Induction of Resistance to the Mitoinhibitory Effects of Orotic Acid During Hepatocarcinogenesis

By

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A thesis submitted in conformity with the requirements
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To

Bhagawan Sri Sathya Sai Baba

My Eternal Teacher, and Continual Source of Inspiration

"The end of wisdom is freedom.
The end of culture is perfection.
The end of knowledge is love.
The end of education is character."

-BABA

Key words: Liver cancer; Resistant phenotype; Induction of resistance; Orotic acid; Uridine phosphorylase; P-glycoprotein; PSC 833; Cyclosporin A.

The overall objective of the thesis is to characterize the phenomenon of “resistance” during hepatocarcinogenesis. Results obtained indicate that a majority of initiated hepatocytes are not a resistant phenotype; however, resistance can be induced upon exposure to certain tumor promoters and genotoxic agents. For example, majority of hepatic foci generated by diethylnitrosamine in the absence of exogenous liver tumor promoters are not resistant to the mitoinhibitory effects of orotic acid (OA) and 2-acetylaminifluorene (2-AAF). However, they expressed resistance upon exposure to the resistant hepatocyte model of liver tumor promotion, 2-AAF and CCl4. These results indicate “induction of resistance” as a new step in the carcinogenic process. Hepatic nodules are resistant to the mitoinhibitory effects of OA largely because they do not accumulate uridine nucleotides upon exposure to OA, a condition necessary for OA induced mitoinhibition. Two defects which could account for the lack of accumulation of uridine nucleotides are: increased degradation of uridine nucleotides and decreased uptake of OA. Nodules promoted by OA expressed increased levels of a variant form of uridine phosphorylase (URPase) an enzyme that degrades uridine nucleotides, and thus escape from the mitoinhibitory effects of OA. URPase is also upregulated in nodules and cancers generated by the resistant hepatocyte model. Furthermore, URPase acts on 2-deoxyuridine to generate 2-deoxyribose, an angiogenic factor. These results suggest a
much broader role for URPase in carcinogenesis. PSC 833, an inhibitor of P-glycoprotein (P-gp), induces hepatic levels of P-gp, increases the urinary excretion of OA and decreases the OA induced hepatic uridine nucleotides. These results indicate the possibility that P-gp or P-gp-like molecules may be involved in the efflux of OA. Thus, hepatic nodules which express increased levels of P-gp may escape from the mitoinhibitory effects of OA by increasing the efflux of OA. In summary, the results identified “induction of resistance” as a new step in the carcinogenic process. In addition, increased expression of URPase and P-gp may contribute to the resistance of hepatic nodules to the mitoinhibitory effects of OA, by increasing the degradation of uridine nucleotides and by increasing the efflux of OA respectively.
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CHAPTER 1

INTRODUCTION
1.1 Carcinogenic Process

Cancer is a broad term used to define a group of more than 200 different diseases. It is a slowly developing, complex disease process occupying about one-half to two-thirds of the life span of the organism (Foulds 1969). The hallmarks of overt malignant cancer are: (1) autonomous growth, (2) invasion and (3) metastasis. Histologically, cancer is characterized by several morphological alterations, including changes in tissue architecture, cytological abnormalities of both the nucleus and cytoplasm and the presence of abnormal mitoses. Several studies have shown that the process usually starts in one or a few discrete cells which acquire in a stepwise manner, several biochemical, genetic and biological alterations that eventually result in a cancer.

Essentially all proliferating tissues in the body are susceptible to the formation of cancer presenting challenges to both the prevention and ultimate cure. Primary liver cancer although rare in the western world, is the second most common cancer worldwide (Munoz and Bosch 1987). Viruses and chemicals have now been identified as the most important etiological factors associated with the development of human liver cancers and with the aid of animal models, exciting advances on the mechanism of hepatocarcinogenesis have begun to emerge. These advances may provide valuable insights into understanding and possibly preventing this disease. In this chapter I will focus mainly on the biochemical and molecular mechanism of early stages of cancer development in the liver.
1.2 Stepwise Development of the Carcinogenic Process

Cancer is a slow multi-stage, multi-step process which involves the appearance of discrete cell populations at different stages in the process (Farber and Magee 1960; Foulds 1969; Farber and Cameron 1980). Each stage signifies a distinct biological endpoint which is relevant to the process and permits both the proposal of a hypothesis and the design of experiments to test the hypothesis. Although all the steps involved in the development of cancer have yet to be elucidated, seminal studies using the mouse skin carcinogenesis model (Friedewald and Rous 1944; Berenblum and Shubik 1947) have identified three major stages - initiation, promotion and progression (Figure 1.1). These stages have now been extended to many other organs such as liver, colon, mammary gland and urinary
bladder (Farber and Cameron 1980). This discussion will give emphasis to studies of these three stages as they pertain to liver carcinogenesis.

