Preneoplastic Phenotype and Chromosome Changes of Cultured Human Bloom Syndrome Fibroblasts (Strain GM 1492)

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ABSTRACT

The Bloom syndrome fibroblast strain, GM 1492, was examined for phenotypic properties generally associated with neoplastic cells. A serial clonogenicity assay indicated that these cells can proliferate in culture, achieving approximately twice the number of population doublings as compared to normal human skin fibroblasts. Strain GM 1492 appeared to be partially transformed in that these cells showed a slight degree of anchorage independence when grown in methylcellulose, and also appeared to have relaxed growth requirements compared to normal fibroblasts. GM 1492 cells are heteroploid, with 20 to 80 chromosomes/cell and a modal chromosome number of 44. Cytogenetic analysis of G-banded metaphase chromosomes indicated that most cells contained at least one copy of each normal human chromosome, and many cells exhibited only aneuploidies with no detectable chromosomal rearrangements. Minute chromosomes were seen in a few of the metaphase cells examined. GM 1492 cells did not form tumors in athymic nude mice. Since many of the characteristics of GM 1492 cells are similar to those seen only in tumor cells, but the strain is nontumorigenic, we suggest that GM 1492 cells are preneoplastic and thus represent an ideal system for the in vitro study of human neoplastic progression.

INTRODUCTION

Individuals with the rare autosomal recessive human disorder, BS, are highly predisposed to various forms of cancer (1). Dividing cells derived from BS patients show a greatly increased number of SCEs (2), and BS fibroblasts show an elevated spontaneous mutation rate over that of normal human fibroblasts (3). Although this elevated SCE frequency and increased mutation rate have been postulated to be involved in the etiology of neoplasia (4), no specific mechanisms have been shown to link them with cellular changes that lead to the neoplastic phenotype.

One particular BS fibroblast strain, GM 1492, has been suggested to be atypical of BS strains since in addition to elevated SCEs it shows an increased frequency of chromosome aneuploidies and rearrangements (5). Initial characterization of early passage cells of this strain, as reported by the Human Genetic Mutant Cell Repository, Camden, N.J., indicated that these cells were 46,XY. Some recurring chromosome rearrangements were observed in GM 1492 cells (5, 6); however, these cells generally tended to generate many variable karyotypes.

Work in this laboratory has involved the analysis of the evolution of the malignant phenotype in different lineages of cultured Chinese hamster cells (7, 8). Although spontaneous neoplastic progression is frequently observed in rodent cell cultures, it is rarely seen in cell culture of normal human cells. Cells from human tumors, however, usually have similar neoplastic characteristics as cells from cultured animal tumors (9). These characteristics include: (a) unlimited proliferative ability ( immortalization); (b) transformed phenotype as defined by anchorage independence and relaxed growth factor requirements; (c) formation of tumors in nude mice; and (d) an unstable karyotype.

The few cases of spontaneous transformation of human cells in culture that have been reported were from “normal” cells derived from patients with neoplasia (10–12). Since BS patients tend to develop cancer and the BS fibroblast strain, GM 1492, has been reported to have an unstable karyotype, we chose to examine these cells in culture to determine if the cellular characteristics of GM 1492 can be related to the neoplastic process.

In this report, we describe the results of our examination of GM 1492 cells for the four characteristics described above that are associated with cultured tumor cells. These data suggest that the BS fibroblast strain, GM 1492, is preneoplastic because the cells exhibit many of the phenotypic characteristics commonly associated with tumor cells.

MATERIALS AND METHODS

Cell Strains. BS fibroblasts, GM 1492D and GM 2520A, were obtained at passages 19 and 12, respectively, from the Human Genetic Mutant Cell Repository, Camden, NJ. The normal human fibroblast strain, HSF-7, was initiated from a primary foreskin culture by Dr. David Chen (Los Alamos National Laboratory), and obtained for use in these studies at passage 4. The CHO line, which has been carried in this laboratory for many years, was originated from Chinese hamster cells in the laboratory of Dr. T. Puck (13).

Cells were grown in αMEM (Grand Island Biological Co.) containing penicillin-streptomycin plus 10% fetal bovine serum. Cultures were maintained at 37°C in 5% CO2–95% air. Cells were split before they became confluent by gentle treatment with 0.125% trypsin and 0.2% EDTA. All cells were routinely tested for mycoplasmal contamination as described previously (14). No contamination was found for any cell type reported here.

