Giemsabanding Karyotype of Rat Hepatomas of Different Growth Rates¹

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ABSTRACT

The Giemsa-banded karyotypes were studied in slowly and rapidly growing transplantable Morris solid hepatomas and in their in vitro stabilized cell lines. In the slower-growing solid hepatoma 8999, all chromosomes of the normal rat complement were readily identifiable. In the rapidly growing solid hepatoma 3924A, the mitotic cells exhibited a range of 54 to 120 chromosomes/cell with a stem-line of 64. In this liver tumor, extensive structural rearrangements occurred. Twenty-four structurally abnormal chromosomes were identified, and the origin of eight of these was suggested. Of the structurally abnormal chromosomes, six occurred in sufficiently high frequency that they can be used as markers for the identification of hepatoma 3924A solid tumor. Structural rearrangements were observed for autosomes 1 to 13. The first three pairs of autosomes of the rat complement were frequently found in chromosomal rearrangements in 3924A tumor cells. The most striking abnormality was in the marker chromosome, designated M1, which has a long homogeneously staining region. The observation of such a homogeneously staining region in human tumors and in rat hepatomas suggests a tumor-specific occurrence of a homogeneously staining region. The presence of this alteration may be connected with the altered enzymic pattern of 3924A tumor.

In hepatoma 8999 in tissue culture, the normal rat chromosome complement was observed with some numerical alterations. The cultured hepatoma 3924A cells contain the three autosomal markers found in the in vivo tumor karyotype. New chromosomal arrangements in which chromosome 2 was preferentially involved occurred in this cell line.

INTRODUCTION

Much of the work in this laboratory concerns the pattern of gene expression and regulation in transplantable rat hepatomas of different growth rates, biological malignancy, and degree of differentiation (25,26). These studies have demonstrated the operation of an ordered metabolic and enzymic imbalance that was linked with neoplastic transformation and progression (25,26). Current work is directed to elucidate the linking of enzyme activity with chromosome loci and the role of enzymic imbalance in the determination of growth rate by use of the technique of somatic cell hybridization. For this purpose, the karyotype and banding pattern were determined in slower-growing and rapidly proliferating tumor lines in solid tumors and in hepatoma tissue culture cell lines established in vitro.

Because of the markedly different enzymic pattern, we selected a tumor of slow to medium growth rate, hepatoma 8999, and the rapidly growing hepatoma 3924A for our biological model systems. Hepatoma 8999 developed in a male Buffalo inbred rat after treatment with N'-fluoro-4-biphenylacetamide (15). Karyotype studies on this tumor were reported in 1967; however, the prebanding results were limited to morphological and numerical evaluation (17). The rapidly growing hepatoma 3924A had been initially induced in a female ACI/N inbred rat after treatment with N-2-fluorenylidacetamide (19). This tumor had a characteristic karyotype with at least 8 abnormal chromosomes (7,17). The biological and growth properties (15) and main biochemical aspects (25,26) of these tumors have been reported. Some of the biological, biochemical, and chemotherapeutic characteristics of tissue culture cell lines produced from hepatomas 8999 and 3924A were described (5, 6). This paper presents the ASG¹ karyotype in the slow-growing hepatoma 8999 and the rapidly growing 3924A in solid tumors and their tissue culture lines.

MATERIALS AND METHODS

Solid Tumor Systems. Hepatomas were transplanted s.c. in 6- to 8-week-old Buffalo (Simonsen Labs, Inc., Gilroy, Calif.) or ACI/N (Laboratory Supply Co., Indianapolis, Ind.) rats. To avoid possible host cell contamination in the karyotype studies, the neoplasms were transplanted into a rat of the sex opposite to that in which the primary tumor had been induced.

Tissue Culture Lines and Conditions. For short-term tissue culture, tumors were collected under sterile conditions. After one washing in phosphate-buffered saline (containing in g per liter, KCl, 0.20; KH₂PO₄, 0.20; NaCl, 8.00; Na₂HPO₄·7H₂O, 2.16) supplemented with 500 units penicillin per ml and 500 µg streptomycin per ml, the tissue was minced and digested with 0.25% trypsin (Grand Island Biological Co., Grand Island, N. Y.). The supernatant was centrifuged for 5 min at 500 rpm, and the cell suspension was seeded into 25-sq cm Falcon tissue culture flasks in McCoy's 5A medium supplemented with 15% FCS, 100 units penicillin per ml, and 100 µg streptomycin per ml. Cells were cultured till near confluency; then they were used for chromosome preparations.