**1.2.1 Initiation**

Initiation is defined as the process by which a given organ or target tissue acquires the ability to be promoted or selected to develop focal lesions, one or more of which can act as sites of origin for the subsequent development of malignant neoplasia (Farber and Sarma 1987).

The essential features of initiation are that, it is an irreversible process which occurs rapidly usually after a brief exposure to a small dose of a carcinogen and affecting a small population of cells (1 in $10^5$ - $10^6$ hepatocytes; (Farber and Cameron 1980)). It is induced by the carcinogen and in general not by agents which subsequently are used to promote their focal proliferation.

In the liver, at least two known obligatory steps are required for the formation of an initiated cell. The first major event involves the generation of a biochemical/molecular lesion(s) and the second a fixation or “making permanent” of the lesion(s) by at least one round of cell proliferation (Cayama et al., 1978; Columbano et al., 1981; Ying et al., 1982; Farber and Sarma 1987). Although the nature of the lesion(s) is not completely known, DNA has been strongly implicated as the major target of the carcinogen during the initiation process (Miller and Miller 1974; Rajalakshmi et al., 1982; Farber and Sarma 1987). The result of such alterations in DNA include formation of adducts, translocations, deletions and the activation of proto-oncogenes among others. The second important step in the formation of an initiated cell is the requirement of cell proliferation. Experiments have shown that a single non-necrogenic dose of a hepatocarcinogen rarely results in hepatocellular carcinoma in an adult animal. Nonetheless, liver cell cancer can be induced in situations where there is
high cell proliferation, as in neonatal animals (Della Porta and Terracini 1969; Peraino et al., 1984), or when the carcinogen is given in association with a liver cell proliferative stimulus such as 2/3 partial hepatectomy (PH) as demonstrated by Craddock (Craddock 1971). Several investigators (Cayama et al. 1978; Colombano et al. 1981; Ying et al. 1982; Kaufmann et al., 1991) subsequently substantiated these initial observations and showed that some chemicals which were not carcinogenic became carcinogenic when their administration was coupled with PH. Delaying the proliferative stimulus after carcinogen administration resulted in a lack of initiation (Scherer et al., 1977; Cayama et al. 1978; Ishikawa et al., 1980; Ying et al. 1982). It was proposed that the lack of initiation in these cases was due to the repair of the carcinogen induced critical lesion(s) in DNA by one of several DNA repair mechanisms. Since replication of the carcinogen damaged DNA results in a transfer of the damage to daughter cells, initiation is referred to as an irreversible step. The type of cell proliferative stimulus has now been identified as an important factor for initiation. The compensatory type of cell proliferation such as that induced by 2/3 PH (Craddock 1971; Cayama et al. 1978; Pitot and Sirica 1980; Colombano et al. 1981) or liver cell necrosis induced by cytotoxic agents such as CCl₄ (Ying et al., 1981) supports initiation. Direct mitogenic type of cell proliferation induced by compounds such as lead nitrate, nafenopin, cyproterone acetate and ethylene dibromide however, do not support initiation (Columbano et al., 1982a; Colombano et al., 1982b). The nature of the differences between compensatory and direct mitogenic cell proliferation in their ability to support initiation still remains obscure. It must be highlighted that initiated cells are by no means capable of independent, autonomous growth, invasion or metastasis unless they undergo clonal expansion and possibly other alterations during the tumor promotion and progression phases of carcinogenesis.
1.2.2 Promotion

Promotion is operationally defined as the selective, clonal amplification of the initiated cells into focal proliferations such as papillomas in the skin and urinary bladder, nodules in the liver and polyps in the colon, one or more of which may act as precursors for the development of cancer (Farber and Sarma 1987). Tumor promoters by definition are not carcinogenic and there is no current implication of any biochemical and/or molecular event(s) associated with them. Many aspects of tumor promotion were first described in the skin model of carcinogenesis in mice. Whereas initiation usually occurs in response to a single brief exposure to a given carcinogen, promotion is dependent on repeated or long-term exposure to the promoting agent to induce focal proliferations in the skin (Berenblum and Shubik 1947), liver (Peraino et al., 1971; Shinozuka et al., 1979; Farber and Cameron 1980; Shinozuka and Lombardi 1980; Laconi 1988b) and other organs (Pitot 1979; Farber and Cameron 1980; Dragan and Pitot 1992). In comparison to initiation, promotion is a relatively a slow process, requiring anywhere from a few weeks to a few months for the generation of grossly visible focal lesions. Further, unlike initiation it is a reversible process, at least in the early stages since most papillomas in the skin, polyps in the colon and nodules in the liver have been shown to regress or redifferentiate into normal looking cells upon withdrawal of the promoter. The fact that initiated cells per se can grow to form focal proliferations, albeit very slowly, suggests that they have a growth advantage over the surrounding normal tissue. However, in the presence of promoting regimens they grow faster. Thus the fundamental requirement in tumor promotion appears to be the creation of a selective environment or differential, which permits greater amplification of initiated cells over the non-initiated ones (Farber 1982). Several working hypotheses exist to operationally define how a differential can be achieved for the development of focal proliferations. These include, differential stimulation (Ohde et al., 1979; Dragan and Pitot 1992), differential killing (Farber et al., 1976), differential recovery (Yuspa and Morgan 1981), and most pertinent to this dissertation, differential mitoinhibition (Farber 1982).
1.2.3 Progression