Assays for Proliferative Ability. A serial clonogenicity assay was done for GM 1492 and HSF-7 cells by methods described elsewhere (28). Briefly, the earliest passage cells available were plated on twenty 100-mm tissue culture plates (Coming) at 1000 cells/plate in 20 ml medium each. These plates were placed in the incubator and were not moved for a minimum of 10 days. Examination on an inverted microscope allowed identification of the largest colonies on each plate. At least twenty of the largest colonies were then transferred individually to separate 100-mm plates, and the assay was continued in a likewise fashion until no more colonies grew. At least three plates at each step in...
the assay were stained for quantitation of colonies. Medium was re-
moved, the plates were rinsed twice in 0.9% NaCl, fixed in 100% methanol, and then stained in 10% Giemsa (Gurr's; Biomedical Special-
ties). Five large colonies were counted for cell number from one Giem-
sa-stained plate in each series to give the mean number of cells per colony for that series. Likewise, the number of large colonies per entire Giem-
sa-stained plate was determined for each step in the series. Colony forming
efficiency was then determined at each step of the assay by dividing the
mean number of cells per colony into the number of large colonies per
plate at the subsequent step in the series (see Table 1).

Transformation. To detect cell growth in 1 versus 10% fetal bovine
serum, cells were resuspended after trypsinization in αMEM containing
1% fetal bovine serum and cell density was determined by counting an
aliquot on a Coulter Counter. The mean number of cells per flask after 7 days' growth in
αMEM containing 1% fetal bovine serum was added to three of the flasks and 25 ml of αMEM
containing 1% fetal bovine serum was added to the other three flasks. Cells were grown at 37°C in 5% CO₂ for 1 wk, and the total number of
cells per flask was determined by counting an aliquot on a Coulter Counter. Cells (5 x 10⁴) were then dispersed into
0.1 ml αMEM containing 10% fetal bovine serum and 1.3% methyl-
cellulose (Matheson, Coleman, and Bell) over a layer of 0.9% agar (Difco-
mine the percentage of growth for both serum concentrations.

Table 1

<table>
<thead>
<tr>
<th>Step in serial clonogenicity assay</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
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<td>0.25</td>
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<td>52.3</td>
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a PD, population doubling.

b Colony forming efficiency in C = A/B = no. large colonies at subsequent step in series

chromosome changes are discussed below.

Proliferative Ability. A serial clonogenicity assay was used to
detect colony forming potential from a low density cell population
of GM 1492 or HSF-7 cells. Table 1 shows the results of this
serial clonogenicity assay. Cellular morphology of the large
colonies remained "normal" for each fibroblastic strain until the final
step in the clonogenic assay. Even though the number of steps in the series varied between the two cell strains examined,
cells from both strains showed classically senescent character-
istics at the final stage of the assay; colonies contained few cells,
all of which were large and granular compared to the earlier
colonies. A single exception to this observation was in the last
series of plates of GM 1492, where one colony was observed
after staining to contain many cells that did not appear senescent in
morphology.

The number of population doublings can be estimated from
the number of cells per colony at each step in the serial clono-
genicity assay, as shown in Table 1. Adding these numbers to
the approximate number of doublings of the cell strain before
the start of the assay, one obtains an estimate of the maximum
number of population doublings. In this manner, we calculated
the maximum number of population doublings for GM 1492 cells
to be approximately 98, and for HSF-7 cells to be about 50. In a
similar experiment, we examined another BS fibroblast strain,
GM 2520, and determined the maximum number of population doublings to be 36. These numbers appear to be consistent with our observations of senescence in HSF-7 and GM 2520 in mass culture. Likewise, GM 1492 cells grown in mass culture appear to senesce at approximately 110 population doublings.

**Transformation.** Two criteria used to define cells as "transformed," are (a) the ability of the cells to grow equally well in media containing 1% fetal bovine serum as in 10% serum, and (b) anchorage independence of the cells, i.e., the ability of the cells to form colonies in methylcellulose. By these criteria, GM 1492 cells can be defined as "partially transformed." These cells grew 30% as well in 1% fetal bovine serum as they did during the same time-span in 10% serum, while HSF-7 cells did not grow at all in 1% fetal bovine serum and (transformed) CHO cells grew equally well in 1 and 10% fetal bovine serum. When grown at low density in 1% fetal bovine serum, GM 1492 cells produced colonies at an efficiency of approximately 5%. Some of these colonies appear to be expandable in low serum concentrations.

Fig. 1 shows the formation of intermediate sized colonies from GM 1492 cells in methylcellulose as compared to the large colonies of a transformed cell (CHO) and no colonies from the (untransformed) HSF-7 cells. These intermediate sized colonies resulted from 0.16% of the GM 1492 cells 3 wk after plating; the assay yielded 100% large colonies from CHO cells in the same time, indicating that the methylcellulose assay was valid and not toxic to cells. The colonies formed from GM 1492 cells did not appear to increase in size, nor did more appear, even after an additional 3-wk incubation with fresh methylcellulose and media.