The 8999 and 3924A cell lines were initiated in our laboratory 5 and 10 years ago, respectively; carried periodically in McCoy's Medium 5A supplemented with 10% FCS; and incubated in a 95% air:5% CO₂ atmosphere. Hepatoma 8999 cells were subcultured once a week, and 3924A cells were subcultured twice a week to keep them in logarithmic growth. Under these in vitro conditions, hepatoma 8999 cultures have a doubling time of 55 to 70 hr, and the rapidly growing hepatoma 3924A has a doubling time of 15 to 18 hr.

Primary tissue cultures from the lung of Buffalo and ACI/N rats were prepared from 14- to 18-day-old embryos. Trypsinized cell suspensions from individual fetal lung were seeded with 8 × 10⁴ cells/25-sq cm Falcon tissue culture flask in McCoy's Medium 5A plus 10% FCS and were incubated at 37°C

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for 24 hr. To stimulate cells to divide, the flasks were washed with phosphate-buffered saline, and fresh medium supplemented with 20% FCS was added. Chromosome preparations were made after a 24-hr incubation.

**Chromosome Preparation.** Chromosome preparations were made according to conventional procedures after arresting cells with 0.2 μg Colcemid per ml (Grand Island Biological Co., Grand Island, N. Y.) for 2 hr. Cells were harvested by a rubber policeman, swollen in 0.9% NaCl solution:deionized water (1:4) at 37 °C twice for 15 min each, and fixed in freshly prepared methanol:glacial acetic acid (3:1). Cells were dropped onto clean, cold, wet slides and air-dried. After a 24-hr incubation at 37°, slides were stored in the fixative used above for 2 to 4 days. Giemsa banding was carried out using a modification of the ASG method described by Sumner et al. (23). Slides were treated at 65° with 10× standard saline citrate (1.5 M NaCl: 0.15 M sodium citrate, pH 7.2), rinsed, and then stained for 5 to 7 min with 2% Giemsa Gurr’s “R-66” at pH 6.8. Fifty to 100 chromosome spreads were scored to determine the modal chromosome number. Banded chromosomes with the modal chromosome number were used to prepare the karyotypes.

**Normal Karyotype Patterns of Buffalo and ACI/N Rats.** To provide a comparison with the tumor, the normal ASG-banded karyotypes of male Buffalo and female ACI/N rats were studied in this laboratory. The chromosomes were arranged according to the scheme set up by the Committee for a Standardized Karyotype of *Rattus norvegicus* (3) and as analyzed by other authors (8, 12). We observed no alteration from the karyotype of *R. norvegicus*.

### RESULTS

**Hepatoma 8999**

**Karyotype of the Solid Tumor.** In hepatoma 8999, the predominant cell type was diploid (Chart 1), and grouping of the 42 chromosomes yielded a morphological appearance typical for the normal rat karyotype. Analysis by the ASG banding technique revealed some minor variations that were characteristic to this cell population, but the banding pattern of this tumor seemed to be the same as that of the standardized *R. norvegicus* (Fig. 1). Satellites were observed occasionally on chromosome 4. The Y chromosome was unpaired, small, andacrocentric, in agreement with a previous report (17). Aneuploidy was observed in about 45% of the cells. Of the nondiploid cells, 24% were hypodiploid and showed an apparently random chromosome loss. Approximately 22% were near-tetraploid. There were no morphologically abnormal chromosomes observed in the nondiploid cells.

**Karyotype of the Tissue Culture Cell Line.** In the hepatoma 8999 tissue culture line, the predominant cell type was diploid (Chart 2, top), with an average of 40.75 normal chromosomes/cell. Both X and Y chromosomes were present, indicating derivation from the male.

