ONCOLOGY

Different Tumorigenicity of Cell Clones Derived from Stromal Fibroblasts of Human Colon Cancer Xenografts

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The tumorigenicity of cell clones derived from fibroblast lines isolated from colon cancer xenografts is studied in thymus-free animals. During cloning of the cell line obtained from the 3rd passage of the xenograft about 20% of the clones proved to be nontumorigenic, whereas such cells were not found in the line obtained from the 89th passage. Cytogenetic analysis of nontumorigenic clones revealed monosomy for the 13th chromosome with no alterations in the other chromosome pairs. Hybridization for the presence of Alu sequences was negative.

Key Words: *fibroblasts; xenografts; thymus-free animals: transformation; immortalization*

The use of immunodeficient animals as model systems for human tumors has demonstrated that transformation of animal stromal tissues into tumors occurs in some cases (although quite rarely) [5,7,9]. This phenomenon was generally revealed in by accident and was observed both during tumor passaging on animals and after graft transfer under *in vitro* conditions [9]. Investigation of the mechanisms leading to the induction of fibroblast transformation in stromal xenografts has shown that cell fusion (tumor cell/fibroblast) [5], primary transfection of DNA [7], and retrovirus activation [3,6,14] occur in some cases. However, there is evidence indicating that not these mechanisms are involved, but rather humoral factors secreted by tumor cells, which may affect the transformation of stromal elements in close proximity [9,11]. Two fibroblastlike cell lines were isolated from the stroma of human tumor xenografts passaged in thymus-free animals [1]. Cell line FM-7 was derived from a strain of colon cells (C_c-7m) passaged on thymus-free mice. Injection of these cells into thymus-free mice induced tumors histologically characterized as fibrosarcomas. The murine genesis of these cells was confirmed by karyotyping. On the assumption that the interaction between C_c-7m cells and stromal cells results in transformation, these cells were injected into thymus-free rats, and the fibroblast like cell line FK-7 was obtained. These cells proved to be tumorigenic in thymus-free mice and produced fibrosarcomas. Karyotyping supported by negative results of hybridization of the Alu sequence confirmed the rat genesis of these cells and indicated that their genome contains no human DNA.

In the present study we investigated the heterogeneity of the lines FK-7 and FM-7 using cloning followed by testing for tumorigenicity. For this purpose we performed karyotypic analysis with staining for G-bands of the tumorigenic clone FK-7k13.

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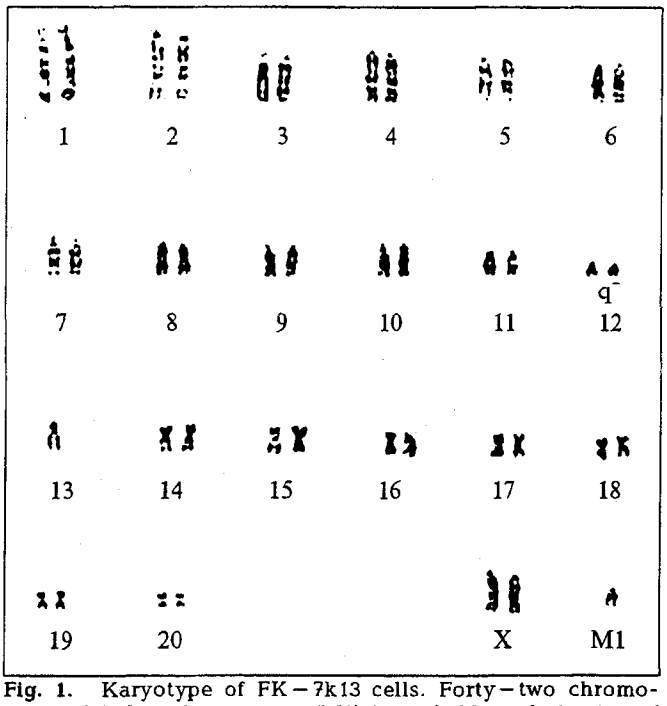

Fig. 1. Karyotype of FK−7k13 cells. Forty−two chromosomes. Marker chromosome (M1) is probably a derivative of chromosome 13.

