SHORT COMMUNICATION

Development of resistance during the early stages of experimental liver carcinogenesis

Aroon Yusuf, Prema M.Rao, Srinivasan Rajalakshmi and Dittakavi S.R.Sarma

Department of Laboratory Medicine and Pathobiology, Faculty of Medicine, University of Toronto, Medical Sciences Building, Toronto, Ontario, Canada M5S 1A8

The present study was designed to determine whether the resistant phenotype is acquired at the initiated cell stage itself or requires further exposure to a promoting regimen to express resistance. Male Fischer 344 rats were initiated with diethylnitrosamine (DENA) (200 mg/kg i.p.) and were subjected to either no further treatment or to the resistant hepatocyte (RH) model of liver tumor promotion. Six weeks later, the resistance of the focal lesions generated in these two groups to the mitoinhibitory effects of 2-acetylaminofluorene (2-AAF) was determined by subjecting the rats to two-thirds partial hepatectomy (PH) in the presence of a mitoinhibitory dose of 2-AAF (5 mg/kg i.p.) given at the time of PH. Labeling index was determined by administering multiple injections of [3H]thymidine. All rats were killed 48 h post-PH. While only a small percentage (23%) of the glutathione S-transferase-positive foci generated by DENA in the absence of an exogenous liver tumor promoting regimen were resistant to the mitoinhibitory effects of 2-AAF, a majority (85%) of the foci became resistant to 2-AAF following exposure to the RH model of liver tumor promotion.

Further, initiated rats exposed to either 2-AAF or CCl₄ alone, the two components of the RH model, resulted in 71% of the foci being resistant to the mitoinhibitory effects of 2-AAF. Similar patterns of results were obtained when the resistance of the foci to the mitoinhibitory effects of orotic acid, a liver tumor promoter and an inhibitor of DNA synthesis in normal hepatocytes, was monitored. These results suggest that the majority of initiated hepatocytes are not of resistant phenotype, however, they have acquired a unique ability to express resistance upon exposure to certain agents such as 2-AAF and CCl₄ or to a promoting regimen such as the RH model of liver tumor promotion.

One of the characteristic features of the neoplastic cell is its resistant phenotype. In addition, in certain systems it has also been demonstrated that neoplastic cells acquire a unique ability to express a multi-resistant phenotype upon exposure to certain chemicals, including cancer chemotherapeutic agents (1–3). Experimental models of carcinogenesis, especially rat liver models, not only substantiated the concept that the neoplastic cell is of resistant phenotype, but also provided evidence that ‘resistance’ is expressed early in the carcinogenic process (4). Further, hepatic nodules, a precursor population for hepatocellular carcinoma, exhibited alterations in the biochemical machinery that are compatible with the concept that the neoplastic cell is of resistant phenotype, expressing resistance to cytotoxicity and mitoinhibition induced by a wide variety of structurally diverse chemicals and also to negative growth regulation (4–14). However, it is not clearly established whether different components of resistance are acquired at the initiated cell stage itself or acquired at different phases of the carcinogenic process. The present approach was developed to address these questions.

In this study, expression of the resistant phenotype was monitored by determining the resistance to the mitoinhibitory effects of 2-acetylaminofluorene (2-AAF) or orotic acid, a liver tumor promoter. Male Fischer 344 rats were initiated with a single administration of diethylnitrosamine (DENA) and later exposed to the resistant hepatocyte (RH) model of liver tumor promotion (2-AAF coupled with CCl₄) or to no further treatment. Several weeks later the rats were subjected to two-thirds partial hepatectomy (PH) in the presence of mitoinhibitory levels of 2-AAF or orotic acid. Proliferating hepatocytes were labeled with [3H]thymidine. We arbitrarily defined the resistant focus as the focus that had a labeling index higher than the highest labeling index in the surrounding liver. If the foci generated by DENA alone (in the absence of exposure to the RH protocol) are resistant to the mitoinhibitory effects of 2-AAF or to orotic acid then it will be reasoned that this resistance was acquired at the initiated hepatocyte stage itself. On the other hand, if the foci becomes resistant upon exposure to the RH protocol then it will be considered that initiated hepatocytes may not be of resistant phenotype but have acquired an ability to express resistance upon exposure to the promoting regimen. In the present discussion, unless otherwise stated, resistance is defined as resistance to the mitoinhibitory effects of 2-AAF or orotic acid.