Progression is defined as the process through which one or more focal proliferations, such as papillomas, polyps or nodules undergo a slow, but increasingly malignant cellular evolution to cancer without any external stimulus or intervention (Farber and Sarma 1987). This definition also includes a series of changes involved in the final processes of invasion and metastasis by the neoplasm. Although there is no clear demarcation between where promotion ends and progression begins, in the rat liver, this process is commonly characterized by the appearance of nodules within nodules (Popper et al., 1960; Farber and Cameron 1980; Farber 1982; Laconi 1988b) with cancer eventually occurring inside these nodules (Foulds 1969; Solt et al., 1977). This material continuity between the nodules and the end point cancer establishes that hepatic nodules are one precursor lesion for hepatocellular carcinoma. During progression several important changes occur within the persistent nodules. Foremost, there is an increase in the proliferating cell population and an increased rate of cell loss. However, the overall balance favours cell proliferation, thus accounting for a slow rate of enlargement of the nodules (Enomoto and Farber 1982; Tatematsu et al., 1983; Rotstein et al., 1986). The basis for progression is not clearly known at either the biochemical and/or the molecular level. However, production of growth factors, chromosomal changes, genetic instability (Nowell 1976; Fearon and Vogelstein 1990; Dragan and Pitot 1992), hormones and dietary influences are all postulated to play an important role during progression (Farber and Sarma 1987; Sirica 1989).

One of the challenges presented by the carcinogenic process is to understand the mechanism by which initiated hepatocytes grow to form focal proliferations, foci and nodules. Several experimental models have been designed to answer this question. These models offered different perspectives and contributed to an understanding of the carcinogenic process. In the context of my thesis, I will discuss in some detail two of the
most notable ones, the resistant hepatocyte (RH) model of liver tumor promotion; and the orotic acid model of liver tumor promotion on which our laboratory has been working.

1.3 RESISTANT HEPATOCYTE MODEL OF LIVER TUMOR PROMOTION:

Farber and his associates proposed that during initiation, carcinogens induce a resistant phenotype in a few rare initiated hepatocytes which enables them to grow in a mitoinhibitory environment in which the majority of normal uninitiated cells are inhibited. In the presence of such a mitoinhibitory environment if a cell proliferative stimulus is given, these resistant cells respond to growth stimuli and form focal proliferations (Solt and Farber 1976). Accordingly, the model consists of a brief exposure of initiated rats to the mitoinhibitory agent, 2-acetylaminofluorene (2-AAF) coupled with a cell proliferative stimulus such as 2/3 PH or carbontetrachloride. Under these conditions, an intense selection pressure is created such that uninitiated cells are inhibited from growing, while the mitoinhibitor-resistant initiated cells proliferate in a synchronous fashion to form foci and nodules within a few days (Solt et al. 1977). This model is one of the most well characterized models of multistage carcinogenesis in the rat liver. The salient features of the RH model include the proliferation of initiated hepatocytes in a synchronous fashion to form foci and nodules within a few days (Solt et al. 1977). In addition, this is the only model that generates such a series of changes in a synchronous fashion and has enabled an analysis of the sequential steps in the process (Farber 1984b). Finally it also provides major insights into the dynamics of cell cycle kinetics in hepatocyte nodules during the promotion phase of the process (Rotstein et al., 1984; Rotstein et al. 1986).

