PRODUCTION OF A RAT EMBRYONIC FIBROBLAST CELL LINE BY DNA TRANSFECTION OF THE EARLY REGION OF SIMIAN ADENOVIRUS SA7

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UDC 612.6.-014.2-06:[
578.826.1:578.264.2

KEY WORDS: immortalization; oncogenes; transfection; superoxide dismutase.

Normal mammalian cells are known to have a limited life span *in vitro* [9]. After a certain number of divisions the cell passes into a "crisis" stage and dies. Sometimes some cells survive and acquire the ability for unlimited division, forming so-called stable or immortalized cell lines [14]. The formation of a line from a normal cell population is a rare event, and for that reason the conditions making such formation possible are being intensively studied. One approach to the production of immortalized lines is transfection of certain genes of retroviruses and DNA-containing viruses into primary normal mammalian cells. These genes include the MC-29 (myc virus oncogene [10]), the gene of the large T antigen of polyoma virus and of SV-40 [14], and also the gene of the early EIA region of human adenoviruses [15]. The products of these genes are known to be able to interact with cellular DNA, to cause immortalization of the cells [10, 14, 15].

In this investigation an attempt was made to obtain an immortalized cell line of normal rat fibroblasts after transfection with DNA from the early region of simian adenovirus SA7.

EXPERIMENTAL METHOD

A primary culture of embryonic rat fibroblasts was obtained by trypsinization of 18-24-day Fisher rat embryos. The cells were maintained on DMEM medium (from Gibco) with the addition of 10% embryonic calf serum. At the second or third passage the cells were transfected with DNA from the early region of simian adenovirus SA7 from plasmid pAA13, generously provided by B. S. Naroditskii.

DNA pAA13, in a quantity of 250 ng, was mixed with 100 mg of NIH 3T3 cellular DNA as coprecipitant and precipitated with $CaCl_2$ by the method in [16]; 0.5 ml of the precipitate was applied to a 70% monolayer of primary rat fibroblasts at the rate of $5 \cdot 10^5$ cells per 60-mm dish. The cells were incubated with the precipitate for 4 h at 37°C in medium with 5% CO_2. The medium was changed after incubation. The cells were maintained on the dishes for 14 days, the medium being changed periodically. After 14 days the cells were trypsinized and subjected to prolonged passage with a coefficient of 1:4. DNA from NIH 3T3 cells was used as the control.

The efficiency of growth of the cells in semisolid agar was verified by determining their ability to form colonies in 0.38% agar with the addition of 10% embryonic serum; the cells were counted after 2 weeks.

The tumorigenicity of the cells was determined by transplantation of the transformed cells into 14- and 30-day nude rats and 2-month nude mice, at the rate of $2 \cdot 10^6$ cells per animal.

Superoxide dismutase (SOD) activity was determined by the usual method [5] in cell homogenates prepared by the method described previously [2].

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0007-4888/86/0002-0219$12.50

Fig. 1. Morphology of cell line REF-1 (40×).

EXPERIMENTAL RESULTS

To obtain a stable cell line of normal rat embryonic fibroblasts, plasmid pAA13, containing an insert of the early region genome of simian adenovirus SA7 [1], was used. On the 14th day after transfection of the primary embryonic rat fibroblasts with plasmid DNA the cells were trypsinized and then subjected to continuous passage. Control cells, transfected with NIH 3T3 cell DNA only, reached the "crisis" stage at the 7th-8th passage and died. By contrast, cells transfected with pAA13 DNA divided continuously and are still being maintained in culture (line REF-1).

Cells of this line have fibroblast-like morphology which is almost indistinguishable from the original embryonic culture, their density of growth is low, and they possess contact-inhibited properties (Fig. 1).

Cells of line REF-1 have gone thrhough more than 40 passages (about 160 duplications) which is more than the conventional limit (120 duplications) after which cells are considered to be immortalized [14].

Ability to divide indefinitely is one of the main characteristics of transformed cells. There are several criteria by which cells can be divided into normal and transformed. The principal biological criteria of transformed cells are: 1) ability to grow in semisolid agar [11]; 2) ability to grow in medium with a low serum concentration [4]; 3) tumorigenicity for syngeneic or nude animals [7]. Cell line REF-1 was accordingly tested by the above criteria.

These cells were shown not to form colonies in 0.38% agar. This is evidence that they have preserved their status of normal cells.

A study of the ability of REF-1 cells to grow in medium with 1% embryonic serum showed that they were virtually indistinguishable from the original primary rat fibroblasts as regards their requirement of growth factors contained in serum (Fig. 2). These results are in good agreement with those obtained by other workers, who also showed that good growth of immortalized Syrian hamster cells depends on a high concentration (10%) of embryonic serum [12].

After transplantation of REF-1 cells into nude animals, irrespective of the times of *in vitro* passage, these cells did not induce tumor formation in either rats or mice. This distinguishes them from the NIH 3T3 cell line, widely used in transfection experiments, which can still induce tumors after prolonged *in vitro* passage, 8-10 weeks after injection into nude mice [3].

