The Transformation by Simian Virus 40 of Cells from Patients with Mucopolysaccharidosis and from Normal Controls

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SUMMARY

Fibroblasts derived from individuals with mucopolysaccharidosis, an inborn error of metabolism, have been found to be more easily transformed by simian virus 40 than are cells derived from normal individuals. The increased susceptibility does not seem to depend upon changes in glycoprotein at the cell surface. Repeated observations were necessary to demonstrate these differences, and we do not believe that this test is suitable for routine screening for cancer susceptibility.

INTRODUCTION

It has been suggested that individual susceptibility to cancer can be measured by subjecting cultured skin fibroblasts to a transformation assay involving SV40, since cells derived from some patients who have an inborn susceptibility to cancer may transform more easily than those derived from normal subjects. First described in 1962 (6, 13), the transformation of human fibroblasts by SV40 was quantitated by Todaro et al. (16) who were able to show that cells from patients with Fanconi's anemia, a disease associated with a high risk of certain kinds of cancer, were many times more susceptible to transformation than were cells from normal control patients or those with diseases not associated with cancer. Fibroblasts derived from patients with Down's syndrome (trisomy 21), which is associated with a high risk of leukemia, also proved to be more susceptible to transformation by SV40 (17, 20) as did cells obtained from patients with trisomy 18 (17) or trisomy 13 (12) and from cancer patients with either XY/XXY mosaicism (9) or XY gonadal dysgenesis (10). High levels of transformation have also been associated with colon cancer (11), myelogenous leukemia (14), and a "sarcoma-prone" family (15), although an anomalous "transformation-prone" family (3) has also been reported.

Studies on xeroderma pigmentosum, however, yielded inconsistent results (2, 18), and those on cells derived from patients with primary immunodeficiency diseases indicated that the latter do not have a greater susceptibility to transformation (5, 7). This is in agreement with the hypothesis that the increased incidence of cancer manifest in these patients is due to poor immunosurveillance.

Thus syndromes associated with an increased SV40 transformation rate are associated with abnormalities of the chromosomes manifested as trisomy, chromosome aberrations, or inappropriate chromosomal sex. Aaronson (1) has, however, reported that differences in cell susceptibility disappear when SV40 DNA is used as the transforming agent instead of the intact virion suggesting that interaction with the cell genome may not be the most important factor in determining differential susceptibility to transformation. It was thought to be of interest, therefore, to study fibroblasts with altered cell surfaces rather than with large errors of chromosome content. Fibroblasts from patients with gangliosidosis or mucopolysaccharidosis present an altered biochemical environment to any invading virion. As interaction with the cell surface must be the first event in any virus replication mechanism, such an alteration must affect the initial infection step. It was also thought to be important to test the efficacy of the method as a potential tool for the detection of cancer-susceptible individuals by examining its reproducibility in greater depth than had hitherto been reported.

MATERIALS AND METHODS

Cell Culture. Fibroblast cultures were established as outgrowths from skin biopsies obtained from children clinically diagnosed as suffering from MPS and from normal children of comparable age. In general the latter biopsies were obtained from patients undergoing cosmetic surgery. Initial establishment of the culture involved maintenance of 0.5-sq mm skin fragments held under a glass coverslip in a 5-cm Petri dish containing 5 ml of Ham's F-10 medium supplemented by 20% FCS and antibiotics (penicillin and streptomycin, 100 IU/ml each). After approximately 3 to 4 weeks the outgrowth was sufficiently advanced for routine trypsinization and monolayer maintenance in medium containing 10% FCS. Routine 1:2 subcultures took place at confluence at approximately weekly intervals, and all transformation experiments were performed on cells between the 5th and 12th passes. Methanol-fixed fibroblasts obtained from patients with MPS I, II, or III (but not MPS IV or control patients) showed metachromatic granule-containing vacuoles when stained with metachromatic or toluidine blue (4). Fibroblasts before virus infection were chromosomally normal.

Virus Stocks. SV40 stocks were obtained by infection of...
SV40 Transformation of Human Fibroblasts

BSC-1, an established line of African green monkey kidney cells. The virus pool, titrated in BSC-1 cells in roller tubes and also by plaque titration, was diluted to $2 \times 10^5$ PFU/ml and stored in 1-ml aliquots at $-70^\circ$. The original SV40 virus seed was a gift from Dr. C. W. Potter.

