Carcinogenesis by Nonmutagenic Chemicals: Early Response of Rat Liver Cells Induced by Methapyrilene

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ABSTRACT

Although methapyrilene (MP) produces hepatocellular carcinomas in rats, it does not elicit many of the cellular responses induced by other hepatocarcinogens. We have investigated the early changes induced in rat liver epithelial cell cultures by MP using morphological, cytotoxicity, and cytofluorometric techniques. Within 2 h of MP treatment, inclusion bodies which were stainable with lipid stains were observed in the cytoplasm. Ultrastructurally, they resembled lamellar bodies with alternating light and dark lamellae. These bodies were transient in nature, since they disappeared within 24 h of removal of MP. They were, however, retained in the cytoplasm as long as MP was present in the medium. Lamellar bodies appear to be induced in the presence of histamine H1 receptor-blocking agents, since methaphenilene and diphenhydramine produced this reaction, but cimetidine, an H2 antagonist, did not.

Morphologically, mitochondria of control cells were long and rod-like, whereas they were short or bizarrely shaped in the MP-treated cells. Moreover, a quantitative increase was observed in the mitochondrial content of the treated cells, when intact liver cells were exposed for 10 to 12 days with MP. Collectively, these results suggest that MP might perturb the cytoskeletal elements leading to an alteration in the nuclear and mitochondrial makeup of rat liver cells.

INTRODUCTION

It is now well established that many nongenotoxic chemicals induce tumors in different species and tissues (11, 12, 18, 21, 24). One such agent, MP, has been shown to be a potent hepatocarcinogen in rats after long-term feeding (13, 24, 28, 31). Some of the early responses induced by MP in rat liver cells in vivo have already been reported. These include an increase in the number of mitochondria and other morphological changes in periportal cells (28, 31-33), some of which may be a reflection of toxicity. MP is generally considered to be a nongenotoxic chemical, since it does not produce mutations in bacteria or in mammalian cells (2, 8), and does not induce unscheduled DNA synthesis (6, 25, 30) or sister chromatid exchanges (20) in a number of mammalian cells. It has also been reported by Lijinsky and Muschik (22) that mitiated MP does not bind to genomic DNA of rat liver to any significant extent. However, Althaus et al. (1) have shown that DNA damage can be induced by this carcinogen in primary cultures of rat liver cells. Therefore, it appears that, although MP may not directly affect nuclear DNA, it may still produce at least minor alterations in the genomic makeup and/or on gene expression of target cells.

In this study, we report the immediate effects of MP and other related compounds on their target cells. Similar experiments, using well-characterized, diploid epithelial cells from rat liver, were also done with other hepatocarcinogens and drugs chemically related to MP or which produce similar biological responses in animals. It was found that MP and other histamine H1 receptor-blocking agents induced an accumulation of lipid particles which were retained in the cytoplasm until the drug was removed. Moreover, a distinct increase in the total mitochondrial content and in the percentage of binucleated cells was noted in the MP-treated liver cell cultures.

MATERIALS AND METHODS

Test Chemicals. MP, clofibrate, DMBA, and AFL were purchased from Sigma Chemical Co. (St. Louis, MO). Diphenhydramine hydrochloride (Benadryl) was a product of the Parke-Davis Co. (Ann Arbor, MI). Nitrosodisethanolamine, methaphenilene, and cimetidine were supplied by Dr. W. Lijinsky (Frederick Cancer Research Facility, Frederick, MD), and Compound Wy 14643 was obtained from Dr. A. Palmer (Frederick Cancer Research Facility). Rhodamine 123 was purchased from Eastman Kodak Co. (Rochester, NY).

Cell Cultures. Two well-characterized liver epithelial cell cultures were used in these studies. One (FNRL-1), derived from a normal 12-day-old Fischer rat, and the other (FPHL-1), derived from an 18-month-old partially hepatectomized Fischer rat, were established and kept frozen in this laboratory and used between passages 10 and 20 in these studies. The establishment, lineage, and characteristics of these liver cell cultures have been described in detail previously (17). Both are diploid epithelial cells and expressed none of the characteristics of transformed liver cells during the period of this study. In addition, a malignant liver cell line, derived from FNRL-1 by APL treatment in vitro (19), and primary liver cells, isolated by the methods described (17) from rats gavaged with test chemicals (see below), were also used in some of the experiments.