Analysis by ASG banding revealed minor variations characteristic of this population but within the range of variation among normal diploid cells (Fig. 2). Approximately one-third of the cells with 42 chromosomes were pseudodiploid, sometimes one member of a chromosome pair was missing, and an increase in the number of chromosomes 17 to 20 was observed. The No. 1 pair in this cell line appeared asymmetric in length. Four of the 20 cells had satellites on a single chromosome, No. 4, and 2 had apparent satellites on No. 7. Of the nondiploid cells, 8% were near-tetraploid. Morphologically abnormal chromosomes were not observed in the nondiploid cells.

**Hepatoma 3924A**

**Karyotype of the Solid Tumor.** In marked contrast to the minimal numerical changes observed with the hepatoma 8999 solid line were the structural changes in the rapidly growing hepatoma 3924A. Fifty cells were counted, and 20 were karyotyped, with results as shown in Chart 1 (bottom) and Figs. 3 and 4. The total number of chromosomes per cell ranged from 54 to 118, with 21 of the 50 cells containing 64 chromosomes. There were 1 to 4 copies of each chromosome per cell. Chromosomes 3, 6, and 14 were monosomic, while chromosomes 1, 2, 4, 7, 8, 12, and 15 to 20 had 3 or 4 homologs in the cells. One pair of sex chromosomes, XX, was present in every cell (Table 1). In addition to variability in number of the normal chromosomes, each cell had structurally abnormal ("marker") chromosomes. Altogether 24 different markers were seen. Only those alterations present in 3 or more cells...
were considered as markers. The origin of some of these markers could be established from the banding pattern. Table 2 lists 8 structurally abnormal chromosomes, the origin of which could be suggested; describes the alterations which were identified; and lists the frequency with which each occurred. These structurally abnormal chromosomes are compared with their normal counterparts in Fig. 4. There are some chromosomes which have not been identified thus far.

The most striking abnormality was the marker chromosome designated M, probably involving chromosomes 4, 5, and 7. This marker contained a long HSR on the interstitial part of the chromosome. M, chromosome itself is the result of translocations (Table 2), but HSR is the result of the physical increase in chromosome material of either the 5q36 or 7q31–36 region. HSR-containing chromosomes were of 2 types, involving the same chromosomes. In most cases, there was a single copy of the terminal region of the long arm, but in 40% of the cells M, had an additional tandem duplication of this segment, i.e., the region originally derived from 7q22–7q33. M, was the most frequently occurring of the marker chromosomes, and the most frequent mechanism for formation of marker chromosomes appeared to be translocation (Table 2).

The Karyotype of Tissue Culture Cell Line. Chromosome counts of 100 individual hepatoma 3924A metaphases yielded a range of 44 to 120 chromosomes/cell (Chart 2, bottom). About 90% of the cells had between 54 and 69 chromosomes, with a modal number of 60. Fourteen % of the cells had more than 69 chromosomes, with a modal number of 60. Forty-four % of the cells had chromosomes in the tetraploid region. Fig. 5 shows the representative karyotype of a hepatoma 3924A cultured cell with 60 chromosomes. Every chromosome of the normal rat except chromosome 2 was represented in this cell line. This chromosome appeared as a part of new marker chromosomes (m, and m3) in the tetraploid region. Fig. 5 shows the representative karyotype of a hepatoma 3924A cultured cell with 60 chromosomes.

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The comparison of HSR’s occurred in only 3% of the cells; however, various telocentric, submetacentric, and dicentric chromosomes with HSR’s were observed in the tissue culture line, suggesting further rearrangements of this chromosome.

**DISCUSSION**

Comparison of Karyotypes from Normal Rat Liver Cells and Solid Hepatomas 8999 and 3924A: Numerical and Qualitative Aspects. Our results appear to be the first ones describing the ASG banding pattern of the solid tumor, hepatoma 8999. Unbanded studies on earlier generations of hepatoma 8999 reported 80 chromosomes as the stem-line with standard Giemsa staining method (17). In contrast, the present generation of 8999, after a lapse of over a decade, has only 22% of the metaphases with chromosomes in the tetraploid region. The differences between the earlier and the present generation of the solid tumor karyotype may indicate (a) tumor progression due to a drift in the cell population over many generations of transplantation, (b) initial endoreduplication in an early generation of the neoplasms followed by chromosome loss during 10 years of transplantation, and (c) selection during transplantation of cells with diploid or pseudodiploid chromosome complements.

