Carcinogen-induced Drug Resistance in Rat Hepatocytes

Brian I. Carr and Brian A. Laishes

ABSTRACT

The relative resistance of liver cells in primary monolayer cell culture to the cytotoxic effects of methotrexate, Adriamycin, cycloheximide, or aflatoxin B₁ was studied using cells derived from normal rats, rats subjected to two-thirds hepatectomy, or rats fed dietary carcinogen. Normal rat liver cells were highly sensitive to the toxic effects of methotrexate, Adriamycin, cycloheximide, and aflatoxin B₁. In contrast, liver cells from carcinogen-treated rats were resistant to the toxic effects of these agents. Cells derived from rats at 24 hr post two-thirds hepatectomy were sensitive to Adriamycin but not to cycloheximide or aflatoxin B₁.

INTRODUCTION

It has been proposed that exposure to chemical carcinogens might be associated with the appearance of a new cell phenotype(s) which is resistant to the known toxic effects of carcinogens exhibited by the cells of normal tissue (8). This general hypothesis has received support through 2 categories of experiment in rat hepatocarcinogenesis, both of which revealed the development of hepatocytes that are highly resistant to known cytotoxic effects of chemical carcinogens (7, 12, 17, 26). The first type of experiment focused on a cytotoxic effect that is cytotoxic to normal rat hepatocytes; i.e., the hepatocyte undergoes lysis (7, 12, 17, 29). The second type of experiment analyzed a cytotoxic effect by which normal rat hepatocytes are rendered incapable of cell proliferation although the cells remain intact (26, 29).

Experiments of the first type have been conducted both in the intact animal and in cell culture. It was shown by Farber et al. (7) that carcinogen-induced neoplastic liver nodules (see Footnote 4 for an explanation of the term 'neoplastic liver nodules') were resistant to the necrogenic effect in vivo of the hepatocarcinogen dimethylnitrosamine under conditions causing necrosis in surrounding liver tissue. Cell culture experiments conducted in this category demonstrated that liver cells prepared from rats that had been fed aflatoxin B₁-contaminated diet were resistant to the cytotoxic effect of aflatoxin B₁ in culture (12). Subsequent in vitro experiments produced a quantitative assay for resistance to the cytotoxic effect of aflatoxin B₁ using cells derived from neoplastic nodules of livers of rats fed 2-AAF* (17).

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* The abbreviations used are: 2-AAF, 2-acetylaminofluorene; DEN, diethylnitrosamine; PH, primary monolayer cultures of cells derived from the livers of rats subjected to two-thirds hepatectomy 24 hr prior to tissue dissociation and containing a high proportion of proliferating hepatocytes; NPH, primary monolayer cultures of normal rat liver cells containing a high proportion of nonproliferating hepatocytes; NLH, primary monolayer cultures of cells derived from livers, containing numerous neoplastic (commonly called hyperplastic) nodules (see Ref. 27 for a discussion of terminology of specific hepatocellular lesions), from rats fed dietary 2-acetylaminofluorene (nodular liver hepatocytes); yGT, y-glutamyltranspeptidase.
which, following isolation, survived doses of toxic 2-AAF derivatives that were cytotoxic to normal rat hepatocytes in vitro (14). These results are consistent with the proposal that a common cellular alteration may be responsible for the expression of the 2-AAF-resistant phenotype in vivo and for the phenotypic property demonstrating resistance to the cytotoxic effect of toxic 2-AAF derivatives in vitro. This proposal forms the basis of the present paper.

In the present paper, we use the quantitative in vitro assay that was originally developed to measure the size of rat hepatocyte populations that were resistant to the cytotoxic effects of chemical carcinogens (17). Thus, we systematically search for nonhepatocarcinogenic chemicals to which hepatocytes isolated during hepatocarcinogenesis are selectively resistant. The most likely candidates for testing in this first stage of our search include several of the chemicals that have been clinically tested against hepatocellular carcinoma (5, 20). Observations of the survival of treated versus untreated patients reveal that clinical results are poor and imply that hepatocellular carcinoma cells may actually be more resistant than normal cells to the cytotoxic action of these drugs. Agents which prove to be selectively cytotoxic to normal rat hepatocytes but not to hepatocytes from carcinogen-treated rats will then be chosen for an in vivo testing program to search for drug-resistant, proliferating hepatocyte phenotypes that develop during carcinogenesis.

