IN VITRO MODEL SYSTEMS OF CARCINOGENESIS: INTERACTION BETWEEN c-Ha-ras ONCOGENE AND IMMORTALIZED CELLS

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UDC 616-006.6-092-092.4

KEY WORDS: immortalization; oncogenes; transfection; transformation.

In vivo carcinogenesis has been shown to be a multistage process [4]. There is sufficiently convincing experimental evidence that malignant transformation of cells in vitro is also the result of successive activation of several (at least two) oncogenes [7, 11]. Attempts have been made to create model cell systems with which to study this process at the molecular level. One of the most convenient models for this purpose consists of primary rodent cells transformed through transfection with two oncogenes [7, 8]. In a simplified form the functions of oncogenes which participate in transformation processes can be reduced to the following: an immortalizer gene of the myc type, the early gene of adenoviruses, and the gene of the large T-antigen of polyoma and SV40 virus bring about "immortality" of the cell, whereas the transforming gene, ras for example, terminates this process, inducing malignant conversion of the immortalized cells [7, 11]. Data obtained in various world laboratories are evidence that transformation of primary rodent cells by one oncogene, such as c-Ha-ras, is impossible [9, 14], although morphologically normal NIH3T3 mouse cells are readily transformed by this gene [5]. Despite the fact that much experimental evidence on the cooperative action of two oncogenes has been gathered, the mechanism of transformation of the primary cells by them has been inadequately studied. In particular, tumors arising in syngeneic and athymic (nude) animals after receiving injections of cells transformed by two oncogenes, are small in size and rapidly regress [8].

The aim of this investigation was to create an experimental cell system simulating the separate stages of the multistage process of carcinogenesis, using a selection of tumorigenic variants of transformed cells in animals. Primary rat embryonic fibroblasts were chosen as the initial cells, for we know that they have an extremely low frequency of spontaneous transformation in cell culture [10], and a transformation inducer, namely the c-Ha-ras oncogene, for it is one of the most active transforming genes [8]. As a result it was shown that a single insertion of the c-Ha-ras oncogene into the genome of rat cells of the Fisher REF-1 line, subjected to long-term transplantation, and immortalized by the early gene of simian adenovirus SA7, does not induce their morphological transformation. On repeated transfection of this oncogene complete morphological transformation is observed and the cells possess high oncogenic potential for animals.

Preparation of an immortalized Fisher rat embryonic cell line as a result of transfection with plasmid containing genes of the early region of simian adenovirus SA7 was described previously [3]. REF-1 cells were transfected with plasmid pEJras 6,6 [12], containing the c-Ha-ras oncogene, by the usual method [15]. Plasmid DNA was used in a dose of 3-4 μg per 10^6 cells. NIH3T3 cells were used for the control. The transformed cells were selected by their ability to form tumors in nude mice after injection of the whole pool of transfected cells into them ($1 \cdot 10^6$-$2 \cdot 10^6$ cells/mouse).

As a result, rapidly growing tumors (NIH3T3 pEJras 6,6) appeared after 12 days in nude mice which had received injections of control NIH3T3 cells, transfected with plasmid pEJras 6,6 DNA. By contrast with this, in mice into which REF-1 cells, transfected by this same plasmid DNA, were implanted, no tumors appeared even after 3 months of observation.

Some of the transfected REF-1 cells were left on the dishes and incubated with a change of medium for 3 weeks. During this time changes were observed in the character of growth and the morphology of these cells, namely the appearance of foci of denser growth, consisting

Research Institute of Carcinogenesis, All-Union Oncologic Scientific Center, Academy of Medical Sciences of the USSR, Moscow. Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 105, No. 3, pp. 329-332, March, 1988. Original article submitted November 12, 1987.