Culture Methods. Stock cultures were maintained in Ham's F-12 medium (14), supplemented with 10% fetal bovine serum (Sterile Systems, Inc., Logan, UT), and were subcultured at weekly intervals using 0.05% trypsin (206 units/mg protein; Millipore Corp., Millipore, CA). Cells were plated in 60-mm plastic culture dishes (Falcon Plastics, Oxnard, CA) at a seeding density of 4 × 10^3 cells/cm^2, which produced a confluent monolayer in 7 days.

Experiments In Vitro. FNRL-1 and/or FPHL-1 cells were plated either at very low seeding density (20 cells/cm^2), so that they would produce distinct cell colonies in 10 days, or at the regular seeding density (4 × 10^3 cells/cm^2),
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10³ (sq cm), to produce a confluent monolayer in 7 days. Cells were treated with MP in concentrations from 0 to 100 µg/ml (MP stock solutions in distilled water were diluted with the culture medium) either 1 day after plating or when the cells were nearly confluent. Cells were examined and photographed using a Zeiss inverted-phase-contrast microscope at different times after treatment. Similar experiments were also done using DCF, DMBA, nitrosodiethanolamine, clofibrate, Wy 14643, methaphenilene, diphenhydramine, and cimetidine.

Cell colonies formed from the sparse cultures were projected on a television screen through a video camera fixed on a Zeiss microscope with a ×10 objective. Four colonies selected at random from each quarter of the control and MP-treated dishes (4 dishes each) were used for counting the total as well as binucleated cells.

Cells grown on Formvar-coated coverslips and fixed with 3% glutaraldehyde plus 2% p-formaldehyde in 0.1 M cacodylate buffer (pH 7.35) were also examined with Nomarski interference optics. In addition, samples were also fixed at various times (2 to 24 h) and processed for electron microscopy. Cells which had been pretreated with MP for 24 h and then allowed to "recover" for various periods in the regular culture medium without MP were also processed for TEM.

In some experiments, the control and MP-treated cells were vitally stained with the mitochondrial-specific stain Rhodamine 123 (9). The cells were incubated for 15 min with 6 µg of Rhodamine per ml in the culture medium and then postincubated for 15 min in the medium without the dye. The cells were examined and photographed using a Zeiss Universal microscope with epifluorescence attachment with an excitation-barrier filter and a reflector combination (excitation, 400 to 490 nm; barrier filter, 520 nm; reflector, 510 nm). In addition, Rhodamine-stained cells were trypsinized, and the cell suspensions were analyzed using a fluorescence-activated cell sorter (FACS IV; Becton and Dickenson, Sunnyvale, CA) with an argon laser in the 488-nm band. A total of 1 × 10³ cells from both the control and MP-treated cell populations was used for the analyses.

Experiments In Vivo. Fischer F-344/Cr rats (6-month-old males; average weight, 375 g) from the Animal Resources Program of the Frederick Cancer Research Facility were used. Rats were treated with either MP (dissolved in water; 100 µg/g body weight) or water by gavage (3 times at 9-h intervals) and used 3 h after the final gavage for the isolation of hepatocytes. The livers were perfused with collagenase-dispase as described in detail before (17). Hepatocytes from both the control and MP-treated rats were examined under a phase-contrast microscope, soon after the isolation and again at 24 h after maintaining them as primary culture.