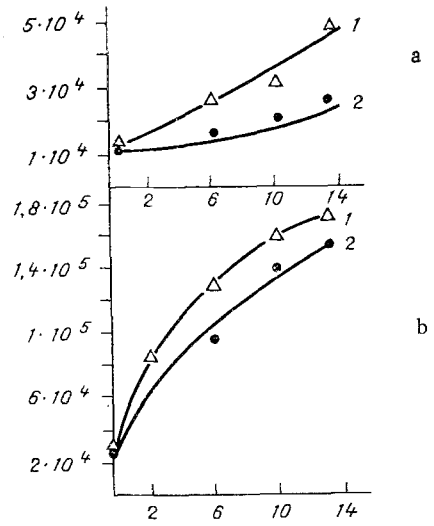

Fig. 2

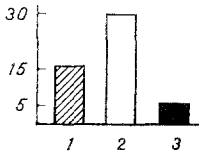

Fig. 3

Fig. 2. Effect of serum concentration in medium on growth of cells. Abscissa, time of counting cells (in days); ordinate, number of cells in 1 ml. 1) Cell line REF-1, 2) original primary rat embryonic fibroblasts. *a*) 1% embryonic serum in medium, b) 10% embryonic serum in medium.

Fig. 3. SOD activity (in units/mg protein) in normal, immortalized, and transformed cells. 1) Original primary embryonic rat fibroblasts, 2) cells of line REF-1, 3) transformed NIH 3T3 cells after transfection with DNA from human tumor V-251 MG.

Immortalized cells, according to the biological criteria, are thus normal cells which have crossed Hayflick's limit [9].

SOD activity is evidently another criterion by which normal, immortalized, and transformed cells can be distinguished. This enzyme metabolizes superoxide radicals in accordance with the reaction:

$$O_2^- + O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \qquad (8)$$

Previously, when SOD activity was compared in normal and transformed cells it was shown that activity of this enzyme is depressed in the latter [13]. It is an interesting fact that in NRK rat cells, transformed by oncogenic viruses, depression of SOD activity also is observed, wheras the SOD level returns to normal in revertants of these cells [6].

Analysis of SOD activity in immortalized REF-1 cells showed that on the formation of the cell line SOD activity in them increased, which distinguishes them from both normal and transformed cells (Fig. 3).

It can be concluded from the results that cells transfected with the early region gene of simian adenovirus SA7 become capable of unlimited growth and, as a result, of forming a cell line. The phenotype of the cells remains normal under these circumstances. It will be noted that the REF-1 cell line provides a good model with which to study the biological, biochemical, and molecular-biological characteristics of cells at one of the intermediate stages of malignant transformation, and, because of its biological properties, it can also be used as recipient cells in transfection experiments on various eukaryote genes.

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CORRELATION BETWEEN CHANGES IN DNA STRUCTURE AND SYNTHESIS INDUCED IN MOUSE L1210 LEUKEMIA CELLS BY 1-METHYL-1-NITROSOUREA AND 1,3-BIS-(2-CHLOROETHYL)-1-NITROSOUREA

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UDC 616.155.392-07:616.155.32-008.
939.633.2-02:615.277.3:547.495.4

KEY WORDS: L1210 leukamia; DNA synthesis and structure; 1-methyl-1-nitrosourea; 1,3-bis-(2-chloroethyl)-1-nitrosourea.

Experimental data showing significant differences in the molecular mechanisms of action of the methyl and chloroethyl derivatives of N-nitrosourea, which differ in the spectrum of their antitumor activity, have been published in recent years. Methyl derivatives are most effective in the treatment of first generation spontaneous mouse mammary gland tumors, whereas chloroethyl derivatives are most effective in the treatment of experimental leukemias [6, 12].

Methyl and chloroethyl derivatives of 1-alkyl-1-nitrosourea (NAU) are nowadays widely used in combination chemotherapy of malignant neoplasms. Many years of systematic study of the mechanism of action of these compounds has shown that NAU in aqueous solutions, and under close to physiological conditions, are unstable and decomposed spontaneously to form chlorodiazohydroxides and isocyanates, which can alkylate and carbamoylate biomacromolecules [3, 6, 11]. In addition, the possibility of enzymic degradation of NAU (hydroxylation and denitrosation) has been demonstrated experimentally [2].

This paper describes a comparative study of changes in DNA structure and synthesis in mouse L1210 leukemia cells induced *in vitro* by 1-methyl-1-nitrosourea (MNU) and by 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU).

EXPERIMENTAL METHOD

Experiments were carried out on (C57BL × DBA2)F_1 hybrid mice weighing 18-21 g, inoculated intraperitoneally with mouse L1210 leukemia cells ($2 \cdot 10^5$-$3 \cdot 10^5$ cells per mouse). MNU was dissolved in 0.9% NaCl and BCNU in 10% ethanol immediately before injection. MNU was injected in doses of 20 and 80 mg/kg and BCNU in a dose of 8 or 20 mg/kg, by a single intraperitoneal injection on the 5th day of leukemia development. DNA synthesis was studied by recording incorporation of 2-^{14}C-thymidine or deoxy-2-^{14}C-uridine. The experiments and analysis of the results were carried out in accordance with the scheme described previously [1, 5]. Defects in the secondary structure of DNA (single breaks and alkali-labile regions) after injection of MNU and BCNU were determined by centrifugation in an alkaline sucrose gradient [13]. To analyze

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 0007-4888/86/0002-0222$12.50