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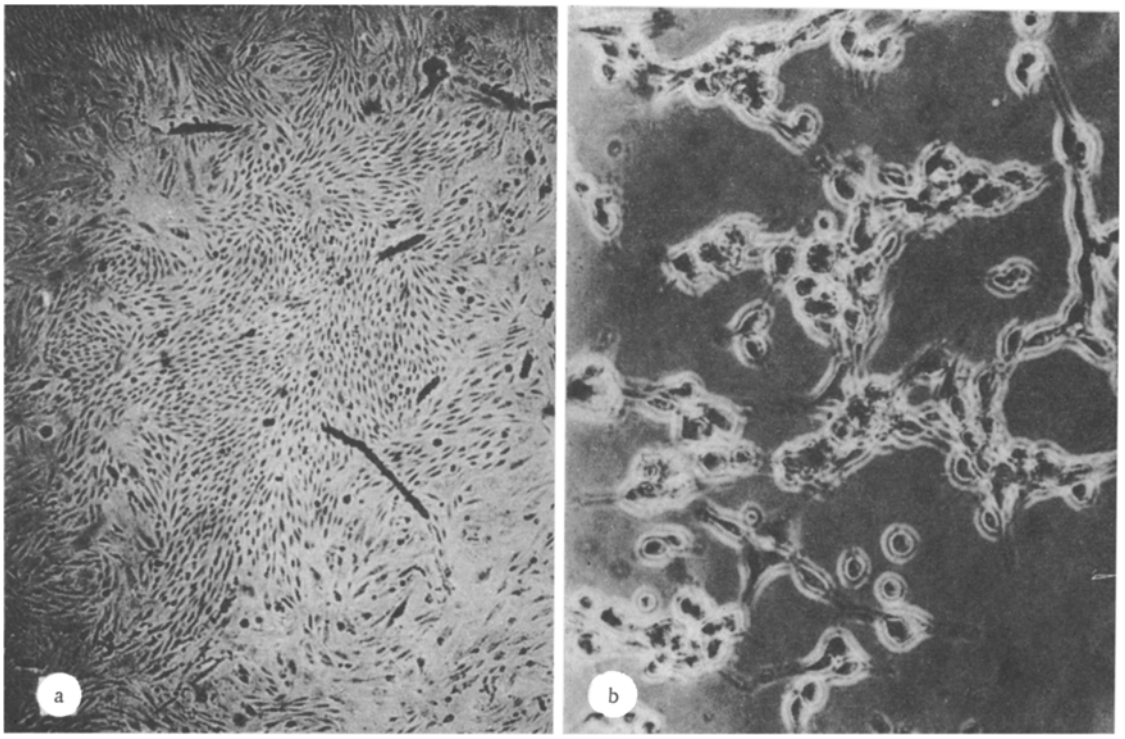
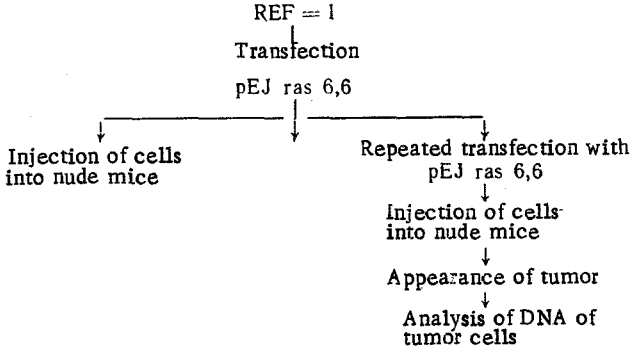


Fig. 1. Cell morphology: a) REF-IEJ (28×); b) REF-2EJ (140×).

of elongated cells (Fig. 1a). These cells, designated REF-1EJ, were seeded after 3 weeks with a coefficient of 1:4 and subcultured continuously for 5 months.

Analysis of the biological properties of the REF-1EJ, cells revealed that, like REF-1 cells, they are unable to grow on semisolid agar. After long-term subculture in vitro the REF-1EJ cells were transfected again with plasmid pEJras 6,6 DNA. The levels of transfection were similar to those described above, and the whole pool of transfected cells was injected into nude mice. After 16 days the experimental animals developed rapidly growing tumors, readily transplantable into animals. Cells of one of the tumors were transferred into an REF-2EJ culture and maintained by passage in vitro. Their morphology was characteristic of transformed cultures (Fig. 1b). To rule out the role of long-term passage in vitro of intermediate REF-1EJ cells as a factor increasing sensitivity to the ras oncogene, the initial REF-1 cells were transfected with plasmid pEJras 6,6 DNA, and 3 days later, the whole pool of transfected cells was divided into two parts without any additional passage (Scheme 1).

SCHEME 1



One part of the cells was injected into nude mice, the other was transfected with plasmid pEJras 6,6 DNA, after which these cells were inoculated into nude mice.