Ultrastructural Studies. Liver epithelial cells plated on a plastic surface or directly on Formvar-coated gold grids were treated with MP (25 µg/ml). Samples were fixed with a fixative containing 3% glutaraldehyde plus 2% p-formaldehyde in 0.1 M cacodylate buffer (pH 7.35) for 1 h or with 1% tannic acid in 1% glutaraldehyde for 20 min at room temperature. The samples were postfixed with 1% osmium tetroxide for 1 h, rinsed 3 times with buffer and distilled water, stained en bloc with 1% aqueous uranyl acetate for 45 min, and then processed (35). Thin sections were stained with Reynold’s lead citrate for 3 min and examined in a Hitachi Hu-12A at an accelerating voltage of 75 kV.

Cells grown directly on Formvar-coated gold grids were fixed using the osmium thiocarbohydrazide osmium procedure and critical point dried (5). The gold grids with whole mounts were then examined in an Hitachi Hu-12A at an accelerating voltage of 100 kV and in Hitachi H-800 at an accelerating voltage of 200 kV in both the transmission and STEM modes. Some of the whole-mount and thick sections (0.25 to 0.5 µm thick) were also examined using the high-voltage electron microscope at the NIH Biotechnology Resource Facility at Albany, NY.

RESULTS

Phase-contrast microscopy of living epithelial liver cells revealed a large number of cytoplasmic granules in the perinuclear area of MP-treated samples compared with the control (Fig. 1, a versus b). Such granules were seen 24 h after treatment with MP, within a dose range of 10 to 100 µg/ml. Even treatment with doses as low as 2.5 µg/ml produced granulation, but only after a longer period of incubation (48 h). A dose of 100 µg/ml has been shown to be toxic to the liver epithelial cells because it inhibited the colony-forming ability of these cells (20). In all further experiments in this study, we used a concentration of 25 µg/ml, a dose which produced considerable granulation within 24 h without any appreciable effects on the viability of the cells, as studied by colony-forming ability and/or acute morphological changes. These granules were stainable with the lipid stains, Sudan Black B and Oil Red O. When fixed cells were examined with Nomarski optics, the untreated control cells showed distinct nucleus, nucleolus, and rod-shaped mitochondria in the perinuclear area (Fig. 1c). In contrast, cells that had been treated with MP for 24 h showed numerous granular structures in the perinuclear area of the cytoplasm, and rod-shaped mitochondria were seldom seen.

Further experiments on the induction of lamellar bodies by MP were done using only phase-contrast microscopy. Cytoplasmic granulation could be induced by MP in both proliferating (spare culture) and nonproliferating (confluent monolayer) liver cell cultures. When sparse cultures of FNRL-1 cells were treated continuously with MP for 12 days, the cytoplasmic lipid granules persisted throughout the period, but they still disappeared within 1 to 2 days after MP removal. To find out whether this response is a specific characteristic seen only in the 2 diploid liver epithelial cells in culture, we have done similar qualitative in vitro experiments using other cell cultures (primary cultures of rat liver cells, secondary cultures of rat embryo fibroblasts, a malignant liver cell line derived from the FNRL-1 cells following AFL treatment), but no electron microscopy was performed. All these cells did produce cytoplasmic granules when examined by phase-contrast microscopy, and the granules disappeared a day after the cells were fed with the regular culture medium. Moreover, freshly isolated liver cells from a rat gavaged with MP (see "Materials and Methods" for details) also showed lipid granules in the cytoplasm, which was not seen in cells isolated from untreated control rats. As in the in vitro experiments, these granules induced by MP in vivo also disappeared when the cells were maintained for 1 day in vitro without added MP. A number of chemicals were then tested (within a dose range of 10 to 100 µg/ml) to see whether they would induce lipid accumulation. These drugs included hypolipidimic agents (clofibrate, Wy 14643), "genotoxic" carcinogens (AFL, DMBA), another nongenotoxic carcinogen (nitrosodiethanolamine), and other antihistaminics (methaphenilene, diphenhydramine, cimetidine). Of these, only methaphenilene and diphenhydramine produced lipid accumulation in the liver cell cultures with a dose >5 µg/ml.