MATERIALS AND METHODS

Cell cultures were grown at 37°C in MEM medium with a double set of amino acids and 8% fetal calf serum, 2 mM L-glutamate, and 0.08 mg/ml gentamicin. Cells were cloned by the method of limiting dilutions in conditioned medium supplemented with 20% fetal calf serum with the subsequent addition of the suspension to 96-well plates and monitoring of the cell count 2 h later. The wells containing one cell were used in further studies. Cell cultures in the logarithmic phase of growth preincubated for 2 h in medium containing 0.2 ng/ml colchicine were used in cytogenetic analysis. After hypotonic treatment with 0.56% NaCl for 35-40 min the cells were fixed with an acetic acid: ethanol (1:3) mixture, and the modal class of chromosomes was determined on 100 metaphase plates after conventional Giemsa staining. Differential staining for G-disks was performed as described [10]; cytogenetic analysis was performed according to the recommendations for rat karyotype standardization [4]. For tumorigenicity studies cells (10^7) were inoculated in thymus-free mice (subcutaneously, the subscapular area), and tumor formation was followed-up during an 8-week period. Thymus-free mice bred on the basis of BALB/c mice aged 4-6 weeks were used in the experiments. DNA for blot hybridization was isolated by cell lysis with 1% SDS followed by deproteinization with phenol-chloroform. Restriction, electrophoresis, blotting, and immobilization of DNA were performed was described elsewhere [2]. Hybridization with pBlur8 plasmid (^{32}P-dNTP labeling during nick-translation) with a specific activity of 10^8 cpm/μg DNA was performed in the presence of 50% formamide. Prehybridization, washing, and autography were performed as described previously [2].

RESULTS

Since in our previous studies we did not find human DNA in the genome of FK-7 cells [1], we assumed that there is an epigenic interaction between tumor and stromal cells with their subsequent transformation. In this context it can be speculated that the stromal cell population of C_c-7 grafts is heterogenic in tumorigenicity. To test this possibility the 8th generation of FK-7 cells and 16th generation of FM-7 cells were cloned, and isolated clones were tested for tumorigenicity on thymus-free mice (Table 1). After a 2-month observation period all clones isolated from the FM-7 line proved to be tumorigenic, and 4 clones isolated from the 18th FK-7 line were not tumorigenic, and their immortalized status was demonstrated after further passaging (more than 30 generations).

In order to determine possible genetic alterations we performed cytogenetic analysis of the FK-7k13 clone. The modal class of the cells was represented by 41 chromosomes in 65% of the analyzed cells. The modal region with the variation interval of 41-42 chromosomes included 80% of the cells.

Karyotyping of FK-7k13 cells with the use of differential staining for G-bands showed that morphologically unaltered chromosomes of all pairs were present in practically all cells, with the exception of chromosomes 12 and 13: one of the homologs was either lost or involved in transformation. The culture was characterized by monosomy of chromosome 13. It can be assumed that one of the markers is a derivative of this chromosome. Only one morphologically unaltered chromosome 12 was present in some cells. Deletion of part of the long arm 12q- occurred in the second chromosome or, probably, translocation of part of the long arm of chromosome 12 to its short arm had taken place (Fig. 1).

Thus, karyotype analysis after differential staining of chromosomes for G-bands revealed that the studied cell culture is characterized by a hypodiploid karyotype with markers of varios origin.

Heterogeneity of cell clones did not exclude the possibility of transformation via cell immortalization as the first step of transformation. There-

fore, taking into account the small proportion of nontransformed cells in the FK-7 cell population (according to the results of cloning), we have to consider whether the negative dot-hybridization of the Blur probe with total DNA [1] might not be due to the impossibility of revealing human DNA owing to the limitations of the method. Therefore, DNA from the nontumorigenic clone FK-7k13 was analyzed for the presence of Alu sequences. Blot-hybridization with plasmid Blur-8 demonstrated the presence of hybridizing sequences only in the positive control. Autography revealed no signal in the DNA of FK-7k13 cells, which indicated the absence of human DNA in the studied cell genome.

Human tumor xenografts growing in thymus-free and immunosuppressed animals have a mixed cell population consisting of malignant primary tumor cells and animal stromal cells. Animal fibroblasts acquire a malignant phenotype that can be revealed both *in vivo* and *in vitro* with subsequent fibrosarcoma formation [3,9].

