect".

TRANSFORMATION OF INFINITE LIFE SPAN HUMAN FIBROBLASTS BY TRANSFECTION OF ONCOGENES

Since these *ras* transfectants were approaching the end of their life span when injected into athymic mice, we reasoned that they may simply not have possessed sufficient replicative capacity to form tumors. Therefore, we and our colleagues set out to generate an infinite life span human fibroblast cell strain that would be otherwise normal. The most common method of generating infinite life span human cell lines is to infect them with SV40 (Sack, 1981) or transfect them with plasmids, such as pSV3, that contain the early region of SV40, including the region coding for large T-antigen (Chang, 1986). However, human fibroblasts that express T-antigen exhibit changes in morphology, become aneuploid, and exhibit anchorage independence, and since these are also the characteristics of tumor-derived cells, such altered cells have limited usefulness in studies designed to gain insight into the step-wise changes required for a normal cell to become a malignant cell.

In our attempt to generate an infinite life span human fibroblast cell strain that had undergone only minimal changes, we were guided by the work of Weinberg and his colleagues (Land *et al*, 1983) who showed

that transfection of rat embryo fibroblasts with a v-myc oncogene increased the frequency at which the cells developed into infinite life span cell strains. We transfected early passage, foreskin-derived normal human fibroblasts with a plasmid carrying the neo gene and a v-myc gene. The transfectants were selected for Geneticin resistance and clonally-derived cell strains were isolated and propagated for many generations. Eventually all cell strains senesced, but among the senescing progeny of one cell strain clones of viable cells could be seen. These eventually gave rise to an infinite life span cell strain that we designated MSU-1.1 (Morgan et al, 1991). These cells have a normal fibroblastic morphology, do not form foci or colonies in soft agar, display a near-diploid karyotype of 45 chromosomes including two distinctive marker chromosomes, and do not form tumors in athymic mice. The karyotype has remained stable, still showing the identical pattern more than 200 generations since its origin. The MSU-1.1 cells were analyzed using a battery of "paternity tests" and shown to be derived from the parental cell strain that had been used for transfection. They were also shown to express the v-myc gene (Morgan et al, 1991).

The infinite life span MSU-1.1 cells were then used as the recipient cells for transfection with plasmids containing H-ras (Hurlin et al, 1989), N-ras (Wilson et al, 1990), or K-ras oncogenes (Fry et al, 1990). Because the recipient MSU-1.1 cells express the neo gene, the transfectants were identified by their ability to form foci of morphologically transformed cells on a background monolayer of fibroblastic cells. Cells isolated from the foci were analyzed and found to express the ras protein of the transfected gene. The transformed cells exhibited the

same altered characteristics found in the ras-transformed finite life span diploid fibroblasts described above, but in addition they made progressively-growing, invasive sarcomas when injected into athymic mice. Cells isolated from the tumors had a human karyotype, contained the two distinctive marker chromosomes of MSU-1.1 cells, and were Geneticin resistant as expected.

Our interpretation of these experiments was that a suitable expression level of a ras oncogene in this infinite life span human fibroblast cell strain was sufficient to bring about malignant transformation. To be sure that the MSU-1.1 cell line was not unique, we (Hurlin et al, 1989) transfected two other infinite life span human fibroblast cell strains (KMST-6 and GM637) with the plasmid carrying the H-ras oncogene in the same vector construct. The KMST-6 cell strain, which arose following repeated radiation treatment (Namba et al, 1985) and the GM637 strain which arose following SV40 infection and subsequent immortalization, are highly aneuploid, morphologically transformed, and capable of forming foci and colonies in soft agar, but they do not form tumors in athymic mice. Following transfection with a plasmid carrying the H-ras oncogene and a neo gene, the transfectants were selected for resistance to Geneticin. When the transfectants were expanded into large populations and injected into athymic mice, they formed progressively-growing, invasive sarcomas. Since the KMST-6 and GM637 cells do not express myc, the results suggested that it was the infinite life span phenotype of the MSU-1.1 cells, rather than their expression of myc, that complemented the expression of the H-ras oncogene and allowed malignant transformation.

The results of these studies demonstrate that human fibroblasts are not refractory to transformation, as was previously thought. They suggest that for such cells to become malignantly transformed in the human body, they must undergo repeated clonal selection to yield cells that express the appropriately activated proto-oncogenes. The use of transfection techniques to transform these cells in culture was especially helpful since it allowed us to directly identify a specific transformed phenotype with the expression of a specific dominantly-acting oncogene.