1.4 OROTIC ACID MODEL OF LIVER TUMOR PROMOTION:

Orotic acid has been shown by our laboratory to promote rat liver carcinogenesis when initiated by a variety of chemical carcinogens (Columbano et al. 1982a; Rao et al., 1983;
Laurier et al., 1984; Rao et al., 1984; Laconi et al., 1987; Laconi 1988a). In this model initiated rats exposed to a diet containing 1% orotic acid (OA), showed a nearly 100% incidence of hepatocellular carcinoma at the end of one year. A minimum of 20 week exposure (Laconi et al., 1993c) to a 1% OA diet following initiation was found to be sufficient to achieve maximum incidence of hepatocellular carcinoma with metastasis to the lung in 60% of the animals within one year (Denda et al., 1985; Laconi 1988a; Bradley et al., 1992). OA has also been shown to promote carcinogenesis in organs other than the liver such as the duodenum (Rao et al., 1986a; Rao et al., 1987), the mammary gland (Elliot and Visek 1989) and in the pancreas (Kokkinakis and Albores-Saavedra 1994). OA can also potentiate tumorigenesis in the lungs and kidneys of rats treated with N-nitroso-(2-hydroxypropyl)(2-oxypropyl)amine (Kokkinakis et al., 1991). These studies reveal that even though orotic acid is a normal cellular constituent, yet when ingested in excess it can be a multi-organ tumor promoter. There are several metabolic and genetic disorders that can produce excess amounts of orotic acid endogenously. Could these conditions pose an increase risk of cancers in humans? This important question has never been addressed in the past and warrants investigation.

1.4.1 The biochemistry of orotic acid
OA, a pyrimidine 2,4-diketo-6-carboxylic acid, is an intermediate in the de novo synthesis of pyrimidine nucleotides. It is normally found in the milk of many species and most significant amounts are found in cow’s milk. However, only trace amounts are found in the milk of humans (Kobata et al., 1962; Okonkwo and Kinsella 1969). A schematic representation of the series of steps in the synthesis of OA and subsequent pyrimidine nucleotides is presented in Figure 1.2. A brief overview illustrates that the synthesis of OA begins with the formation of carbamoylphosphate (CP) in the cytosol from bicarbonate, glutamine and ATP catalyzed by carbamoylphosphate synthetase II (CPS II). The next step is the addition of aspartic acid which results in the synthesis of carbamoylaspartate (CA). This molecule then
undergoes cyclization to form dihydroorotic acid, which in turn is dehydrogenated to give orotic acid. In the cytosol these reactions are catalyzed by the multienzyme complex CAD (carbamoylphosphate synthetase II, aspartyltranscarbamoylase and dihydroorotate dehydrogenase). Orotate then reacts with 5-phosphoribosyl-1-pyrophosphate (PRPP) to form orotidine-5-phosphate, which in turn undergoes decarboxylation to yield uridine monophosphate (UMP), the direct precursor of other pyrimidine nucleotides. These latter steps, i.e., the synthesis of UMP from orotate is catalyzed by a second enzyme complex called UMP synthase and contains orotate phosphoribosyltransferase and orotidine-5’-phosphate decarboxylase (Jones 1980; Floyd and Jones 1985).
Figure 1.2  Schematic representation of the metabolic pathway for the production of orotic acid and pyrimidine nucleotides. Details of the pathway are given in the text.
1.4.2 Role of Urea Cycle in orotic biosynthesis

The urea cycle is connected to the OA pathway through the production of mitochondrial CP (Figure 1.2). Hence any blockage in the utilization of mitochondrial CP for urea synthesis can result in an overproduction of OA and uridine nucleotides. As pointed out earlier an important consideration in the study of the OA model is the fact that there are genetic and metabolic disorders which are associated with increased levels of orotic acid. These disorders may pose an increased risk of tumor promotion. Increased excretion of OA has been noted in children with hyperammonemia due to ornithine transcarbamoylase deficiency (Levin et al., 1969) as well as in animal models (Qureshi et al., 1979; Vasudevan 1989). Other urea cycle enzyme deficiency disorders also result in increased OA production. These include deficiency in argininosuccinate synthetase (Beaudet et al., 1986), argininosuccinate lyase (Kalumuck et al., 1985) and arginase (Dizikes et al., 1986). Diets lacking in ornithine, citrulline or arginine (Milner and Visek 1973; Hassan and Milner 1979) have also been shown to result in the elevated excretion of OA. Interestingly, an arginine deficient diet is also associated with nucleotide pool alterations very similar to that induced by OA (Milner and Visek 1973; Hatchwell and Milner 1978b; Vasudevan 1989) and is also known to exert a liver tumor promoting effect in rats and mice (Vasudevan 1989). High protein diets have also been shown to increase OA excretion by up to three fold (Hatchwell and Milner 1978a; Nishio et al., 1986). Orotic aciduria can also result from hepatic insufficiency due to partial hepatectomy, carbontetrachloride toxicity (Visek 1985), portacaval shunt (Steele 1984), hepatic cirrhosis and alcoholic hepatitis in rats and humans (Visek et al., 1984; Visek 1985). Epidemiological data about the aforementioned diseases and their cancer incidences are poor, however few reports have begun to emerge about hepatocellular carcinoma in patients with orotic aciduria and hypercitrullinemia due to a deficiency of argininosuccinate synthetase (Jeffers et al., 1988; Nakayama et al., 1990). Whether OA has a role in the increased incidences of liver tumors in these diseases is only speculative at present and further study is required.
Orotic acid has been shown to be metabolically converted to uridine nucleotides; and exposure to OA results in a several fold increase in the levels of hepatic uridine nucleotides. (Fig. 1.3) (Rajalakshmi et al., 1961; von Euler et al., 1963; Marchetti et al., 1964). Several biochemical studies indicated that OA needs to be metabolized to uridine nucleotides to exert its biological and biochemical effects. Adenine (Rajalakshmi and Handschumacher