**Tumorigenicity.** Approximately $1 \times 10^7$ GM 1492 cells at passages 20, 30, and 45 were injected into preimplanted gelatin sponges in athymic nu/nu mice. There is no evidence of any tumor formation within the 8-mo observation period routinely used in this laboratory.

**Chromosome Changes.** In our initial characterization of the cell strain GM 1492 we confirmed the elevated frequency of SCEs that is typical of BS fibroblasts (Fig. 2a). After counting 15 metaphase cells with SCEs for each cell strain, we calculated the frequency of SCE per chromosome to be 1.22 (SE = 0.077) for GM 1492 and 0.12 (SE = 0.005) for HSF-7.

GM 1492 cells were heteroploid in chromosome number, with 20 to 80 chromosomes/cell observed. The cells also tended to be hypodiploid, with a modal chromosome number of 44 (Fig. 3). Analysis of 60 metaphase cells in Fig. 3 indicates that the majority of cells are near-diploid (2N = 46) while a few cells are near-tetraploid. This near-tetraploid population was also observed by flow cytometric analysis of DNA content (data not shown).

Further demonstration of the chromosome variability from cell to cell in strain GM 1492 is seen in the G-banded karyotypes shown in Fig. 4. With the exception of missing Y chromosomes in Fig. 4, cells a and c, at least one of all normal human homologues are represented in these karyotypes. Fig. 4, cells a, c, and d represent near-diploid cells, while cell b is a near-tetraploid. Table 2 shows chromosome complements of 15 G-banded karyotypes. From Table 2, it can be seen that more than one-half (8 of 15) of the cells analyzed resembled those in Fig. 4a in that they differed from the normal karyotype only in chromosome number; rearranged chromosomes were seen in the other 7 cells examined. In comparing the cells karyotyped for
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Table 2, it appears that the chromosomes most frequently missing are 13, 16, and 22. The karyotypes in Fig. 4c and d show the only examples of recurring chromosome changes observed: 16 q+ and 17 dup(q12 → q25). It should also be noted that these are the only cells observed in which chromosome 2 is involved in a simple (Fig. 4c) or a complex (Fig. 4d) translocation. All other chromosome rearrangements observed were seen a single time.

In a small frequency of GM 1492 metaphase cells observed, we found some dot or minute chromosomes present (Fig. 2b). We confirmed that this material was chromatin since it bound the DNA-specific fluorochrome Hoechst 33358. Preliminary results indicate that the presence of these minute chromosomes is inheritable, and individual substrains of GM 1492 may have higher frequencies of minutes than the parental strain. Our analysis, after further studies of the evolution of this material, along with its molecular characterization will be presented elsewhere.

DISCUSSION

Although the BS fibroblast strain, GM 1492, was nontumorigenic, the data suggest that these cells are preneoplastic. The other cellular parameters examined, proliferative ability, transformation, and chromosomal stability, all indicate that GM 1492 cells differ from normal human fibroblasts (HSF-7) and have many similarities to neoplastic cells.

In the study of cell proliferative ability (Table 1) it can be seen that the assay used showed that HSF-7 cells had a potential of approximately 50 population doublings. This is in complete agreement with what has long been believed to be the life span of normal human diploid cell strains (20). GM 1492 cells, however, exhibited approximately twice that number for population doublings in the same assay, and the single colony observed but not passaged in the ninth step of the assay could very well have exhibited further proliferative ability (and may have even been immortalized). This increased proliferative ability is not typical of BS fibroblasts; strain GM 2520 had a maximum number of potential doublings of about 36, which is lower than normal.

One can postulate that the elevated spontaneous mutation rate in BS fibroblasts (3) could be related to the observed increase in proliferative potential of GM 1492 cells; i.e., the frequency of a mutation at a locus controlling senescence would be greater in BS cells than in normal cells. Shall and Stein (21) have proposed that a single mutation could cause a failure in the senescence process of cells. We suggest that since an increased cellular life span has been observed in strain GM 1492, but these cells are not immortalized, that more than one gene may be responsible for the senescence process.

The serial clonogenicity assay allows for the calculation of the "colony forming efficiency" (Table 1, C) at each step in the series. The theoretical value of C should approach and remain at the integer 1.0 in an immortal cell line. For both GM 1492 and HSF-7 cells this value decreases in the series until no more colonies can be detected. The value for C in step 4 of the assay for GM 1492 cells is unusually low. This is attributed to a decrease in cell doubling time without correction as is done in the next step in the series by increasing the growth time in step 5.

