Further Studies on SV40-Induced Transformation in Human Renal Cell Cultures. I. Eventual Failure of Subcultivation Despite a Continuing High Rate of Cell Division.* (28986)

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Earlier reports(1,2,3) have described induction by simian virus 40 (SV40) of a reproducible transformation in primary cultures of human renal (HK) tissues which is characterized by an abnormal morphology and growth pattern, a greatly accelerated growth rate, chromosomal aberrations, and an altered cytopathic response to infection with several RNA viruses. Previously(2,3) we reported that the morphology and growth characteristics of SV40-transformed HK cells remained essentially unchanged after 10-20 serial subcultivations. Subsequently we have noted that after 20-30 passages, subcultures of 7 transformed lines included in these initial studies could be effected only at progressively longer intervals and in each case failed after 25-42 passages. Similar observations were made with 7 transformed lines derived from more recent experiments(4), including a cloned derivative of one line, and with 2 different transformed lines of the earlier study that were frozen and stored at —70°C and then thawed and passaged. Accordingly, experiments here described were undertaken to determine possible factors that might account for the eventual cessation of growth of the transformed HK lines.

Materials and methods. Transformed human renal (HK) cell cultures. SV40-transformed HK cells were derived from SV40-inoculated primary human fetal, newborn, 3-week and 6-week old renal cell monolayer cultures as previously described(1,2).

Maintenance and subcultivation of transformed cells. Procedures were the same as previously described(2).

Procedures for karyological analyses were as described elsewhere(5).

Results. Morphological changes in transformed HK cells after prolonged subculture. It was noted that transformed cells in later passages presented a characteristic morphology which differed strikingly from that observed in earlier passages. To compare in a systematic fashion morphological features of cultures at different stages of subculture, cells of several transformed HK lines were fixed and stained with haematoxylin and eosin at different passage levels.

(a) *Early passages.* In the earlier passages, nuclei varied widely in size and shape, ranging from circular to slightly oval and containing from one to 6 or more nucleoli (2). The cells uniformly exhibited an epithelioid morphology and, although cells varied considerably in diameter, variations exceeding the average by 3- or 4-fold were rare. The mitotic index was less than 5% as determined by counts in 4 lines, and the great majority of mitotic figures appeared normal. Multinucleated cells were extremely rare and nuclear "budding" (see below) was not observed.

(b) *Later passages.* From passages 10-20, certain new abnormalities appeared which increased in frequency as passages were multiplied. Of these the most striking was a marked increase in numbers of multinucleated cells and cells with single giant nuclei which were larger than any previously observed (Fig. 1). The nuclear outline in certain cells exhibited out-pocketings which became progressively larger and often appeared to be pinched off ("budding"), giving rise to multiple daughter nuclei, frequently of different size, which usually remained clumped together (Fig. 2a, 2c). In the terminal passages, "budding" and multinucleated cells were the most common abnormalities noted, comprising an estimated 10-30% of the cell population. These phenomena tended to occur more frequently in certain areas in which multilayering was absent. In contrast with those in earlier passages, many cells assumed

* Supported in part by research grant from U.S.P.H.S.

an elongated fibroblastoid configuration in which the nucleus was either round, oval, or greatly elongated in outline (Fig. 1). In many areas of terminal passages cell size and morphology varied so widely that not more than 2 cells of similar appearance appeared contiguously. The proportion of mitotic figures in later passages varied widely from line to line and even in different areas of single cultures. Mitotic figures were more frequent in heaped-up than in monolayered areas, were not seen in budding nuclei, and only very rarely in multinucleated cells. As determined by repeated counts in terminal passages of 5 lines, mitotic figures comprised from 5 to more than 10% of the cell populations. In about half of the transformed cell lines, most of the mitotic figures did not appear abnormal except for the wide variation in size and extreme rarity of anaphasic and telophasic forms. In the other lines, however, the majority of mitotic figures exhibited a wide range of abnormality including multiple metaphase plates and displacement of chromosomes distant from the metaphase plate (Fig. 3). Despite the increasingly sparse growth of later passages, the cellular pattern remained random(2,3), and even in the last passages cells in scattered loci continued to form microscopically apparent multilayers. The overall impression gained by examination of the cells in later passages was of an increasing rate of aberrant nuclear division associated with a declining rate of growth.