**Pyrimidine Nucleotide Synthesis**

Carbamoyl phosphate + Aspartate  
\[ \rightarrow \]  
Dihydroorotate  
\[ \rightarrow \]  
Orotic Acid  
\[ \rightarrow \]  
Adenine  
\[ \rightarrow \]  
OMP  
\[ \downarrow \text{Allopurinol} \]  
UMP

\[ \text{Ribose-5'-PO}_4 + \text{ATP} \]  
\[ \rightarrow \]  
Glutamine  
\[ \rightarrow \]  
5-Phosphoribosylamine  
\[ \rightarrow \]  
IMP  
\[ \rightarrow \]  
AMP, GMP

**Purine Nucleotide Synthesis**

**Figure 1.3** Metabolic pathways of pyrimidine and purine nucleotide biosynthesis involving 5-phosphoribosyl-1-pyrophosphate (PRPP). Details are provided in the text
1968) competes with OA for PRPP and allopurinol (Rao et al., 1986b; Rao et al. 1987) inhibits orotidine decarboxylase. These inhibitors not only inhibit the metabolic conversion of OA to uridine nucleotides but also inhibit OA induced liver tumor promotion as well as its effects on DNA synthesis and lipid accumulation (Rajalakshmi et al. 1961; von Euler et al. 1963; Marchetti et al. 1964). These results suggest that creation of an imbalance in nucleotide pools is critical for OA to exert its biological adverse effects. In this context it is interesting to note that there are genetic and metabolic disorders which are associated with an alteration in uridine nucleotide pools without an increase in the levels of OA. For example, the Lesch Nyhan syndrome, an X-linked trait, is characterized by a deficiency of hypoxanthine guanine phosphoribosyl transferase. This enzyme is required for the synthesis of inosine and guanosine mononucleotides in the presence of PRPP (Nuki et al., 1977). A six-fold increase in uridine nucleotides and decreased levels of adenosine nucleotides is observed in this disease. This is due to an increased rate of conversion of OA to orotidine monophosphate as a result of increased PRPP concentrations as well as increased activity of UMP synthase. Folic acid deficiency also results in up to a 10-fold increase in uridine nucleotides due to the lack of their conversion to thymidine nucleotides. Interestingly, folic acid deficiency is known to enhance cell transformation (James et al., 1994). Whether an imbalance in nucleotide pools plays a role in the pathogenesis of these diseases is only speculative. The effect of correction of the nucleotide pool imbalance on the pathogenesis of these disorders should be interesting.

1.4.3 Mechanism of tumor promotion by orotic acid
OA does not appear to be mitogenic to the liver (Rao et al. 1983), nor does it induce ornithine decarboxylase, an enzyme associated with cell proliferation (S. Vasudevan and D.S.R. Sarma, unpublished observations). This suggested that OA may not promote by providing cell proliferative stimulus. However, it has been shown that OA can inhibit DNA synthesis in normal hepatocytes both in vitro in response to a variety of growth factors (Laconi et al., 1988; Pichiri-Coni et al., 1990; Manjeshwar et al., 1992b) and in vivo
following growth stimuli induced by both compensatory cell proliferation (Sheikh et al., 1993b) and direct mitogenic stimulation (Laconi et al., 1997) of hepatocytes. The proposed mechanism for OA induced mitoinhibition rests on the accumulation of uridine nucleotides within the hepatocyte. This accumulation results in the disruption of ribonucleoside diphosphate reductase (RNR, E.C. 1.14.4.1) a key rate limiting enzyme involved in DNA synthesis. RNR, has been shown by Manjeshwar et. al (Manjeshwar et al., 1993b; Manjeshwar et al., 1999) to be sensitive to imbalances in nucleotide pools induced by OA. This imbalance has been associated with decreased stability of the mRNA for RNR resulting ultimately in the shut off of DNA synthesis. Most interestingly, hepatic nodules promoted by several models were relatively resistant to the mitoinhibitory effects of OA (Manjeshwar et al., 1993a; Sheikh et al. 1993b). Taken together, these observations suggested that OA promotes carcinogenesis via differential mitoinhibition where the initiated hepatocytes by virtue of their resistance to the mitoinhibitory effect of OA respond to the growth stimuli forming foci and nodules, while normal hepatocytes remain inhibited without responding to the growth stimuli.