Accordingly, to determine whether the initiated hepatocyte is a resistant phenotype, male Fischer 344 rats (Charles Rivers Breeding Laboratories, St Constant, Quebec, Canada) weighing 120–130 g were initiated with a single necrogenic dose of DENA (200 mg/kg i.p.; Sigma Chemical Co., St Louis, MO). Two weeks later the rats were divided into two groups. The rats in the first group (group 1) were not subjected to any further treatment, whereas those in group 2 were subjected to the RH model of liver tumor promotion (15): 20 mg/kg intragastral 2-AAF daily for 3 days and on day 4, 2 ml/kg intragastral CCl₄, 1:1 with corn oil. Six weeks later all animals were subjected to PH in the presence of 2-AAF (5 mg/kg i.p.) given at the time of PH. This dose of 2-AAF inhibits DNA synthesis by nearly 100% in normal hepatocytes. [3H]Thymidine (50 µCi/injection, sp. act. 84.6 Ci/mmoll) was administered every 4 h beginning 16 h after PH to determine the labeling index in the surrounding liver and the foci. At 48 h post-PH,
Fig. 1. Percent labeling index of hepatocytes exposed to a mitoinhibitory dose of 2-AAF (5 mg/kg) in foci/nodules and surrounding non-nodular liver in animals initiated with a single necrogenic dose of DENA (200 mg/kg) and exposed to no further treatment (A) or to a complete RH protocol of liver tumor promotion (B). Every focus/nodule in the section and 30 random fields in the surrounding non-nodular liver in group A and 60 random fields in the surrounding liver in group B were counted. Every hepatocyte, both labeled and unlabeled, in the focus/nodule and on average 100 hepatocytes in each field in the surrounding non-nodular liver were counted. Percent labeling represents the percent of labeled hepatocytes per total hepatocytes within the focus or within the field. Each triangle represents one focus or one field from the surrounding liver. Data shown are for three animals in group A and six animals in group B. The dashed line represents the highest labeling index in the surrounding liver. Further details are given in the text.

all animals were killed and livers were sectioned and fixed in cold acetone for immunohistochemical staining for glutathione S-transferase (GST-7,7). The stained sections were then processed for autoradiography (16).

The results presented in Figure 1 indicate that 23% of the foci generated by DENA alone (group 1) in the absence of an exogenous liver tumor promoting regimen were resistant to the mitoinhibitory effects of 2-AAF. In contrast, 85% of the foci initiated by DENA and promoted by the RH model were resistant to the mitoinhibitory effects of 2-AAF (Figure 1). The latter observation is in agreement with the conclusions drawn from the RH model of liver tumor promotion (4). Since the RH model has two components, i.e. 2-AAF and CCl₄, it became of interest to determine whether an exposure to either 2-AAF or CCl₄ itself is sufficient for the initiated hepatocyte to express the resistant phenotype. To answer this question, in the next experiment male Fischer 344 rats (120–130 g) were initiated with DENA as described in the previous experiment and were then divided into two groups. Two weeks after the administration of DENA, rats in group 1 received only 2-AAF (20 mg/kg intragastrically, daily for 3 days), whereas the rats in group 2 received only CCl₄ (1:1 with corn oil, 2 ml/kg intragastrically). Six weeks later, all the rats were subjected to PH in the presence of 5 mg/kg 2-AAF. Rats initiated with DENA alone without any further treatment (group 1 in the previous experiment) were used as the control for this experiment. Resistance of the foci was determined as described above. The results presented in Figure 2 indicate that a large number of foci (71%) exhibited resistance to the mitoinhibitory effects of 2-AAF. Resistance or lack of it did not appear to depend on the size and zonal distribution of foci.
suggests that the acquisition of resistance may reflect a metabolic conversion of orotic acid or 2-AAF as an index to monitor the resistant phenotype. The fact that a similar pattern of resistance was observed to two different mitoinhibitory agents, orotic acid and 2-AAF. These two agents require entirely different metabolic pathways for them to exert mitoinhibitory effects. Orotic acid inhibits DNA synthesis by nearly 100% in normal hepatocytes. The values are generated from six rats in each group.

In the second series of experiments, the above-described studies were repeated by determining the resistance of foci to the mitoinhibitory effects of orotic acid, a liver tumor promoter and a mitoinhibitor for normal hepatocytes. Similar to the results obtained above, only a small percentage (5%) of foci generated by DENA alone in the absence of any further treatment were resistant to the mitoinhibitory effects of orotic acid, whereas a majority (80%) of the foci initiated by DENA and promoted by the RH model of liver tumor promotion were resistant to the mitoinhibitory effects of orotic acid (Table I). Further, exposure of initiated hepatocytes to 2-AAF alone or to CCl<sub>4</sub> alone resulted in a greater percentage (60 and 40%, respectively) expressing resistance to the mitoinhibitory effects of orotic acid (Table I).