NUMBER OF CHANGES REQUIRED FOR MALIGNANT TRANSFORMATION

One of the major interests of workers in the field of carcinogenesis is to determine the number and kinds of independent changes required for normal cells to become malignant. Studies using human cells in culture can be useful for answering such questions. But if results obtained in culture are to be applied to the problem of the mechanisms that operate to cause human cancer, it is necessary to demonstrate that the process in culture recapitulates what occurs in humans. From our studies and that of many other investigators it seems clear that acquiring an infinite (or very greatly extended) life span in culture is a prerequisite if a cell is to acquire sequentially all the changes needed to become malignant. Whether this is the case for cells in the human body is not known for certain. What is known is that cells derived from malignant human tumors sometimes give rise to infinite life span cell lines when placed in culture, but cells from normal tissues never do so.

If the process we are studying can be applied to human cancer, then our results suggest that at least five changes must take place before a cell becomes malignant. This number derives from the fact that the finite life span diploid human fibroblast cell lines transformed by ras oncogenes were not malignant, whereas the MSU-1.1 cells transformed by the same ras constructs were malignant. Two changes were involved in converting the H-ras or N-ras gene into a transforming oncogene for these cells. First, the proto-oncogene had to acquire a mutation in a specific codon, and second it had to be overexpressed so that the level of ras gene product present in the cell was three to seven-fold higher than normal. The H-ras or N-ras oncogene in a low expression vector was ineffective (J. J. McCormick, unpublished studies). Generation of the MSU-1.1 cell strain from its normal diploid parental cell line involved acquisition of at least three changes. We do not yet know if expression of the transfected v-myc gene in these cells played a role in generating their infinite life span phenotype. But if it did, it was not directly responsible since the clonally-derived cell populations that went into crisis and senesced expressed the same level of myc protein as the clonal cell population that survived senescence and gave rise to the MSU-1.1 cells. What is clear, however, is that MSU-1.1 cells possess two marker chromosomes which apparently arose independent of one another, and one of these arose as a result of partial chromosomal trisomy, a third event (Morgan et al, 1991). Studies to determine the necessity of such changes if a cell is to be malignantly transformed are in progress (see below).

In the course of the above studies with the MSU-1.1 cell strain, we examined stocks of cells from the original myc-transfectant that had

been frozen during the time that the cells were senescing to determine when the cells with the two unique marker chromosomes first appeared. We found that in a stock frozen early there was a pure population of diploid cells. These cells were designated MSU-1.0. An intermediate passage taken from the freezer was found to contain two populations, one diploid, like MSU-1.0; the other identical to MSU-1.1. Both the MSU-1.0 and MSU-1.1 cell strains have undergone more than 200 population doublings since their siblings senesced, without any change in chromosome complement. Both express the v-myc protein and have the same integration site for the transfected v-myc and neo genes. The MSU-1.0 cells, like normal diploid fibroblasts, cannot grow without exogenously-added growth factors. The MSU-1.1 cells grow moderately well under the same conditions, and grow to a higher saturation density than MSU-1.0 cells. Since the chance of human cells acquiring an infinite life span in culture is very rare, the data suggest that MSU-1.1 cells are derived from MSU-1.0 cells. We recently began ras oncogene transfection studies with this diploid cell strain. The data obtained so far are not conclusive, but they suggest that transfection of a ras oncogene in a high expression vector is not sufficient to transform MSU-1.0 cells into the malignant state.

ROLE OF GENETIC INSTABILITY IN MALIGNANT TRANSFORMATION

All of the malignant H-, K-, or N-ras transfected derivatives examined have exhibited the stable karyotype of the parental MSU-1.1 cells. The cells form characteristic malignant tumors in athymic mice and have proven positive in experimental metastatic studies (Hurlin et al, 1989; Fry et al, 1990; Wilson et al, 1990). The cells derived from these tumors have the same stable karyotype as the transfectant precursor cells injected.

We have also found rare spontaneous clonal variants of MSU-1.1 that are malignantly transformed and have shown that malignant variants can also be induced by carcinogen treatment. Exposure to carcinogen caused a dose-deperdent increase in foci formation, and cells from such foci grew to a higher density in medium containing 1% serum than did the MSU-1.1 cells they were derived from. A substantial fraction of these focus-derived strains proved to be malignant. Unlike the H-, K-, or N-ras-transfected malignant MSU-1.1 cell strains or the cells from their tumors, the carcinogen-induced focus-derived or spontaneous malignant transformants each exhibited unique chromosome changes in addition to the marker chromosomes of the parental strain. These data indicate that activated ras oncogenes, even when expressed at high levels, do not cause genetic instability of the parental strain. They further suggest that spontaneous and/or carcinogen-induced oncogene activation (or tumor suppressor gene inactivation) commonly takes place as a result of major chromosome alterations.

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