We then investigated the effect of MP on the diploid rat liver epithelial cells in greater detail using TEM. In thin sections from the control cultures, hardly any inclusion bodies were seen, but they did contain a number of rod-like mitochondria (Fig. 1e). In the MP-treated cells, there was a definite increase in the cytoplasmic granules which resembled lamellar bodies with alternating light and dark lamellae (Figs. 1f and 2b). Apparently, there was also an increase in the number of mitochondria in the MP-treated cells (Fig. 1f), but they were smaller and were ovoid, in contrast to the rod-like mitochondria seen in the control. From
these sections, it was not possible to determine whether the small ovoid mitochondria represented tangential sections of rod-like mitochondria which had been placed in a different spatial configuration as a result of the treatment with MP. Therefore, we prepared whole mounts of intact cells (and also thin sections where needed) and analyzed them using HVEM. Using these techniques, we then investigated the time course of the appearance and disappearance of lamellar bodies as well as the relationship between these cytoplasmic inclusions and mitochondria.

Lamellar bodies could be seen in TEM of whole mounts of cells as early as 2 h after treatment with MP (Fig. 2, d versus c), and their number increased considerably throughout the incubation. After 6 h in the presence of MP, small multiple bodies enclosed in double membranes were seen (Fig. 2a). An abundance of lamellar bodies was observed both in the whole mounts (Fig. 2e) and in thin sections (Fig. 2b) of liver epithelial cells treated for 24 h with MP. However, 24 h after the removal of the chemical, hardly any lamellar bodies were visible (Fig. 2f). Even as soon as 6 h after removal of MP, the number of lamellar bodies was reduced considerably (Fig. 3a).

MP also produced appreciable changes in the morphology and number of mitochondria. Whole mounts of normal liver epithelial cells in culture exhibited long rod-like mitochondria when examined by HVEM (Fig. 2c). Pleomorphic mitochondria were observed within 2 h after MP treatment (Fig. 2, d versus c), and some bizarre forms were seen in some of the cells treated for 24 h (Fig. 2e). Many more smaller ovoid mitochondria were also present in the 24-h sample (whole mounts), compared with the untreated control (Fig. 2, e versus c). Therefore, some of the small ovoid mitochondrial profiles seen in the thin sections of cells treated with MP for 24 hr (Figs. 1f and 2b) might represent cut edges of the bizarre (branched or hairpin-shaped) mitochondria present in the intact cells (Fig. 2e). There does seem to be a real increase in the number of ovoid mitochondria in the treated cells. Within 24 h after removal of MP, the morphology of almost all the mitochondria had reverted to the rod-like form (Fig. 2f) seen in the control cells (Fig. 2c). However, 6 h after MP treatment, both the small, as well as the rod-like, forms of mitochondria were visible (Fig. 3a). To determine whether there is any connection between the mitochondria and the lamellar bodies, stereo photographs of high-voltage (1000 kV) electron micrographs were prepared. When viewed stereoscopically, these 2 organelles were found to be located in different planes (Fig. 3b).

We then stained mitochondria in the intact, living liver cells with Rhodamine 123, a vital stain for these subcellular particles (8), to determine whether there was any increase in the actual mass of mitochondria in the MP-treated cells and not just in the number of small mitochondria. When examined with a fluorescence microscope, cells treated with MP for 24 h appeared to contain a greater amount of mitochondria (Fig. 3, c versus d). Similarly, an increase in the Rhodamine 123-stained mitochondria was observed in the primary cultures of hepatocytes, derived from a rat that was gavaged with MP. When the control and MP-treated FNRL-1 cell cultures were analyzed with the fluorescence-activated cell sorter, it was found that, while the cell size remained similar in both cell populations (Chart 1a), there was a definite increase in the number of cells with a higher level of fluorescence in the MP-treated cell population (Chart 1b). This increase in the number of cells with a higher mitochondrial content was statistically significant ($p > 0.005$) if the cells were treated for 48 h with MP (25 μg/ml).