The OA model and in particular the RH model of liver tumor promotion thus indicated that differential mitoinhibition is one mechanism of liver tumor promotion. Differential mitoinhibition has been postulated to be a mechanism of tumor promotion by phenobarbital (Barbason et al., 1983; Eckl et al., 1988), by ethynyl estradiol (Yager and Yager 1980), and by α-hexachlorocyclohexane (Tsai et al., 1991). However, it should be pointed out that the hypothesis that phenobarbital promotes by differential mitoinhibition was challenged by Manjeshwar et. al. (Manjeshwar et al., 1994) because phenobarbital inhibited DNA synthesis both in the nodules as well as in the non-nodular surrounding liver almost to a similar extent. Further, even though ethynyl estradiol and α-hexachlorocyclohexane inhibit DNA synthesis in normal hepatocytes, it has not been
demonstrated that hepatic nodules are resistant to the mitoinhibitory effects of these two agents.

1.5 Development of Resistance During Carcinogenesis

As pointed above, since initiated hepatocytes and hepatic foci/nodules proliferate in an otherwise cytotoxic and mitoinhibitory environment created by the carcinogens and tumor promoting agents, it may be concluded that these lesions are relatively resistant, compared to the non-initiated surrounding hepatocytes, to the mitoinhibitory and cytotoxic effects of the carcinogens and tumor promoting agents. Since expression of resistance is so intimately associated with the development of cancer, a brief discussion on the development of resistance during carcinogenic process is appropriate.

As early as six decades ago Haddow (Haddow 1938) postulated that the ability of the neoplastic cell to proliferate in a toxic environment was in fact due to its resistance. Since then, several investigators especially Farber and his co-workers have studied the biochemistry of hepatic nodules and concluded that hepatic nodules exhibit properties relatable to the resistance to the cytotoxic and mitoinhibitory effects of a wide variety of xenobiotics including carcinogens and tumor promoters (Solt and Farber 1976; Feo et al., 1978; Hanigan and Pitot 1985; Roomi et al., 1985; Tsai et al. 1991; Yager et al., 1992).

1.5.1 Hepatic nodule as a multi-resistant phenotype

In this section a brief discussion on the nature of the growth and survival advantage acquired by hepatic foci/nodules will be presented (Table 1.1).

Liver being the major organ responsible for activation and detoxification of a wide variety of cytotoxic and/or mitoinhibitory xenobiotics, also becomes the primary target for these cytotoxic and mitoinhibitory agents. Hepatic nodules therefore have altered their
biochemical machinery such that they protect themselves from the cytotoxic and/or the mitoinhibitory effects of several xenobiotics.

**Table 1.1** Some of the biochemical properties that confer growth and survival advantages in the preneoplastic and neoplastic hepatocyte lesions.

**Alterations relatable to the resistance to cytotoxicity and/or mitoinhibition:**

- decreased uptake (a)
- increased expression of Pgp (b)
- decreased Phase I system of microsomal xenobiotic metabolizing components (c)
  (cytochromes p 450, mixed function oxygenases)
- increased Phase II system (d)
  (GST, gamma glutamyl transferase, UDP-glucuronyl transferase, epoxide hydrolase)
- increased glutathione (e)
- decreased lipid peroxidation (f)
- increased expression of heat shock proteins (g)
- increased expression of N-acetyl glucosaminyl transferase III (h)

**Alterations relatable to the growth advantage:**

- increased expression of genes relatable to cell cycle (j)
  (c-fos, c-myc, c-Ha-ras, HMG Co A reductase, etc.)
- low threshold for growth stimuli (j)
- altered carbohydrate metabolism (k)
  (increased glucose-6-phosphate dehydrogenase, decreased glucose-6-phosphatase, increased pyruvate kinase-type M2, etc.)
- refractory to negative growth regulation (l)

**Alterations relatable to survival advantage:**

- mutated p53 (m)