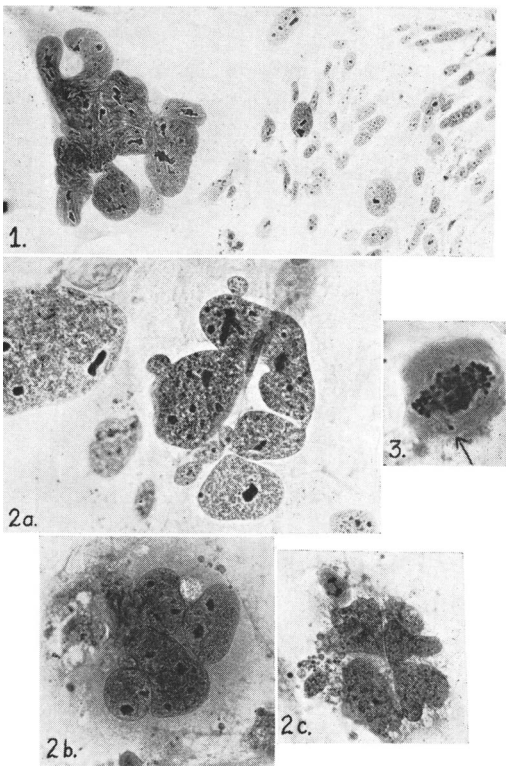

FIG. 1. Characteristic area from 32nd passage of an SV40-transformed HK line in which subcultivation failed after 42nd passage. Contrast the multiple clumped giant nuclei of varying sizes apparent on one side of microphotograph with the smaller, more uniform H. and E. stain nuclei on the other. (160×)

FIG. 2a-2c. Abnormal nuclear morphology in terminal passage (42nd) of the same SV40-transformed HK line as in Fig. 1. H. & E. stain. (460×). 2a. Budding, multiple nuclei and a giant single nucleus. A smaller single nucleus in one corner of microphotograph approximates the size characteristic of the more uniform nuclei observed in earlier passages. 2b. Clumped, budding nuclei. Note pinching-off of nuclei of widely varying sizes. 2c. Clumped nuclei undergoing degenerative changes. Abnormal mitotic figure is also apparent.

FIG. 3. Abnormal mitotic figure from 40th passage of an SV40-transformed HK line. Note disorganized pattern of chromosomal separation and displacement of one chromosome distant from the others. H. & E. stain. (460×)

Presence of infective SV40 in later passages. Supernatant fluids from 12 of the 14 lines studied were assayed in grivet monkey kidney cultures for the presence of infectious SV40(1). In the initial passaged lines, infective virus persisted in each to the terminal passages in titers varying from $10^{-1.0}$ to $10^{-2.5}$. In the more recent lines, infective virus was demonstrated consistently in 2 lines, in one of 2 attempts in a third line and not in either of 2 attempts in each of 2 other lines.

An exceptional transformed cell line.† One

† Subsequent studies have confirmed that this "exceptional" line is also SV40-transformed. In collaboration with Dr. Albert B. Sabin, an SV40-specific complement-fixing antigen distinct from that contained in the virus has been demonstrated in these cells. In addition, karyological studies on this line in collaboration with Dr. Yerganian have revealed a human chromosomal complement, with a subdiploid modal frequency of 40 chromosomes absent from groups 21-22 and 13-15. Infectious SV40 has been demonstrated at several passage levels in this line which has now been carried for 65 passages without apparent diminution of growth potential.

apparent exception was observed to the limited capacity of transformed HK cells to undergo serial subculture. In the 25th passage of a line of transformed newborn HK cells, the population was so small that subculture was not feasible. After about 2 months there appeared among the cells of the kind previously described a mass of smaller, more rounded and homogeneous cells with vacuolated cytoplasm which rapidly overgrew their neighbors. These newly appearing elements were readily maintained throughout 20 subcultures and have continued to exhibit a low, apparently non-increasing incidence of the nuclear aberrations observed in other lines.

Chromosomes in terminal passages of transformed HK cells. Previously reported karyological studies of cells derived from 3 transformed HK lines during passages 6-19, a period of rapid growth, indicated that, although the total number of chromosomes in these cells remained normal or nearly normal (*i.e.,* 46 or 45 chromosomes), most of the cells exhibited monosomy in one or more chromosomal types and trisomy in others so that the chromosomal complement was either pseudo-diploid or aneuploid(2,5). It was of interest to determine whether the decreased growth rate of transformed HK cells during later passages was associated with more extreme chromosomal aberrations. To this end, in collaboration with G. Yerganian of the Children's Cancer Research Foundation, karyological studies of transformed HK cells in terminal passages were initiated. These will be reported elsewhere. Preliminary karyological observations on cells derived from terminal passages of 2 such lines have revealed more marked deviation from the normal chromosomal complement than was observed in the previous studies. The majority of metaphase figures thus far examined from both lines have contained 38-42 chromosomes, a strikingly subdiploid complement. Marker chromosomes have not been observed in either line.