A study of tumor-induced transformation of stromal fibroblasts in xenografts yields no definite conclusion regarding the mechanisms of this phenomenon, which may be quite rare [3,12]. For example, possible fusion of tumor and normal cells with the subsequent loss of certain chromosomes and selection of tumorigenic cells has been demonstrated in a number of studies [5,8]. Primary transfection of the DNA of a human small-cell lung cancer xenograft in murine stromal cells was also shown [7]. However, this mechanism of transformation was not revealed in a number of other cases [9,11], including the studied cell lines [1]. It could be that human DNA was not detected in these cell lines due to its elimination from the genome of stromal cells; however, from the present study of early generations of the xenograft C_c-7k and the cell line FK-7 it can be concluded that these mechanisms are not likely to operate in these cells, since Alu sequences are not revealed by hybridization.

Malignant transformation may be associated with epigenic factors. It is likely that transforming growth factors produced by numerous human tumors [13] together with cell-cell interactions may cause phenotypic transformations of fibroblasts and human tumor xenografts [9]. This suggestion is supported by the evidence obtained by researchers who had immortalized normal fibroblasts from thymus-free mice by long-term passaging using growth medium conditioned by a small-cell lung cancer cell line [11]. Phenotypic transformations were observed upon culturing of mouse fibroblasts with human tumor cells [9]; however, transformed cells rapidly reverted to the original normal phenotype after the tumor cells were removed from the culture. From this we can conclude that the transformed cell lines FM-7 and FK-7 are heterogeneous regarding their tumorigenicity. In fact, cloning of FK-7 cells showed that 4 out of 18 clones exhibit no tumorigenic potential on thymus-free mice and are immortal, as was revealed upon their subsequent passaging *in vitro*. The lack of heterogeneity of the FM-7 cell population in terms of tumorigenicity in thymus-free mice, which was revealed upon cloning, may be due to the number of generations the xenograft passed through before a primary culture was obtained. Since substitution of stromal cells occurs only at the first passages of a xenograft, while during further passaging stromal cells grow from the cells passaged along with the tumor, they are exposed to the influence of tumor cells during the entire period of strain passaging. The marginal zone cells may be the sole exception; however, their percentage is small. The fact that the FM-7 cell line was isolated from the 89th generation of the strain, whereas the FK-7 line was isolated from the 3rd generation, accounts for these differences in the proportion of tumorigenic and immortal clones.

There is, however, another possibility: the cells of nontumorigenic clones attained the transformed status by progressing from the immortalized status that they had acquired either as a result of fusion with human tumor cells or due to primary transfection followed by selection of transformed cells. In this case hybridization with Alu sequences of DNA isolated from the entire population could have been negative due to limitations of the method and the small proportion of immortalized cells in the FK-7 cell population. However, blot-hybridization with the nontumorigenic clone

TABLE 1. Tumorigenicity of Clones from Cell Lines FK−7 and FM−7 after Injection in Thymus−Free Mice

Initial cell line	Number of isolated clones	Number of injected cloned cells per mouse	Tumorigenic/ nontumorigenic clones
FM−7 (the 6th generation)	16	5×10^6	16/0
FK−7 (the 8th generation)	18	5×10^6	14/4

Note. The animals were observed for at least 8 weeks from the day of cell injection. If there was no tumorigenicity, the experiment was repeated on two animals.

FK-7k13 also proved to be negative, providing additional evidence of the absence of human DNA in the genome of FK-7 cells.

Taken together, these findings do not rule out the possibility that these phenomena have to do with the instability of transformed cells. Thus, cytogenetic analysis of the immortalized nontumorigenic clone FK-7k13 showed that this clone is characterized by monosomy for the 13th chromosome and the absence of alterations in other chromosomal pairs. These data suggest that monosomy 13 may be the cause of immortalization of rat fibroblasts. However, we cannot rule out that the specific features of the karyotype of FK-7k13 cells demonstrated in this study result from cell selection during their passaging *in vitro*. For example, there were no changes in the ploidy of three immortalized rat fibroblast lines isolated from the tumor stroma [15].

From our results we can conclude that the interaction between human tumor cells with stromal cells in C_c-7 xenografts may lead to malignant transformation of the latter and that this is not due to the transfer of genetic material from human cells. Study of genetic characteristics of stromal fibroblasts showed heterogeneity of the cell population in terms of tumorigenicity upon their isolation from early passages of the xenograft. This model is interesting for further investigation of the interaction between various cell populations in tumors.

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