Carcinogen-induced Abnormalities in Rat Liver Cells and Their Modification by Chemical Agents

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ABSTRACT

Epithelial cells derived from livers of rats bearing diethylnitrosamine-induced hepatocarcinomas were propagated in vitro and cloned. These cells exhibited acentric nuclei and juxtanuclear cytoplasmic lesions indistinguishable from the Mallory hyaline bodies found in the cirrhotic livers of alcoholics. The hyaline region was found to be comprised of a filamentous aggregate in the cytoplasm which contained components that bound concanavalin A, an interaction that was blocked by α-methylmannopyranoside. This hepatocellular aggregate also bound immunoglobulin G from the sera of normal rabbits and from patients with advanced alcoholic cirrhosis. The immunoglobulin G fraction of sera from normal goats, rats, or mice did not show such an affinity. The morphological appearance of cells containing this Mallory body-like lesion was altered, in a reversible reaction, to one resembling normal rat liver epithelial cells after incubation with dimethyl sulfoxide or sodium butyrate. Such treatment also caused the cells to lose their acquired ability to localize concanavalin A and immunoglobulin G in the juxtanuclear region. The presence of the filamentous aggregate may be responsible for the acentric displacement of the nucleus and might be correlated with the ability of the cells to express certain in vitro properties associated with the transformed state. The cytoplasmic lesion may reflect an impairment of microtubular function and polymerization.

INTRODUCTION

Long-term in vitro propagation of transformed epithelialoid liver cells isolated from rats after p.o. administration of diethylnitrosamine has been reported (6, 7). These unusual cells possessed acentric nuclei and were characterized by the appearance of a juxtanuclear aggregation of filaments resembling the Mallory bodies seen in the livers of patients with advanced alcoholic cirrhosis and other degenerative liver diseases (Ref. 34; for review, see Ref. 19). Cells containing Mallory body-like abnormalities were reisolated from carcinomas following inoculation of the cultured liver cells into newborn rats or nude mice (6). The filamentous aggregate was not modified when the cultures were exposed to cytoschalin B or Colcemid (6), agents known to interfere with the polymerization of microfilaments or microtubules, respectively. The cells and their prominent morphological marker had not been previously described in vitro. They represent a unique model system for the investigation of the relationship between cytoskeletal structure and neoplastic growth characteristics. We now report studies concerning the nature of these carcinogen-induced abnormalities and the ability of certain chemical agents to modify them.

MATERIALS AND METHODS

Cells and Cell Cultures. Cell lines containing Mallory body-like lesions were derived, as described (6, 7), from collagenase-dissociated livers of Wistar rats sacrificed 10 to 14 weeks after daily p.o. administration of diethylnitrosamine (50 ppm) in the drinking water and were established in culture. One of several cloned cell lines (72/22), all of which produced tumors when injected into rats or nude mice, was selected for further investigation. Tumor-producing cells derived from well-demarcated neoplastic liver nodes which did not contain these cytoplasmic lesions were studied for comparison. Long-term cultures of rat liver cells (9) which maintain a normal karyotype, do not grow in semisolid agar medium, and do not produce tumors upon inoculation into newborn rats or nude mice served as control.

Cells were cultured in Ham's F-12 medium supplemented with 10−4 M dexamethasone, penicillin, streptomycin, fungizone (2.5 μg/ml), and 10% fetal calf serum in a humid 5% CO2 atmosphere at 37°. To examine the effect of chemical agents on cell morphology, Me2SO (Fisher Scientific Co., St. Louis, Mo.) were used for binding studies. Con A and the polysaccharides α-methylmannopyranoside and α-methylglucopyranoside (all from Sigma Chemical Co., St. Louis, Mo.) were used for binding studies. Rabbit sera were obtained from New Zealand White rabbits; human sera were supplied by the serum bank of our Institute (courtesy of Dr. Y. Hirshaut).

For tests of anchorage-independent growth, soft-agar culture overlays containing 1 × 105 to 1 × 106 cells in 2.0 ml of 0.33% Difco-Bacto agar in F-12 medium supplemented with 0.3% Tryptose phosphate and 10% fetal bovine serum were prepared and placed on a base of 5.0 ml of 0.5% agar medium in 60-mm plastic Petri dishes. Cultures were incubated for 2 weeks at 37°, at which time colonies were counted. Several agar colonies were isolated, disrupted by

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trypsin, and further propagated in liquid medium.