Another observation on the liver epithelial cell cultures, FNRL1 and FPHL1, was the induction of binucleated cells by MP (Table 1). While MP did not affect the colony size (total number of cells/
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colony), a significant (<0.002) induction was observed in the number of binucleated cells/colony or in the percentage of binucleated cells within any of the colonies. Nested analysis of variance has also shown that there are no significant differences between the data obtained from within or among the experiments. Even in other experiments done with a higher inoculum size of the cells, unit areas of the MP-treated dishes showed a higher percentage of binucleated cells (data not shown). Another qualitative observation was the frequent occurrence of tripolar mitoses in the MP-treated cells, while such mitoses were hardly ever seen in the control liver epithelial cell cultures.

DISCUSSION

A number of nongenotoxic chemicals have been identified, and others are being added to the list (7, 10, 11, 18, 21, 23, 25). Although other cell systems were used to study the possible mechanisms by which MP might induce cancer, we concentrated our efforts to determine the effects of MP on diploid rat liver epithelial cell cultures at early passage levels, before they exhibit any transformed phenotypes. These cultures have been shown (19) to induce the expression of conventional transformed phenotypes after treatment for 8 weeks with the genotoxic hepatocarcinogen AFL. In the present study, which spanned only 2 to 12 days, 3 types of cellular responses to MP were seen: these include the appearance of phase-dense cytoplasmic granules (lamellar bodies) in the perinuclear area; alterations in the morphology and content of mitochondria; and an increase in the number of binucleated cells.

The appearance of lamellar bodies soon after MP treatment indicates that this may be due to a direct perturbation of normal lipid metabolism rather than as a result of genetic expression. However, hypolipidemic agents (clofibrate, Wy 14643) or other carcinogenic chemicals (AFL, DMBA, nitrosodimethylamine) did not induce this change. This cellular response appears to be specific only for histamine H1 receptor antagonists (MP, metapirilene, diphenhydramine), since it was not induced by cimetidine, an H2 receptor blocker. This response was also transient in nature and was not linked to the proliferative status of the cells, since the cytoplasmic granules could be produced in both dividing and nondividing liver epithelial cells.

In previous ultrastructural studies in rats, the presence of lamellar bodies in liver has not been described. However, in one study (31), extensive cytoplasmic vacuolization has been found in the liver of rats fed 1000 ppm of MP in food. In our experiments, we administered divided doses of MP by gavage, so as to avoid too little or too much MP in the body at any given time. The freshly isolated hepatocytes did contain lipid granules, and paraffin sections of liver tissue fixed with 4% formaldehyde did show cytoplasmic vacuoles (data not shown), which does suggest that lipid accumulation has occurred.

Mitochondrial alterations were also seen within 2 h of MP treatment (Fig. 2d), and the morphological changes may have been caused by a direct action on the mitochondria or on the fine fibrillar network in the cytoplasm. It is known that mitochondria are arranged along the cytoplasmic microtubules in monolayer cultures of various normal cell lines (15); it has also been suggested that a subpopulation of intermediate filaments can serve as anchorage sites for mitochondria (26). Mitochondria have been shown to be redistributed in the cytoplasm, if microtubules were disrupted with Colcemid (15). It is possible that MP could have brought about the changes in the mitochondrial morphology by preferentially interacting with the microtubules and/or intermediate filaments or by acting directly on the mitochondria. The latter possibility is consistent with previous reports that autoradiographic grains seen in experiments using [3H]MP were located in the cytoplasm (20) and were associated with mitochondria (32). It is now becoming increasingly clear that a number of carcinogenic chemicals bind preferentially with mitochondrial DNA (3, 27, 40, 41), and the extent of this binding can be even 40 to 90 times greater compared with the binding to nuclear DNA. Therefore, it is conceivable that MP could produce significant alterations in the mitochondrial DNA, which in turn would alter the mitochondrial functions, as has been shown with other hepatocarcinogens (4, 10).

Although mitochondria have been implicated in the formation of lamellar bodies (34), our TEM studies (Fig. 2a) as well as the stereophotographs from HVEM (Fig. 3b) have shown no indications of any association between these 2 inclusions or the direct formation of lamellar bodies from mitochondria.