The results presented above are interpreted to indicate that a majority of initiated hepatocytes are not a resistant phenotype, however, they have acquired a unique ability to express resistance upon exposure to either the promoting regimen or to agents such as 2-AAF and CCl<sub>4</sub>. Since 2-AAF and CCl<sub>4</sub> are genotoxic, it would be interesting to determine whether the second exposure has to be a genotoxic insult for the initiated hepatocytes to express the resistant phenotype. The fact that the surrounding non-initiated hepatocytes under identical conditions did not express resistance to either orotic acid or 2-AAF suggests that the initiated cells are altered in such a fashion that they have acquired a unique potential to express resistance upon exposure to certain tumor promoters and/or genotoxic agents. This unique property, i.e. the ability to express resistance upon exposure to certain chemicals, seen as early as in initiated cells, can be observed throughout the neoplastic process as well. Development of multi-drug resistance during cancer chemotherapy is a classical example of this phenotype. The expression of resistance does not seem to be an adaptive response targeted specifically to the chemical or the drug to which the preneoplastic or the neoplastic cell is exposed. For example, in these studies resistance was monitored to two different mitoinhibitory agents, orotic acid and 2-AAF. These two agents require entirely different metabolic pathways for them to exert mitoinhibitory effects. Orotic acid needs to be metabolically converted to uridine nucleotides and the accumulation of uridine nucleotides is essential for it to exert its mitoinhibitory effects (17,18). Conversely, 2-AAF needs to be hydroxylated and esterified for it to exert its mitoinhibitory effects (19). The fact that a similar pattern of results was obtained irrespective of whether we used orotic acid or 2-AAF as an index to monitor the resistant phenotype suggests that the acquisition of resistance may reflect a significant event in the pathogenesis of the carcinogenic process. It will be of interest to identify the alteration(s) relatable to resistance at the genetic level and to determine by molecular approaches whether a resistant phenotype with a potential to progress to cancer can be induced.

The results of the present study raise several questions. For example, what is the significance of the two populations of lesions, i.e. the foci that express resistance and those that express resistance upon exposure to the tumor promoting regimen, in terms of carcinogenesis? Is it that the lesions that have not yet acquired resistance are promoter dependent for their growth and progression and, by contrast, those that express resistance have perhaps become promoter independent? Another question pertains to the heterogeneity within the population of the resistant lesions. Could it be that the degree of resistance reflects the ability or the inability to remodel, the least resistant ones being those with greater potential to remodel? In other words, this heterogeneity may play an important role in the different fates of these preneoplastic lesions in the pathogenesis of the carcinogenic process. These considerations will also be of significance in developing chemopreventive and therapeutic strategies for liver cancer. The eventual question, however, is whether acquisition of resistance is a prerequisite for the initiated cell to progress through the carcinogenic process. The studies carried out by Farber and co-workers clearly point out that acquisition of resistance is one mechanism for the initiated cell to progress through the carcinogenic process (4,20). Indeed the RH model of liver tumor promotion was developed on the premise that the early putative preneoplastic hepatocyte is a resistant phenotype (4). Furthermore, rats initiated with DENA alone developed no hepatocellular carcinomas, while those similarly initiated with DENA and subsequently exposed to the complete RH protocol or to dietary 2-AAF alone had incidences of 70 and 45% hepatocellular carcinoma, respectively, within 8 months (21). Whether acquisition of resistance is the only mechanism for cancer development is still an open question. Nevertheless, it may be rationalized that acquisition of resistance in its broadest sense provides the preneoplastic and the neoplastic hepatocytes with a growth and survival advantage in an otherwise mitoinhibitory and cytotoxic environment created by tumor promoters and carcinogens.

Based on the results of the present study, it may also be speculated that certain tumor promoters, especially those tumor promoters that are mitoinhibitors, may induce resistance in initiated cells and selectively amplify those that acquired resistance (Figure 3). More importantly, it is likely that there may be agents which are not necessarily initiators or promoters
but still participate in the carcinogenic process by virtue of their ability to induce resistance in initiated hepatocytes. It would be interesting to identify this group of compounds and determine their role in the carcinogenic process.

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References


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