**Immunofluorescence.** For the indirect immunofluorescence assay (36), cells on coverslips were either fixed for 30 min with 3.5% formaldehyde in PBS (pH 7.2); postfixed with acetone: H₂O (1:1) for 2 min, with acetone (100%) for 5 min, and with acetone: H₂O (1:1) for 2 min; and washed with PBS or else simply fixed with methanol for 30 min. Then the cells were incubated for 1 hr in moist chambers with normal rabbit, goat, rat, or mouse sera; with IgG purified therefrom (21); or with sera from normal individuals or from patients with advanced alcoholic cirrhosis. The coverslips were then washed with 2 changes of PBS for 30 min, followed by incubation with the appropriate FITC-conjugated secondary antibody to the primary IgG source. After 1 hr, the coverslips were washed with PBS and mounted in 50% glycerol in PBS. Preparations were viewed with a Zeitz photomicroscope equipped with UV optics.

**Lectin Binding.** The binding of Con A to the surface of viable cells was studied using lectin which had been directly coupled with fluorescein as described (22) according to the method of Ash and Singer (2). Cells were incubated in medium containing FITC-Con A (50 μg/ml) for 5 or 20 min and then were fixed with 2% formaldehyde. In other experiments, cells were first fixed with methanol, followed by incubation for 1 hr with FITC-Con A (80 μg/ml), or else they were incubated with unlabeled Con A (80 μg/ml) prior to exposure to FITC-Con A. In still other experiments, a mixture of FITC-Con A with α-methylglucopyranoside or α-methylmannopyranoside was used to stain methanol-fixed cell monolayers. Coverslips were mounted in 50% glycerol in PBS and viewed with UV optics.

**Electron Microscopy.** For transmission electron microscopy studies, cells were pelleted, fixed in 2% glutaraldehyde, postfixed in 1% osmium tetroxide, and embedded in epoxy resin. Thin sections were stained with uranyl acetate and lead citrate and examined with a Siemens electron microscope.

**Autoradiography.** The effect on DNA synthesis of chemical agents added to the culture medium was examined with the aid of [3H]thymidine. Cells were grown on coverslips in culture dishes in the presence of n-butyrate or Me₂SO for 3 or 5 days, respectively. [3H]Thymidine (final concentration, 0.25 μCi/ml [6 Ci/mmol]) was then added to freshly replaced culture medium containing the agents and in parallel sets of dishes to medium from which the agent had been omitted; the cells were incubated for an additional 18 hr. Unlabeled thymidine (10 μg/ml) was then added, followed after 10 min by a medium wash and fixation with methanol. Coverslip preparations were extracted with cold 5% trichloroacetic acid for 5 min to remove unincorporated label, rinsed, and then coated with Kodak NTB emulsion, diluted 1:1. A total of 300 to 500 cells were scored for labeled nuclei in randomly selected fields.

**RESULTS**

Incubation of methanol-fixed hyaline body-containing rat liver cells (Fig. 1a) with normal rabbit sera or IgG purified therefrom, followed by FITC-conjugated goat anti-rabbit IgG, resulted in the appearance of a brightly fluorescing juxtanuclear area coincident with the Mallory body region when viewed in UV light (Fig. 1b). No fluorescence was observed after incubation with IgG from goats, rats, or mice. Neither neoplastic carcinogen-transformed rat liver cells devoid of Mallory body-like lesions nor normal rat liver cells established in vitro (9) and examined in parallel experiments gave the fluorescence reaction after incubation with nonimmune rabbit IgG.