**Transformation Assay.** Actively growing subconfluent monolayers (5 $\times$ 10$^5$ to 1 $\times$ 10$^6$ cells/5-cm dish) were washed 3 times with PBS and 0.4 ml of SV40 (2 $\times$ 10$^5$ PFU/ml) was added (i.e., approximately 1000 PFU/cell). The monolayers were infected at 37$^\circ$ with “rocking” every 15 min to ensure even distribution of the virus. Control dishes were mock infected with 0.4 ml PBS. After 3 hr the unadsorbed virus was removed, the infected monolayer was washed 3 times with PBS, and 5 ml of medium with 10% FCS were added to each dish. After 24 hr the cells were suspended with trypsin and replated at 5 $\times$ 10$^6$ cells/dish so that the infected fibroblasts were able to undergo several divisions before confluence was reached. The cultures were maintained with weekly medium changes containing 10% FCS at first but dropping to 5% serum after 3 weeks when the monolayers were confluent. After 6 to 7 weeks, when individual foci of transformed cells could be observed with the naked eye, the medium was removed, and the cells were washed with PBS, fixed in Bouin’s fixative for 20 min and stained with Giemsa. After the cells were washed in running water and dried, the transformed foci were counted. Areas of cells where any ambiguity arose were not scored as foci.

**Plating Efficiencies.** Both SV40-infected and control cells were plated at 2 $\times$ 10$^6$ cells/5-cm dish at 24 hr postinfection. After a further 24 hr the cell numbers were estimated using a Coulter counter.

**Adsorption of SV40.** BSC-1 cells (10 $\times$ 9-cm dishes) were infected with stock SV40 in the presence of $[3H]$thymidine, 10 $\mu$Ci/ml. Labeled virus was harvested by cell sonic disruption followed by low-speed centrifugation, and the supernatant was separated on a CsCl cushion between $\rho$ 1.2 and $\rho$ 1.4. The resulting band, which lay at the interface between the layers, was centrifuged in a CsCl gradient from $\rho$ 1.32 to $\rho$ 1.38. The fraction at 1.34 corresponding to the peak of $^3H$ activity was dialyzed against at least 4 changes of 0.01 M Tris-HCl in 0.9% NaCl solution, pH 7.2, for 24 hr. It was then used to infect fibroblasts as described above. After 3 hr the cells were washed 3 times in PBS, removed from the dishes, and adsorbed onto glass fiber filters; their radioactivity was then determined.

**RESULTS**

Transformation assays were carried out on cells from 6 patients with MPS I to III, 6 matched controls, and 9 other subjects who were either homozygous or heterozygous for certain autosomal recessive diseases. Considerable variation was found between replicate dishes and between repeat observations at different times on the same cells. Table 1 shows the mean transformation rates obtained from control patients, Table 2 shows the rates from patients with MPS, and Table 3 shows the rates obtained for other abnormal subjects.

No foci were ever observed on control plates. We should expect that the estimation of numbers of foci on plates follows an approximately Poisson distribution, but as the numbers of individual observations are large then it is possible...
The cells from the individuals with MPS have a mean transformation rate of 4.5 ± 1.4 (S.D.) foci/dish, while the value obtained for the controls was 1.5 ± 0.9 foci/dish. This difference in transformation rate is significant to the 0.1% level, when a simple t test is applied to the mean values obtained for different individuals.

Such an analysis does not, however, reveal the extent of the variability between replicate observations. It can be shown that the controls with the highest and lowest transformation rates do not differ significantly and that the results from MPS patients with the highest and lowest rates also do not differ significantly. All the values obtained for normal individuals can then be treated as one population and all those from the MPS cases as another, and if these 2 populations are compared then the weighting of results obtained by calculating means of means is avoided.

The control patients' value then becomes 1.6 ± 1.8 foci/dish and the value for the MPS patients becomes 4.6 ± 3.1 foci/dish. When the 2 populations are compared they are, however, still significantly different at the 5% level although the considerable variation is now apparent. Transformation frequencies for fibroblasts obtained from patients with Morquio's syndrome, ataxia telangiectasia, Gaucher's disease or GM1 gangliosidosis all lay within the normal range, as did the values obtained for 2 of 3 obligatory heterozygotes for MPS I. The raised values obtained for 2 cases of cystic fibrosis and the 3rd obligatory heterozygote should be further investigated. Cells from the proband that led to the identification of this particular carrier were not sufficiently viable for a study to be made to see whether their transformation rate was higher than that of the other MPS patients, but the raised transformation rate may not be due to her heterozygous state but to some other factor contributing to individual variability as in the transformation-prone family (3).