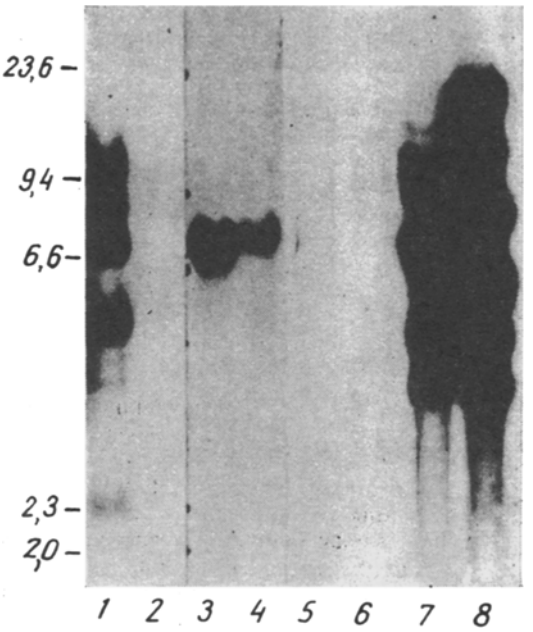


Fig. 2. Autoradiography of blot-hybridization of DNA from various cells. 1) NIH3T3pEJras 6,6; 2) NIH3T3; 3) NIH3T3T24; 4) human lymphocytes; 5) normal rat liver; 6) REF-1EJ; 7) REF-2EJ (1); 8) REF-2EJ (2); probe ^{32}P-pEJ-ras 6,6. Exposure 48 h. 10 μg of each DNA was treated with endonuclease BamHl and subjected to electrophoresis in 0.8% agarose. Hybridization carried out at 42°C in buffer: 50% formamide, 4 × SSC, 2.5 × Deinhard's solution, 100 mg/ml of yeast tRNA, 10% dextran sulfate 40.

As a result of this, REF-2EJ tumors (two) were obtained only in the group of animals injected with REF-1 cells, transfected twice with the ras oncogene. In none of the groups of experimental animals were tumors observed to appear throughout the long period of observation.

For transformation of intermediate REF-1EJ cells, prolonged culture of these cells in vitro was not required.

To determine the fate of the ras oncogene after transfection, DNA was extracted from cell lines and tumors of concern to us, by means of the guanidine thiocyanate method [1]. These DNAs, after restriction by the enzyme BamHl, were analyzed for the presence of Ha-ras sequences by Southern's method [13]. Figure 2 gives the results of hybridization of the test DNAs with the ^{32}P-pEJras 6,6 probe. DNA from NIH3T3 cells, transformed after transfection by DNA from human T24 bladder carcinoma cells (track 3) and DNA from normal human lymphocytes (track 4) were used as positive controls. It will be clear from Fig. 2 that in both cases a diagnostic Ha-ras fragment with mol. wt. of 6600 base pairs was discovered. During hybridization of the probe which we used with DNA from normal mouse (track 2) and rat (track 5) cells, only after a long period of exposure were minor bands of hybridization found at 3800 and 8800 base pairs respectively. These results can be explained by the limited homology between the human Ha-ras gene, used as the probe, and endogenous sequences of the Ha-ras oncogene in the mouse and rat genomes. Because of this, the human Ha-ras gene can be easily discriminated in genomes of rodent cells. Numerous hybridization bands (track 1), due both to plasmid sequences and to the endogenous Ha-ras oncogene, were found in DNA from a tumor arising in a nude mouse injected with NIH3T3 cells after their transfection by pEJras 6,6 plasmid, in agreement with data obtained by other workers [14]. In REF-1EJ cells which, as was pointed out above, differ from the original REF-1 cell line and cannot induce tumors in nude mice, no pEJras 6,6 sequences could be found after hybridization with the above-mentioned probe (track 6). However, during analysis of DNA from REF-2EJ (1) and REF-2EJ (2) tumors, multiple fragments hybridized with the ^{32}P-pEJras 6,6 probe were detected on the autographs (tracks 7 and 8).

The results thus suggest that it is integration of the Ha-ras oncogene that leads to transformation of the REF-1 cells.

The investigations showed that single transfection of REF-1 cells by Ha-ras oncogene does not lead to their transformation. Only after a second insertion of this oncogene does tumorigenic conversion of these cells take place. The problem connected with the absence of this particular oncogene in the genome REF-1EJ cells remains open. There may evidently be two causes leading to this effect. First, comparatively rapid elimination of integrated pEJ-ras sequences. This possibility of disappearance of integrated sequences while the cells preserve their biological characteristics has been demonstrated in principle for a number of systems [2, 6]. Second, primary transfection evidently modifies certain membranous structures, thereby increasing the sensitivity of the cells to subsequent transfection by the Ha-ras oncogene.

The REF-1 — REF-1EJ — REF-2EJ system presented above is thus a convenient model with which to study the multistage process of carcinogenesis in vitro, for primary rat fibroblasts undergo immortalization, which is followed by additional morphological changes during single transfection with Ha-ras oncogene and, finally by complete transformation as a result of a second transfection with this oncogene.

The authors are grateful to Dr. Med. Sci. E. S. Revazova for constant attention and support in the course of this work.

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