To investigate whether the cytoplasmic lesion could be modified by agents known to interact with cell surface receptors (2), cultures of the juxtanuclear aggregate-containing liver cells were grown in the presence of Con A (4 to 20 μg/ml; 2 to 10 × 10⁻⁷ M). This carbohydrate-binding lectin combines with sites most complementary to α-methyl-D-mannopyranosyl residues of polysaccharides or glycoproteins (24). No alterations in cellular morphology or growth characteristics could be observed when Con A was present in the medium. However, when methanol-fixed cells were incubated with 4 × 10⁻⁶ M FITC-Con A a bright fluorescence in the hyaline region was observed (Fig. 2). This binding was partially inhibited by preincubating the cells with 4 × 10⁻⁸ M unlabeled Con A and completely inhibited with 5 × 10⁻⁵ M unlabeled Con A. No fluorescence was observed when methanol-fixed cells were incubated...
with a $4 \times 10^{-6}$ M solution of FITC-Con A which had previously been mixed with $\alpha$-methylmannopyranoside ($5 \times 10^{-2}$ M) 30 min prior to overlaying on the cells. The addition of $\alpha$-methylglucopyranoside ($5 \times 10^{-2}$ M) to a FITC-Con A solution ($4 \times 10^{-6}$ M) only partially prevented Con A binding to sites in the juxtanuclear region. This is in keeping with the finding that Con A binding to the $\alpha$-D-glucans is less specific than for the $\alpha$-D-mannans (24). It was of interest to determine whether viable filamentous body-containing liver cells differed from normal liver cells in the pattern of Con A binding to surface membrane receptors. FITC-Con A was, therefore, added to the incubation medium. Whereas normal rat liver epithelial cells showed a uniformly distributed surface fluorescence after a 20-min incubation with FITC-Con A, hyaline body-containing cells exhibited a patchy clustering of fluorescence (Figs. 3 and 4) at the surface. Similar fluorescent clusters induced by Con A have been observed in virus-transformed rat kidney fibroblasts (3, 18) and in rat kidney or chick fibroblasts and mouse lymphocytes after incubation with colchicine, an agent known to interfere with microtubule assembly (2, 18, 54).

Fluorescence was not observed in either the IgG or Con A incubation experiments if cells had been fixed with formaldehyde rather than with methanol. Methanol-fixed normal or transformed rat liver cells that did not contain Mallory-type hyaline bodies did not fluoresce after incubation with rabbit IgG. Fluorescein-coupled phytohemagglutinin at a concentration of 200 $\mu$g/ml did not stain the Mallory-like bodies.

There have been reports that patients with alcoholic cirrhosis might produce liver-directed autoantibodies (55, 57). Sera from such patients have produced bright immunofluorescence reactions in the Mallory bodies (alcoholic hyalin) of cells in cryostat sections of cirrhotic livers of alcoholics (38, 56). Examination of our cultured liver cells with sera obtained from patients with advanced alcoholic cirrhosis revealed that these cells similarly showed a bright fluorescence in the hyaline body area with 2 of 5 of the cirrhotic human sera tested (Fig. 5). Normal rat liver cells or neoplastic liver cells which did not contain such cytoplasmic abnormalities did not react with these sera. Sera from 4 normal individuals were fluorescence negative with all cell lines tested.

**Reversal Studies.** A series of chemical agents found to induce differentiation in various cell systems (1, 10, 16, 20, 27, 32, 39, 45) were tested for their effects on the hyaline body-containing liver cells and on their growth characteristics. Whereas bromodeoxyuridine, ouabain, hypoxanthine or dibutyryl cyclic adenosine 3'-5'-monophosphate, and testosterone were without significant effect, inclusion of 2% Me$_2$SO in the culture medium for 2 weeks resulted in a marked flattening and a gradual disappearance of the cytoplasmic aggregate in about 60% of the cells (Fig. 6). Although the population doubling time was increased over that of cultures growing in the absence of Me$_2$SO, cells continued their propagation in the presence of this agent for more than 2 months. However, when Me$_2$SO was removed from the medium, cells reverted to their original morphology within 2 to 3 days. Cells in which the cytoplasm appeared normal after growth in the presence of Me$_2$SO no longer bound nonimmune IgG or FITC-Con A in the juxtanuclear area; those cells which had retained the filamentous bodies still showed fluorescence. Reappearance of the Mallory-like hyaline body and subsequent IgG- and Con A binding was again demonstrated 3 days after return of the cultures to normal growth medium.

Sodium butyrate was found to be even more effective in altering the characteristic cytoplasmic lesion. The agent was tested over a concentration range of 2 to 4 mM at pH 7.0 to 7.4. Whereas 4 mM butyrate was toxic to the cells when present in the medium for more than 1 week, 2 mM butyrate was not deleterious, and 3 mM solutions were well tolerated. A concentration of 3 mM, which slowed but did not inhibit proliferation, produced marked cell flattening and the disappearance of the cytoplasmic aggregate in 70% of the cells after 4 to 6 days of incubation (Fig. 7). Incubation with sodium butyrate, inhibited the binding of rabbit preimmune IgG and FITC-Con A to the Mallory body region of methanol-fixed cells. Removal of the butyrate from the medium led to the reappearance of the Mallory-like body after 2 to 3 days, and with it the ability to bind IgG and Con A returned. Half of the cells that had lost their Mallory-like hyaline bodies after incubation with 3 mM sodium butyrate regained this cytoplasmic lesion after treatment with $10^{-6}$ M Colcemid for 3 hr. One might infer that the disappearance of the hyaline body in the butyrate-treated cell was caused by a “normalization” of the cytoskeletal structure, which was again disrupted by Colcemid.