As MP treatment continued, the mitochondrial number (Fig. 3b) and their mass increased (as reflected in the increased fluorescence with Rhodamine 123, which stained the mitochondria specifically; Chart 1b). Previous morphological studies done on the liver of rats fed MP have shown a definite increase in the number of hepatic mitochondria (28, 31–33). In a sequential study, Ohshima et al. (28) described an increase in mitochondria in the MP-treated hepatocytes, but most of the adenomas and premalignant foci showed a decrease in mitochondrial content. In contrast to a decrease in mitochondrial content observed after feeding rats with a number of potent hepatic carcinogens (29), a definite increase was observed earlier in the liver of rats fed the marginally active aminoazo dye, 2-methyl-4-dimethylaminoazo-benzene (29, 39).

Although it has not been proven, the induction of a large number of binucleated cells by MP seems to suggest a perturbation in the microtubules. Recently, a number of nonmutagenic carcinogens or tumor promoters have been shown to induce aneuploidy (12, 36, 37). Our observations, that binucleated cells are induced by MP and that mitochondria which are attached to the fibrillar structure are disrupted by MP treatment, are reasonable indicators that MP may indeed produce aneuploidy after continuous treatment.

The evidence that MP does not affect nuclear DNA has come primarily from a number of short-term in vitro assays (6, 8, 20, 25, 30), each with its own limitation and/or differing sensitivity. None of these assays could identify a chemical that indirectly affects DNA of the target cells. It has been reported recently that nonmutagenic tumor promoters produce sister chromatid exchanges in target cells, when added along with human phagocytes in vitro (38). The differences in published results from assays using hepatocyte primary cultures (1, 6, 8, 25, 30) may be explained if the different preparations of the freshly isolated cells contained liver macrophages (Kupffer cells) and/or leukocytes. Such cells could be activated by MP in vitro (through histamine H1 receptors; 16). Further research into this aspect of the problem is needed for elucidation of the mechanism of action of nonmutagenic carcinogens.
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Fig. 1. a and b, phase-contrast micrographs of live cultures of liver epithelial cells, FNRL-1. a, control untreated cells; b, MP-treated cells. Note the presence of numerous phase-dense granules in the perinuclear area of cells treated with 25 µg of MP per ml for 24 h. a and b, Nomarski interference micrographs of FNRL-1 cells, e and f, TEM of thin sections cut in the perinuclear area of cells treated with 25 µg/ml of MP. a, whole mount of FNRL-1 cells after 24 h of MP treatment. Numerous large osmiophilic lamellar bodies (white arrows) of various sizes are seen in the perinuclear area. Inset, lamellar bodies at higher magnification. Bizarre-shaped mitochondria (black arrows) are sometimes seen in the treated cells. f, whole mount of FNRL-1 cells pretreated for 2 h with MP (25 µg/ml), then grown for an additional 24 h in the absence of MP. The cells have lost most of the lamellar bodies. Mitochondrial morphology resembles that of the untreated control cells in Fig. 2c, except for a few bizarre ones (arrows).

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Fig. 3. a, whole mount of FPHL-1 cells pretreated for 24 h with MP and then grown for 6 h in the absence of MP. Very few lamellar bodies (L) are seen compared with the 24-h-treated sample (Fig. 2e). Numerous small mitochondria (see inset and arrows) are still present. b, stereomicrographs of an area in the cytoplasm showing spatial relationship of mitochondria and lamellar bodies. Mitochondria located at different planes (white arrows) appear to be crossing over each other when viewed in a 2-dimensional plane. Likewise, osmiophilic lamellar bodies (black arrows) that may appear to be touching the mitochondria when viewed in a 2-dimensional plane are in fact located in different planes when viewed stereoscopically. Stereomicrographs were taken with a 1000-kv microscope, NIH Biotechnology Resource Facility, Albany, NY. c and d, microphotographs of live cultures of FNRL-1 cells showing fluorescence from mitochondria stained with Rhodamine 123. MP-treated cells appear to have an increased mitochondrial content in many of the cells in the population (d) compared with those of the control (c).
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