Preliminary evidence indicated that normal adult rat hepatocytes, isolated while in the DNA-synthetic phase of the cell cycle following a two-thirds hepatectomy (PH cells), demonstrated high levels of selective resistance to the cytotoxic effects of certain cytotoxic drugs (3). We therefore considered cell cycle-related resistance in the design of our in vitro screening system since autoradiographic studies of rat livers, conducted during hepatocarcinogenesis, revealed that large proportions of hepatocytes are engaged in DNA synthesis (1, 6, 19). An in vitro screening system using either NPH or NLH might therefore detect hepatocytes that express only cell cycle-related resistance and not resistance expressed as a new, specific phenotypic property developed during hepatocarcinogenesis. To exclude this possibility, we utilized PH cells in the screening program and therefore sought agents that are cytotoxic to both NPH and PH cells and not NLH cells.

The possible utility of this in vitro screening system for the purification of specific subpopulations of hepatocytes during hepatocarcinogenesis has been discussed elsewhere (14).

MATERIALS AND METHODS

Animals and Treatment. Male Fischer 344 rats (Microbiological Associates or Charles River Breeding Laboratory) weighing 150 to 200 g were used. The animals were fed a basal, high-casein diet (Bio-Serv Inc., Frenchtown, N. J.), unless supplemented by carcinogen, and were maintained on a 12-hr light cycle in the animal colony. Water was given ad libitum. For some experiments, animals were fed a diet containing 0.02% 2-AAF (w/w) for 8 to 12 weeks. Where indicated, a PH was acquired 24 hr prior to the experiment, according to the standard Higgins-Anderson technique, involving removal of the median and left lateral liver lobes (9).

Primary Monolayer Cultures. Liver cell suspensions were prepared by the proteolytic enzyme perfusion technique (10, 16, 18, 23). Cell suspensions were passed through sterile gauze filters to remove undissociated fragments, and viability was assessed by trypan blue exclusion. Cells were plated at 1 × 10^6 viable cells per plastic culture flask (Falcon Plastics, Oxnard, Calif.; 25-sq cm surface area) in 4 ml of Medium L-15 with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (3.5 mg/ml) and albumin (2 mg/ml), supplemented with 10% fetal bovine serum, penicillin (100 μg/ml), and streptomycin (100 μg/ml). After a 3-hr attachment period at 37° in a water-saturated 5% CO2-95% air incubator, the cells were twice washed in the above medium and then replaced in 4 ml of the same medium with or without (controls) cytotoxic agents (17).

Quantitation of Cell Resistance. The monolayer cultures were incubated for 24 hr. Then, 0.8 ml of trypsin blue was added to the 4 ml of medium in each flask and incubated for 10 min at 37°. After this, the medium was removed, and the number of viable (nonstaining) cells was counted exactly as described (17). The percentage of resistant cells was expressed as the number of viable, attached cells in the experimental flasks compared to the number of viable, attached cells in control flasks. Under these experimental conditions, none of the cell types proliferates.

Cytotoxic Agents. Chemicals were purchased and handled as follows: aflatoxin B1 (Aldrich Chemical Co., Milwaukee, Wis.) was dissolved in dimethyl sulfoxide, its concentration being determined spectrophotometrically, and was stored at -4°; stock solutions of 10 mg Adriamycin (doxorubicin HCl; Adria Laboratories, Inc., Columbus, Ohio) per 10 ml were kept at -4° and discarded after 3 days; stock solutions of 25 mg methotrexate (Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.) per ml are stable at room temperature, and suitable dilutions were made using water; cycloheximide (Sigma Chemical Co., St. Louis, Mo.) was made as a fresh solution for each experiment.