Changes in the cytoplasmic organization were followed by transmission electron microscopy. Whereas cells grown in normal medium contained a well-demarcated juxtanuclear filamentous cytoplasmatic area surrounded by a ring of mitochondria (6, 7), incubation with Me$_2$SO or butyrate resulted in a more homogeneous distribution of cytoplasmic organelles and in an apparent dispersal of the filamentous aggregate (Figs. 8 and 9).

Examination of the capacity of the Mallory body-containing liver cells and that of their Me$_2$SO- or butyrate-treated companion cultures for anchorage-independent growth in semisolid agar medium revealed that cells with the cytoplasmic lesion could grow in soft agar. Cultures previously incubated with Me$_2$SO or n-butyrate did not grow if the agents were also present in the agar medium; they did form colonies, however, when the agents were omitted. Thus, the transformed cells (72/22) not only assumed a morphologically more normal appearance during incubation with Me$_2$SO or butyrate, but they were also anchorage dependent for growth, as is characteristic of normal cells. However, other transformed cell lines such as HeLa, Meth A, and several of our hepatoma lines, which usually grow in soft agar, also did not grow when Me$_2$SO or butyrate was included in the agar medium, although they were able to replicate (but more slowly) in liquid medium which contained these agents.

**Autoradiography.** The autoradiographic technique was used in order to assess the ability of the Mallory body-containing cells grown in the presence of 3 mM butyrate for 3 to 6 days to synthesize DNA; the results indicate that only 10 to 13% of the cells so treated had incorporated [$^{3}H$]thymidine. However, when normal medium containing [$^{3}H$]thymidine was added to cells which had previously been
incubated for 3 or 5 days with 3 mM butyrate, the labeling index increased to 64 or 80%, respectively (Table 1). Repression of DNA synthesis was dependent upon the concentration of butyrate. In the presence of 2 mM butyrate, 27 to 30% of the nuclei were labeled, and with 4 mM butyrate 6 to 9% were labeled. Increases in the labeling index upon change into normal medium were in the 60 to 80% range for all concentrations tested. The results indicate that the cells were still viable despite the suppression of DNA synthesis induced by butyrate. The DNA labeling index of control, untreated cells containing Mallory body-like lesions when propagated in normal medium was 95%.

**DISCUSSION**

We recently reported a carcinogen-induced juxtanuclear cytoplasmic abnormality in rat liver cells (6, 7). The abnormal cytoplasmic region of these neoplastic cells was characterized by light and electron microscopy as a membraneless but well-demarcated homogenously staining area consisting of an aggregate of 100- to 150-Å filaments surrounded by mitochondria and elements of the Golgi apparatus. An interacting cytoskeletal network of actin filaments, microtubules, and 100-Å filaments (intermediate filaments) is believed to be necessary for the regulation of cellular function. Antisera that are specific for these cytoskeletal components have served to identify them and help reveal their role, distribution, and interrelationship in normal and virus-transformed cells (28). An altered cytoskeleton, observed in rat kidney or chick fibroblasts infected in vitro with tumor viruses, has been attributed to abnormalities of microfilamentous or microtubular structure or to a marked decrease of their components (3, 11, 18, 42, 49, 50). This suggests that cytoplasmic filaments may play a role in the process of transformation and the loss of contact-inhibited growth. The appearance of patchy clusters of cell surface membrane receptors to Con A on transformed but not on normal fibroblasts has been attributed to a transmembrane linkage of the receptors with other cellular constituents. Me2SO has recently been found to induce the polymerization of tubulin in vitro in the absence of tubulin-associated proteins (26). It is intriguing to speculate that tubulin polymerization is enhanced by agents known to interfere with microtubule formation (53).