(a) Backway et al., 1994; Lea et al., 1990.
(b) Thorgerisson et al., 1987; Fairchild et al., 1987; Bradley et al., 1992.
(c) Feo et al., 1978; Astrom et al., 1983; Hanigan and Pitot, 1985; Roomi et al., 1985.
(d) Fiala et al., 1976; Bock et al., 1982; Sato et al., 1984.
(e) Demi and Oesterle, 1980; Alhuwalia and Farber 1984.
(f) Benedetti et al., 1984.
(g) Carr et al., 1986.
(h) Narasimhan et al., 1988; Pascale et al., 1989.
(i) Bhave et al., 1988; Rao et al., 1989; Garcia et al., 1989; Chander et al., 1989; Coni et al., 1992; Rossiello et al., 1994.
(k) Bannasch et al., 1984; Eigenbrodt et al., 1992.
(l) Zhang et al., 1994; Mansbach et al., 1996.
(m) Hsu et al., 1991; Fujimoto et al., 1994.
Hepatic nodules for example exhibit:

(a) A decreased net uptake of certain tumor promoters (Lea et al., 1990; Backway et al., 1994).

(b) An increased expression of p-glycoprotein (Fairchild et al., 1987; Thorgeirsson et al., 1987; Bradley et al. 1992; Nakatsukasa et al., 1992), an ATP driven efflux pump believed to transport large hydrophobic molecules including drugs and other cytotoxic agents (Juliano and Ling 1976). Further, examination of the expression of p-glycoprotein during the stepwise development of liver cancer has shown that p-glycoprotein expression was higher in the large hyperplastic nodules and in hepatocellular carcinomas than in the early microscopic lesions promoted with the OA model (Bradley et al. 1992). Interestingly, it has been documented that many cancers and drug resistant cell lines also exhibit increased expression of p-glycoprotein (Biedler and Riehm 1970; Riehm and Biedler 1971; Carlsen et al., 1976; Beck et al., 1987; Ling 1995).

(c) A decrease in phase I microsomal drug metabolizing systems involved in the first step of metabolic activation of hydrophobic xenobiotics (Cameron et al., 1976; Feo et al. 1978; Astrom et al., 1983).

(d) An increased expression of phase II drug metabolizing enzymes including glutathione-S-transferases, gamma-glutamyltransferase, UDP-glucuronyl transferase involved in the conjugation and detoxification of xenobiotics oxidized or hydroxylated by phase I systems (Fiala et al., 1976; Bock et al., 1982; Sato et al., 1984).
(e) Increased levels of cellular glutathione (Deml and Oesterle 1980; Ahluwalia and Farber 1984) a competing nucleophile which protects DNA from being attacked by the electrophilic derivatives of the xenobiotics.

(f) Increased levels of pyrimidine nucleoside phosphorylases (Backway 1995; Yusuf et al., 1996; Yusuf et al., 1997) that degrade pyrimidine nucleosides which have the potential to inhibit DNA synthesis (Chapter 3 of this thesis).

(g) Hepatic nodules also exhibit increased activity of N-acetylglucosaminyl transferase III (Narasimhan et al., 1988; Pascale et al., 1989) which has been implicated in resistance (Campbell and Stanley 1984). N-acetylglucosaminyltransferase III catalyzes the insertion of a bisecting N- acetylglucosamine in the complex-type N-linked glycans of cellular glycoproteins.

(h) Hepatic nodules have also been shown to be resistant to lipid peroxidation (Benedetti et al., 1984). This type of oxidative damage is one of the major endogenous damages (Fraga et al., 1990) where membrane lipids are especially vulnerable. This apparent resistance to lipid peroxidation may be a reflection of alterations in the fatty acid composition of the membrane lipids. This alteration in membranes may also play a role in decreased drug influx as many anticancer drugs are lipid soluble and can freely diffuse into the membranes. Changes in the phospholipid composition of the plasma membrane (sphingomyelin, cardiolipin, phosphatidyl ethanolamine and phosphatidyl choline) can affect membrane fluidity as well as the efficiency with which membrane transporters function (Bruzik 1988). Changes in the membrane architecture have been observed in Chinese hamster ovary cells and human leukemic cells which are known to be multi-drug resistant (Escriba et al., 1990) and in the membranes of nodules (Tamiya-Koizumi et al., 1985).
Recent evidence has suggested that hepatic nodules also exhibit increased expression of heat shock proteins (Carr et al., 1986; Lindeman et al., 1998).

Hepatocyte nodules also express resistance to a wide variety of compounds for which a biochemical explanation is not clear. For example, hepatic nodules are resistant to fat accumulation by polybrominated biphenyls (Jensen et al., 1983) and by choline deficient diet (Sells et al., 1979); resistant to toxic effects of phalloidin (Mitaka and Tsukada 1988) and megalocytic effects induced by lasiocarpine (Hayes et al., 1985) among others.