RESULTS

Primary monolayer cultures of cells were established from the livers of normal rats (NPH), or from rats which had been subjected to a two-thirds partial hepatectomy 24 hr previously (PH), or from rats fed the dietary carcinogen 2-AAF for 3 months and which had hepatic nodules (NLH). The perfusates of NPH cells had viabilities of 70 to 80%, whereas those of PH or NLH cells ranged from 55 to 65%. No perfusates containing less than 55% viable cells were used. Chart 1 demonstrates that NPH were sensitive to the cytotoxic effects of aflatoxin B1 at concentrations of 1 × 10^-5 M to 1 × 10^-7 M. By contrast, the NLH, derived from the perfusates of livers containing nodules, and PH hepatocytes were relatively resistant to aflatoxin B1-induced cytotoxicity at these concentrations as judged by trypan blue exclusion. Control flasks demonstrated that the observed differences in resistance were not due to selective cell detachment during the experiment since, for each cell type, less than 15% floating cells were found in the medium at 24 hr. Attachment efficiencies were also comparable for the 3 cell types, as judged by the percentage of attached cells at 3 hr after plating, and they were between 65 and 80%. This experiment differed from those published elsewhere in which nodule cells were shown to be resistant to aflatoxin B1 cytotoxicity (17), since NLH cells represent the whole perfusate from nodule-containing livers. Thus, there was a variable mixture of.
Aflatoxin B<sub>1</sub> Concentration (M)

Chart 1. Comparison of the proportion of aflatoxin B<sub>1</sub>-resistant liver cells cultured from a normal adult rat liver (NPH), from the liver of an adult rat 24 hr after a two-thirds partial hepatectomy (PH), and from the liver of an adult rat which had been fed 0.02% 2-AAF for 3 months (NLH). Each point, mean of 3 flasks; bars, S.D. Percentage of survival of attached cells compared to controls after 24-hr exposure to drug. The data in this and subsequent charts represent the results of 6 experiments on normal hepatocytes, 4 experiments on hepatocytes from 2-AAF-fed rats, and 3 experiments on hepatocytes from rat liver 24 hr post-PH.

nodule and nonnodule hepatocytes, yet greater than 80% of the cells appeared resistant to toxicity by aflatoxin B<sub>1</sub> (Charts 1 and 5). Presumably, therefore, resistance to toxicity induced by aflatoxin B<sub>1</sub> is exhibited by both nodule and nonnodule cells. The PH cells were similarly resistant, but these cells were derived from rats that had not been exposed to a carcinogen.

The cytotoxic action of a protein synthesis inhibitor, cycloheximide, was then examined. This drug is also a cancer-inhibiting drug in animals, but it has proved to be too toxic for use in humans (24, 28). It may be seen (Chart 2) that a similar pattern of sensitivity was displayed by nonproliferating hepatocytes but not by hepatocytes from a PH liver nor from a carcinogen-treated (NLH) rat liver. It seemed that the phenomenon of resistance might be a feature of hepatocytes obtained from the livers of rats containing increased numbers of proliferating cells. This explanation, however, was made highly unlikely by the next experiment using Adriamycin.

Adriamycin (14-hydroxydaunorubicin) is a DNA-binding, anthracycline, antitumor antibiotic in wide clinical use as a cancer chemotherapeutic agent. It is also the most useful drug in the management of clinical hepatocellular carcinoma (5, 20). Chart 3 shows that, at 1.8 x 10<sup>-4</sup> M concentration, normal cells that were either resting (NPH) or proliferating (PH) in vivo were sensitive to the toxic effects of this drug in vitro. Thus, for both aflatoxin B<sub>1</sub> and Adriamycin, there was at least a 2-log difference in the concentration of the drug needed to kill 20% of NPH compared to NLH cells (Charts 1 and 3). Adriamycin toxicity in this system is therefore different from that induced by aflatoxin B<sub>1</sub> and cycloheximide in that carcinogen-altered cells were resistant to its action, compared to normal cells, either resting or proliferating (Chart 3).

Chart 4 shows the differential cytotoxic effects of the folic acid analog and antagonist, methotrexate. It may be seen that this cancer-chemotherapeutic agent was toxic to the NPH but not to the NLH at the concentrations that were tested. Hepatocytes that were proliferating in vivo showed an intermediate resistance to toxicity, depending on the concentration which was used.