Biochemical characteristics of the earlier and current generations of hepatoma 8999 are in agreement with the observation of the general similarity of the chromosome complement then and now and the fact that no aberrant chromosomes were detected in the current generations of this tumor. This is the first time that a solid hepatoma with normal banding characteristics has been reported. Other rat neoplasms, primary and transplantable leukemias and lymphomas induced in rats by either chemicals or virus, showed consistent stability at the
diploid level (14, 20), but minor structural rearrangements of chromosomes were consistently found in these tumors.

For hepatoma 3924A solid tumor we were able to compare the earlier Giemsa-stained chromosomes and the more recent ASG-banded preparations (7, 17) with our current generation of 3924A. In spite of the frequent transplantation, approximately every 3 weeks, this hepatoma has retained its range of chromosome number (Table 3) for more than 1 decade since this line was first evaluated in 1967 by Nowell (17). Our current studies indicate that the chromosomes were present in approximately the same number of copies as in the earlier generations, and we noted the constancy of chromosomes 6, 9, and X. Chromosome 6 was always monosomic, and chromosomes 9 and X were in diploid number. The first 3 pairs and chromosomes 15 to 20 were represented by several copies (Table 1).

Qualitatively, 2 of the abnormal chromosomes (M₆ and M₈) were the same markers noted by Kovi and Morris (7). The large marker, M₆, was probably a translocation derived from the large telocentric chromosome with a large heteropycnotic interstitial region (17). The identification of this aberrant chromosome as bearing a HSR is a new observation that may be particularly useful in identification of this chromosome in somatic cell hybrids. In addition, we have observed 6 new markers which probably were formed by translocations and deletions.

Our assignment of other markers differs from that of earlier studies (7). The differences may be due to (a) heterogeneity of the tumor population, (b) alterations in the tumors with concurrent maintenance of essential biological and biochemical characteristics, and (c) the in vitro propagation used in this and the earlier study (7), in which short-term tissue cultures of the tumor were used for characterization of the banding patterns. The retention of the main biological, histological, and enzymic pattern may relate to (a) the relatively constant numbers of individual chromosomes and (b) the maintenance of the gene pool in spite of the new arrangements.

Comparison of Quantitative and Qualitative Aspects of the Solid Tumors and Their Tissue Culture Cell Lines. In the current generations of the solid hepatoma 8999 and its tissue culture cell line, the quantitative and qualitative chromosomal constitution was similar. In addition, the growth characteristics of each tumor are maintained. The 8999 solid tumor in vivo requires 4 months to grow to 1.5 cm in diameter and in vitro requires 64 hr to double the cell population. Hepatoma 3924A, on the other hand, requires 3 to 4 weeks to reach 1.5 cm in diameter in vivo and has a doubling time of 16 hr in vitro. The 8999 tumor in tissue culture was primarily normal diploid or pseudodiploid with occasional satellites in chromosomes 4 and 12. There were random occurrences of monosomic and trisomic cells and occasional terminal deletions of different chromosomes.

In the case of solid hepatoma 3924A, differences appeared when this tumor was subjected to repeated culturing. Both the solid tumor and the cell lines have chromosomes mainly in the near-triploid region. However, there is a slight decrease in stem-line number in the cultured cells. The major differences between the solid tumor and the cell line were in the aberrant chromosomes observed. Chromosome M₆ occurs in only 3% of the cultured cells, but the HSR was observed in 70% of the metaphases as part of abnormal chromosomes. As seen in Fig. 5, m₆ contained HSR. In identifying the marker chromosomes, it appears that M₆ and M₈ in the solid tumor were probably the same as m₆ and m₈ in the cultured lines. Among the unidentified chromosomes, the first and the fourth probably were the same as those observed previously in the solid tumor (7). We observed 5 new markers that were produced in the tissue culture cells. Two of them (m₆ and m₈) involved chromosome 2, which was lost from the chromosome complement (Fig. 5). Chromosome 2 has been frequently observed in rearrangements in cells transformed or tumor induced by adenovirus SV40 or by hydrocarbon carcinogens (9, 11, 13, 18). In rat hepatomas, this chromosome was also preferentially involved in rearrangements; No. 2 with pericentric inversion was observed in 5 rat hepatomas (7). The abnormal banding pattern of No. 2 was described in a "diploid" transplantable Morris rat hepatoma (28), in Novikoff rat hepatoma (24), and in the H4 hepatoma cell line (12, 16).