Discussion. Significance of failure of transformed cells to persist in subculture. The changing morphology of SV40-transformed HK cells on prolonged subcultivation, and the eventual failure of these cells to persist in subculture appear to provide the first example of which we are aware of a tumor-virus transformed cell which cannot be subcultured indefinitely. In this respect these cells resemble certain "normal" human cells which have also been reported to exhibit consistent limitation of growth upon serial cultivation(6). However, the factors limiting subcultivation are apparently not the same in these 2 cases since the eventual failure of "normal" cells to multiply is accompanied by decreasing mitotic activity(6), whereas growth of the SV40-transformed cells is associated with an increasing incidence of nuclear division which is frequently aberrant.

The transformed cells have not yet been shown to be oncogenic in experimental hosts (7) and accordingly, they may also eventually provide an example of cells with morphology and growth characteristics altered by a tumor virus *in vitro* which are not neoplastigenic *in vivo.* In this regard, it is of interest that SV40-transformed Syrian hamster kidney (SHK) cells, which are capable of inducing tumors *in vivo*(8,9,10), and are apparently capable of indefinite cultivation *in vitro*(11), exhibit, under the same conditions of subcultivation as SV40-transformed HK cells, similar bizarre nuclear aberrations, but in lower incidence, which have not been seen to involve progressively the entire cell population(9,11). Accordingly, future analytic comparison of these two cell systems may lead to understanding of factors permitting a stable level of aberrations that may be related to the maintenance of capacities for oncogenesis and for unlimited subcultivation.

Mechanism of eventual failure of transformed HK cells to persist in subculture. The causes of the observed increasing incidence on subculture of abnormalities in transformed HK cells remain obscure. Several possibilities are: (1) Continuing action of the virus, in complete or incomplete form, may further derange nuclear components. (2) The original viral-induced alteration may initiate a progressive nuclear instability, unassociated with further direct action of the virus. Evidence of increasing karyotypic derangement as passages are continued may be regarded as consistent with either of these mecha-

nisms. (3) Conditions of subcultivation employed may selectively favor substrains of transformed cells which are of high instability and eventually non-viable.

It is not known whether SV40 virus remains associated with all transformed HK cells so that it is not possible to decide between the first two possibilities. However, recent reports indicate continued association of the viral genome with all or almost all SV40-transformed Syrian hamster cells(12, 13). Since it seems unlikely that the basic mechanism of transformation is dissimilar in the two systems, these findings suggest that the SV40-genome continues to be associated with all transformed HK cells and accordingly may eventually be responsible for the extremely bizarre nuclear aberrations (nuclear budding, mono and multiple giant nuclei) observed in late passages. This concept is supported by the fact that similar aberrations are noted in SV40-infected Syrian hamster kidney (SHK) cells less than a week after viral inoculation before morphological transformation is clearly evident(9,11), as well as in such cells after transformation has occurred(9,11). These findings, however, do not explain why the incidence of these aberrant forms in transformed human cells continues to increase while remaining constant in transformed hamster cells.

The third possibility mentioned above, *i.e.*, that the specific conditions of subcultivation permitted the selection of unstable, eventually non-viable cell forms, is being explored by studies on the effects of altered conditions of subcultivation‡. That such selection alone is responsible appears *a priori* unlikely since the evidence just mentioned suggests essential participation of the virus.

It remains to be determined whether the "exceptional" transformed cell line represents an unusual type of SV40-induced transformation, a "spontaneous" transformation of the sort occasionally observed in apparently virus-free cultures, or a contamination with other lines carried in the laboratory.†

Summary. With a single exception human renal cells transformed by simian virus 40 exhibited eventual failure to undergo serial subcultivation. An increasing rate of aberrant nuclear division was observed concomitantly and may therefore be the immediate factor responsible for the declining growth rate.

‡ In studies on 2 transformed lines not mentioned in the text, in which the rate of subcultivation was varied and in other studies in which various types of media (Eagle's basal, Enders', BAF and Puck's) were employed, eventual failure of the cells to undergo subcultivation occurred.

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Received October 9, 1963. P.S.E.B.M., 1964, v115.