Thus, normal hepatocytes are shown to be sensitive to 4 structurally unrelated toxins: one a carcinogen and 3 having cancricidal activities. By contrast, hepatocytes from a carcinogen-fed rat were resistant to the cytotoxic effects of these 4 compounds at doses which were toxic to the normal hepatocytes. Cells which were proliferating in vivo (PH) displayed different degrees of resistance, which depended on the toxin.
they were sensitive to the action of Adriamycin but not to aflatoxin B$_1$ or cycloheximide (Chart 5). It would appear that, depending on the choice of toxin used, the differential sensitivity and resistance of normal and carcinogen-altered hepatocytes, which are described above, could be used in the selection of carcinogen-altered cells in a mixed cell population.

DISCUSSION

The experiments described in this paper were intended to survey compounds that might be selectively toxic to normal hepatocytes while not affecting carcinogen-altered hepatocytes, as a first step to selecting neoplastic or possibly preneoplastic liver cells. Most of the compounds which were tested have antiproliferative action and so might be useful in substituting for 2-AAF in the procedure of Solt and Farber for the production of carcinogen-altered foci, and thus using a nonhepatocarcinogenic mitotic inhibitor. Three nonhepatocarcinogenic compounds that might be suitable for this purpose are methotrexate, Adriamycin [although Adriamycin is not known to be hepatocarcinogenic, it has been reported to induce mammary tumors in rats (21, 25)], and cycloheximide. When methotrexate was given acutely, it did not inhibit the proliferative response to PH (13). However, it has not been given chronically to our knowledge, and our preliminary experiments suggest that it does indeed inhibit the mitotic response of normal hepatocytes to a PH. Methotrexate also has the advantage, for chronic administration, of being absorbed by the gastrointestinal tract when administered in the drinking water.

The most notable feature of the experiments that are described here is the general sensitivity to toxins of cells from normal rat liver in primary monolayer culture compared to the resistance of cells from the livers of rats fed the carcinogen 2-AAF. The phenomenon is the more remarkable considering the heterogeneity of the toxins tested, both in structure and mechanism of action. Thus, cycloheximide (or actidione) is a piperidinedione antibiotic and is a potent inhibitor of protein synthetis (24, 28). Methotrexate, which is a folic acid analog, is thought to act mainly by depriving cells of reduced folates and thus of 1-carbon methyl groups by competing with folates for the enzyme dihydrofolate reductase (for review, see Ref. 4). Adriamycin is an anthracycline antibiotic and is regarded as exerting its antiproliferative effect by cross-linking double-stranded DNA and RNA (4).

The in vitro resistance (Chart 1) to aflatoxin B$_1$-induced cytotoxicity, both by NLH and by PH, at first suggested that the resistance seen in vitro might be associated with a proliferative state. However, it can be seen (Chart 3) that PH were sensitive to the toxic action of Adriamycin, but NLH which also contain a high proportion of proliferating cells in vivo were not sensitive to Adriamycin toxicity. This suggested that the phenomenon of resistance seen in vitro is not directly altered per se to the property of cell replication in vivo but rather to the acquisition in vivo of a phenotypic alteration caused by the carcinogen 2-AAF. This alteration resulted in a proportion of the hepatocytes undergoing cell proliferation in vivo, although the proportion was not as high as that seen 24 hr after a normal liver is subjected to a partial hepatectomy.

The high proportion (greater than 80%) of cells from NLH which showed resistance to cytotoxicity in vitro is remarkable. It has been demonstrated previously that the nodule cells from the livers of rats treated with dietary 2-AAF displayed resistance in vitro to toxicity by aflatoxin B$_1$ (17). However, the similar experiment which is shown in Chart 1 was performed on the unselected whole-liver perfusate from a nodule-bearing rat liver and not on the hepatocytes derived only from nodules, as described previously (17). Thus, in this situation, nonnodule hepatocytes were included in the experiment. Smears made from the suspensions of hepatocytes from the carcinogen-treated rat livers, when stained for the marker γ-GT, showed that 30 to 40% of the cells stained positively for this marker, a figure similar to that noted elsewhere (2, 15). Thus, the phenomenon of resistance in vitro, which we have demonstrated using whole-liver perfusates from carcinogen-treated rat livers, indicates a carcinogen-induced phenotypic change that is more extensive than that which is demonstrated by staining for γ-GT alone, and it is not confined to the cells of foci and nodules, as judged by the high proportion of cells demonstrating the re-
Hepatocyte Resistance in Carcinogenesis

REFERENCES


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