On the other hand, alterations of chromosome 2 which were not connected with malignant transformation were also reported (19, 22). The fact that in our 3924A cell line this chromosome was missing and occurred as new rearrangements suggests that certain chromosome segments are predisposed to rearrangements within one species and are connected with nonspecific alteration of this chromosome.

Chromosomes 6 and 9 were present in the same number of copies as in the solid tumor. Other chromosomes represented in the same number in the cell line as in the tumor were No. 1, 8, 9, 10, 12, 13, 19, 20, and X.

**Table 3**

<table>
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<th>Hepatoma</th>
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<th>Chromosome range</th>
<th>Marker chromosomes</th>
<th>Chromosome range</th>
<th>Marker chromosomes</th>
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<td>73</td>
<td>10</td>
<td>332</td>
<td>69–72</td>
<td>69</td>
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<td>54–70</td>
<td>60</td>
<td>8</td>
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* This paper.

b In the case of cultured lines, it refers to the number of subcultures.

c In region where more than 50% of the cells were located.
of defined HSR's in rapidly growing rat hepatoma 3924A. Earlier studies identified a HSR in human neuroblastoma cells (2), and it was suggested that it might represent a mechanism for gene amplification or that it might be involved in the overproduction of one or more proteins characteristic of these malignant neuronal cells (2). This interpretation was first proposed in studies of HSR's in Chinese hamster cell lines which were resistant to antifolates due to markedly elevated levels of dihydrofolate reductase (1). Lev et al. have reported a HSR in rat sarcomas (10). Recently a possible HSR was noted in the rapidly growing 7777 hepatocellular carcinoma in the short arm of a marker chromosome with a "poorly defined banding pattern." It was suggested that this region might be connected with the high levels of α-fetoprotein produced by this rat hepatoma (27). Hepatoma 3924A, however, does not produce a particularly high concentration of α-fetoprotein (21). The rapidly growing hepatomas such as 7777 and 3924A do share a marked imbalance as manifested in the highly increased activities of key enzymes of glycolysis; pentose phosphate production; and purine, pyrimidine, and polyamine biosynthesis and in a concurrent decrease in activities of enzymes involved in the opposing metabolic pathways (25, 26). This imbalance, on the other hand, is a minor one in hepatoma 8999. The observation of such HSR's in human tumors and in rat hepatomas suggests a tumor-specific occurrence of HSR, and this alteration in our cells may be connected with the altered enzymic pattern of the 3924A tumor.

The presence of an essentially normal diploid karyotype and banding pattern in the slower-growing hepatoma 8999 and the presence of well-defined characteristic markers and a HSR in the rapidly growing 3924A, together with the profound differences in the proliferative and enzymic and metabolic aspects of gene expression, make these 2 cell lines of particular interest for testing by somatic cell hybridization the relationship of biochemical characteristics and malignancy (4).

ACKNOWLEDGMENTS

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REFERENCES

Fig. 2. ASM-banded karyotype of a cell derived from the cell culture of hepatoma 8929. Note the satellite on chromosome 4. Random monosomy and trisomy have occurred.
Fig. 3. ASG-banded karyotype of hepatoma 3924A solid tumor from the 374th generation. The total chromosome number including markers is 64. Each normal chromosome is represented in at least one copy (No. 3, 5, 6, and 14) and up to 4 copies (No. 1, 8, and 19).
Fig. 4. Abnormal chromosomes of 3924A tumor with suggested origins. Note M₁, a giant chromosome with a HSR in the interstitial part of the long arm. Chromosomes 4, 5, and 7 are probably involved in the formation of this marker.
Fig. 5. ASG-banded karyotype of a cell derived from the cell culture of hepatoma 3924A. All the normal rat chromosomes are represented except No. 2. This chromosome can be identified as a component of chromosomes m₁ and m₅ (Line 4). Note m₄ with a HSR. There are 9 chromosomes which cannot be identified.
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