The fibroblasts derived from the patient with MPS IV, which is not manifest in skin fibroblasts, lay as would be expected within the normal range.

When a line of cells was allowed to age beyond passage 25 before being examined for susceptibility and compared with early-passage cells from the same line, a considerable increase in mean transformation rate was observed. Over several estimations the transformation rate was found to increase from 0.7 ± 0.2 foci/dish at passage 2 or 3 to 4.6 ± 2.1 foci/dish for passages 25 to 30. These figures are significantly different at the 0.1% level.

Infection of skin fibroblasts with SV40 has little effect on their plating efficiencies at 24 hr postinfection indicating that, even at a multiplicity of 1000 PFU/cell, massive cell death did not result.

Adsorption studies with [3H]thymidine-labeled SV40 indicated that there was no increase in the ability of fibroblasts from MPS or gangliosidosis patients to adsorb virus when compared with the control cells from normal individuals. The BSC-1 line, which is permissive for SV40, showed a 5-fold higher adsorption of [3H]thymidine-labeled SV40. The input multiplicity in the adsorption studies was not 1000 but 5 PFU/cell. At this level of infection approximately 10% of the input virus or 0.5 PFU/cell was adsorbed by the BSC-1 cells and 2% of the input virus or 0.1 PFU/cell was adsorbed by skin fibroblasts. Such a reduction in input virus should, however, serve to emphasize any difference in adsorption by different cell strains rather than to mask it.

DISCUSSION

Although significant differences were found between the transformation rates of fibroblasts derived from patients with MPS and those from control patients, these were measured over a large number of experiments. Considerable variation was observed between replicate cultures and between experiments carried out on different days with the same cells.

When, as a control on the accuracy of the assay method in our hands, we set out to detect differences in transformation rates between young and aged cells derived from the same individual as had been reported but not quantitated by Todaro (15), a 6-fold increase was found for cells at passage numbers greater than 25 when compared to those at passage numbers less than 4. Thus not only must control individuals be age matched in any attempt at screening but also the fibroblasts themselves must be strictly comparable in passage level before results can be taken as meaningful.

A major difficulty encountered in the production of reproducible results was that of maintaining cell monolayers for the 6 to 7 weeks necessary for large unmistakable foci to emerge. While this was relatively easy with the healthy well-growing control cells, the MPS and other metabolically abnormal monolayers required special care to ensure that they survived. In some cases, after 3 to 4 weeks, the monolayers of MPS cells were in an unsatisfactory condition and so were discarded. If cancer susceptibility is accompanied by some fragility of the fibroblasts, then experimental reproducibility of foci may become impossible as it did with several of our poorly growing MPS cell lines.

To be certain that differences of the magnitude observed in these studies are real and not the consequence of variations between experiments, repetition of the observations is essential. It is felt, therefore, that this test could not be used as a screening procedure to detect individuals who are susceptible to cancer. The procedure itself is time consuming and if multiple replications, or even repeat experiments, are required then the whole procedure would be too unwieldy to be used as a routine. Exceptional cases where there are large differences, such as those found in Fanconi's anemia (16, 19), would of course be detected more easily. Another possible drawback of this procedure as a test is that there is no firm evidence of a regular correlation between predisposition to cancer and high transformation rate; e.g., the frequency of cancer in the transformation-prone family of Aaronson and Todaro (3) has not yet been reported.

There is an approximately 3-fold increase in the frequency with which cells from patients with MPS acquire the properties of transformation after infection with SV40, when compared with normal cells. The magnitude of the increase is thus comparable to that found for trisomy 21 fibroblasts (17,
There is, however, no increase in the adsorption of SV4O by these metabolically abnormal cells, nor were their plating efficiencies after infection better than those of control fibroblasts. The increased transformation rate of the MPS cells must arise therefore from an increased sensitivity at some point in the relationship between the cell and the virus subsequent to adsorption.

Transformation studies with SV4O DNA and whole virus adsorption experiments caused Aaronson (1) to conclude that, while an event involving the SV4O coat was responsible for differences in susceptibility of human fibroblast strains to SV4O, such differences must occur at the level of penetration or uncoating of the intact virion, rather than at the adsorption step. Our findings on chromosomally normal cells tend to reinforce the conclusion that the increased susceptibility noted in chromosomally abnormal cells is not necessarily due to the abnormality of the chromosome per se but may be attributable to a cellular imbalance caused by qualitative or quantitative changes in DNA content. The relationship between the viruses and the cell must therefore be complex with both early events after infection and interaction between the virus genome and the cell genome playing important roles in determining the transformation event.

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