The transformation assays also indicated that GM 1492 cells are "partially transformed." The observation of partial cell growth in low serum is consistent with the observation of Lechner et al. (22). These workers suggested that the defect in BS fibroblasts impairs the ability of the cells to respond fully to growth stimulation. Since no cell growth was seen in HSF-7 cells at the same low serum concentration in which significant growth of GM 1492 cells occurred, we suggest that these BS fibroblasts have a somewhat relaxed growth requirement over normal cells, indicating partial transformation. In addition, our observation that certain substrains from GM 1492 selected in low serum are expandable in 1% fetal bovine serum indicates that a subpopulation may exist that may be "completely" transformed. A serial clonogenicity assay, karyotype analysis, and tumorigenicity studies of these substrains are currently in progress. We therefore must consider the possibility that the "partial" phenotypes observed in GM 1492 may represent those of clonal subpopulations within the strain.

The presence of intermediate sized colonies (Fig. 1b) when GM 1492 cells were grown in methylcellulose suggests that many of these cells are not anchorage dependent. Peehl and Stanbridge (23) observed anchorage independent growth of normal human skin fibroblasts under certain conditions. A very low (less than 0.05%) frequency of colonies from HSF-7 cells was observed; however, these were much smaller than those observed from GM 1492 cells. The frequency of intermediate sized colonies from strain GM 1492 was always larger than that from HSF-7 cells, and this frequency varied among but not within several GM 1492 subcolonies examined (data not shown). As of this writing, none of the intermediate-sized colonies has produced expandable populations that consistently grow in methylcellulose.

It is generally accepted that while normal cells show little karyotype variability, neoplastic cell populations are generally heteroploid (24). Our distribution of chromosomes per cell (Fig. 3) indicates that GM 1492 is a classically heteroploid population; it appears that many different karyotypes are constantly being generated as seen in neoplastic cells. This also appears to be the case in our preliminary examination of several cloned sub- strains from GM 1492; chromosome numbers vary from cell to cell.
Fig. 4. G-banded karyotypes of 4 GM 1492 metaphase cells. a, (2N = 40) no aberrant chromosomes; b, (4N = 80) two aberrant chromosomes, a dicentric C group and a submetacentric C-group sized chromosome; c, (2N = 41) three aberrant chromosomes: t(2;6)(q37;q12); 16q+; and dup17 (q21 —> q25); d, (2N = 46) four aberrant chromosomes: dir ins (-7;2)(q11;q13q —> 35), del(17q11 —> q22), a submetacentric F-group sized chromosome, 16q+, and dup17 (q21 —> q25).
cell within individual clones. As shown in Table 2, cells with chromosomal rearrangements are slightly less frequent than those with only aneuploidies. The other "chromosome instability syndromes," Fanconi’s anemia, xeroderma pigmentosum, ataxia telangiectasia (25), and Werner’s syndrome (26) present karyotypes that are diploid or pseudodiploid. (The use of the word "instability" here is not to be confused with unstable karyotypes, as we have described "heteroploidy").

It can be seen from Fig. 4 and Table 2 that most of the chromosomes observed were normal in appearance; chromosome 13s was seen in most cells. In a previous report on GM 1492 cells (6) no normal chromosome 13s or Ys was seen in the 25 cells they studied, and marker chromosomes were frequently seen; a predominance of near-tetraploid cells was also observed. Brat et al. (6) studied GM 1492 cells at passages 15 to 23, while we began our analysis at passage 20. One possible explanation for this discrepancy is that we have a population of cells with a different clonal origin than those cells used in the other experiments. Another possibility is that the cells have changed in culture. It is interesting to note that the most common chromosome missing from cells shown in Table 2 is the 13. We have observed some rearranged chromosomes (see Table 2); however, we refrain from calling these chromosomes "markers" since they are not consistently seen.

In earlier work (7, 8) we suggested that the phenomena of cell immortalization and the onset of karyotype instability are closely coupled. The data presented in this manuscript indicate that karyotype instability of this type (heteroploidy) is insufficient for immortalization. The BS fibroblast strain, GM 1492, shows definite karyotype instability and indications of increased proliferative potential and transformation but has not proved to be immortalized or tumorigenic. While these cells may resemble cancer cells, cell populations by our criteria can only be proven to be neoplastic if they are tumorigenic.

The observation of minute chromosomes (Fig. 2b) is further evidence that GM 1492 cells are preneoplastic. To our knowledge, these extrachromosomal,acentric chromatin bodies have not been observed in any nonimmortalized cell strain and are almost entirely restricted to tumor cells. Since double minute chromosomes represent gene amplification (27) we have begun experiments to determine what sequences (if any) are amplified in the strain, GM 1492; this presumably amplified DNA may be related to the preneoplastic phenotype we have observed. We also are in the process of characterizing several single cell clones obtained from GM 1492. We are hopeful that further research in this area will help answer some of the many unresolved questions regarding the progression of the neoplastic process in humans.

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