The reasons for the fluorescence seen after incubation of the cells with IgG from nonimmune rabbit sera or with sera from patients with advanced alcoholic cirrhosis are currently under investigation. Normal rabbits are known to produce autoantibodies to actin (48) and tubulin (29). However, nonimmune IgG absorbed with actin or tubulin was still reactive in our system (8); these cytoskeletal components thus do not seem to be the active constituents involved. Furthermore, we found that extracts of preparations of the cytoplasmic bodies did not show actin or tubulin bands by polyacrylamide gel electrophoretic analysis (4, 8, 46). Human sera containing smooth muscle autoantibodies, obtained from cancer patients, reacted with vinblastine-induced perinuclear intermediate filaments in human embryonic skin fibroblasts. This reaction, observed by indirect immunofluorescence, was unaffected by purification of actin; it was inhibited by a preparation of isolated intermediate filaments (30).

If the components involved in the Mallory body formation are indeed constituents of intermediate filaments, their abnormal, juxtanuclear aggregation might have an adverse effect on cell physiology and growth patterns. Intermediate filaments are believed to play a role in the anchorage of nuclei (33). They appear to attach to nuclear pores and to the plasma membrane and could thus serve as a link in the transmission of information between the nucleus and the cell surface (37). The retraction of these filaments into a juxtanuclear cap, with a concomitantacentric displacement

**Table 1**  
*Effect of butyrate on incorporation of [3H]thymidine into DNA*

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<th>Concentration of butyrate (mM)</th>
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<td>In presence of butyrate</td>
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of the nucleus, may lead to a disruption in the orderly flow of information.

Since the continuous presence of Me₂SO or butyrate in the culture medium was required for the maintenance of a normal-appearing phenotype, it was not feasible to test the tumorogenicity of these cells in vivo. Examination of their growth potential in soft agar, on the other hand, was possible by including the respective chemicals in the agar medium. Under those experimental conditions, Me₂SO- or butyrate-treated cells did not grow in soft agar medium and, therefore, behaved like normal cells, but they proliferated and formed colonies as expected of transformed cells when the agents were not included in the agar. A suppression of the growth of neoplastic Syrian hamster cells, grown in butyrate-containing agar medium, has recently been shown (31).

It has been reported that n-butyrate shuts off DNA synthesis reversibly in chick embryonic fibroblasts and HeLa cells and that cytosol from butyrate-treated cells will turn off DNA synthesis in control nuclei (25). Butyrate also has been found to inhibit histone deacetylase (12, 44). Although there was a marked decrease of DNA synthesis in our cell system after butyrate exposure, utilization of [³H]thymidine was not impaired cytoskeletal structure and neoplastic transformation of the growth of neoplastic Syrian hamster cells, grown in butyrate-containing agar medium, has recently been shown (31).

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REFERENCES

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Fig. 2. Liver cells (72/22) were methanol fixed and then incubated with FITC-Con A (80 µg/ml) for 1 hr as described. Note fluorescence concentrated in the juxtanuclear area. UV optics. x 430.

Fig. 3. Liver cells (72/22) incubated in the living state with FITC-Con A (50 µg/ml) for 20 min and then fixed with 2% formaldehyde. Note the patchy distribution of Con A-binding sites. UV optics. x 330.

Fig. 4. Normal rat liver cells (9) incubated in the living state with FITC-Con A as in Fig. 3. Note uniform distribution of Con A-binding sites. UV optics. x 330.

Fig. 5. Indirect immunofluorescence. Liver cells (72/22) were methanol fixed and then incubated with serum from a patient with advanced alcoholic cirrhosis and stained with FITC-conjugated goat anti-human IgG as described. UV optics. x 410.

Fig. 6. Liver cells (72/22) grown in the presence of 2% Me2SO for 42 days. Note the absence of aggregate in the cytoplasm. Methanol fixation, Giemsa stain. x 330.

Fig. 7. Liver cells (72/22) grown in the presence of 3 mM sodium butyrate for 12 days. Note the absence of juxtanuclear aggregate in the cytoplasm. Methanol fixation, Giemsa stain. x 330.
Fig. 8. Transmission electron micrograph of a liver cell (72/22) cloned from a culture derived from a rat after daily administration of diethylnitrosamine for 3.5 months. Note the Mallory-type body (MB) next to the nucleus (N) and the ring of mitochondria. Glutaraldehyde fixed. Uranyl acetate and lead citrate. × 6500.

Fig. 9. Transmission electron micrograph of a liver cell (72/22) after incubation for 6 days in the presence of 4 mM sodium butyrate in the culture medium. Note the absence of filamentous aggregate and the random distribution of mitochondria. N, nucleus. × 6500.
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