Irrespective of the initiator or promoter used many of the aforementioned biochemical changes observed in hepatic nodules are common (Farber et al. 1976; Roomi et al. 1985; Sarma et al., 1986; Farber and Sarma 1987; Sarma et al., 1987; Farber and Rubin 1991). For example, orotic acid promoted hepatic nodules are resistant to the mitoinhibitory effects of orotic acid (Manjeshwar et al. 1993a; Sheikh et al. 1993b), nodules generated with the RH model are resistant to the mitoinhibitory effects of 2-acetylaminofluorene (Solt and Farber 1976) and nodules promoted by a choline deficient diet are resistant to the lipid accumulation created by a choline deficient diet (Sells et al. 1979). Perhaps of greater significance is the cross resistance exhibited by nodules generated by exposure to one liver tumor promoter and its observed resistance to several other liver tumor promoters (Farber et al. 1976; Sarma et al. 1986; Sarma et al. 1987; Farber and Rubin 1991). For example, nodules generated with the choline deficient diet model (Columbano and Sarma, unpublished observation), phenobarbital model (Schulte-Hermann et al., 1981) are cross resistant to the mitoinhibitory effects of 2-acetylaminofluorene. Nodules promoted by the RH model are cross resistant to the mitoinhibitory effects of OA (Backway et al., 1993; Backway et al. 1994), fat accumulation induced by a choline deficient diet (Ghoshal and
Ahluwalia, unpublished observation), toxic effect of phalloidin (Mitaka and Tsukada 1988), the necrogenic effects of carbontetrachloride and diethylnitrosamine (Farber et al. 1976) and clofibrate (Nagai 1993). Based on these observations it is apparent that nodules are resistant to a vast array of agents and this is of significance because it indicates that the new pattern may not be in direct response to any one particular xenobiotic initiator or promoter but is turned on during the neoplastic process.

The concept of resistance becomes even more fascinating when survival advantage focuses on the cell cycle and cell death pathways. For example, certain neoplastic cells exhibit increased expression of bcl-2 (Higashiyama et al., 1995; Sierra et al., 1995) and either a decreased expression of p53 or an expression of a mutated p53 (Hsu et al., 1991; Fujimoto et al., 1994). This alteration increases survival value because bcl-2 is known to inhibit apoptosis while p53 induces apoptosis. Interestingly, mutated p53 also appears to increase the expression of Pgp (Chin et al., 1992; Zastawny et al., 1993). The multiresistant phenotype also appears to provide survival advantage to the host. For example, it was observed that rats with hepatic nodules exhibit unusual resistance to carbontetrachloride at doses that are lethal (100%) to normal rats (Harris et al., 1989). Similarly, UV-irradiation induced epidermal thickening provides protection against sunlight (Blum 1959).

In the cell cycle, the G1 phase progression of mammalian cells is mainly controlled by the cyclin-cyclin-dependent kinase (CDK)-CDK inhibitor-retinoblastoma protein (pRb) regulatory pathway. Cell cycle regulators controlling G1 phase progression are frequently involved in the carcinogenesis of many human cancer types. In hepatocellular carcinoma the CDK inhibitor p16INK4 is predominantly inactivated by post-transcriptional regulation and p16INK4 inactivation participates in the early-stage of hepatocarcinogenesis and in disease progression. Reduced p27Kip1 expression is also frequently involved in hepatocellular carcinomas. The CDK inhibitors p16INK4, p21(WAF1/CIP1) and p27Kip1 are
independently affected and a change in the expression of one or more of these inhibitors contributes to carcinogenesis of the majority (nearly 90%) of hepatocellular carcinomas (Liew et al., 1999) Hepatic nodules are also refractory to the negative growth regulatory factors such as TGF-β1 (Zhang et al., 1994; Mansbach et al., 1996).

A message that emerges from the above discussion is that expression of resistance is intimately associated with cancer development. However, it is not clear whether all this consortium of biochemical changes relatable to resistance are expressed at the initiated cell stage itself or acquired at different stages. Is there one master switch or several sub-master switches? As may be seen in almost all the studies characterizing the phenomenon of resistance were carried out on hepatic nodules and hepatocellular carcinomas. The results were then extrapolated to the initiated cell stage. This was largely because there was no reliable tag for initiated hepatocytes. Since several of the agents such as 2-AAF, CCl₄ and OA used in amplifying initiated hepatocytes to hepatic nodules have the potential to induce alterations in the genome either directly or indirectly it is likely that initiated hepatocytes are not a resistant phenotype, but express resistance upon exposure to the promoting regimens. Because of these uncertainties I have undertaken to re-examine the expression of the resistant phenotype during OA induced liver tumor promotion.

In the conventional model of liver tumor promotion by OA, the initiated rats are exposed to dietary 1% OA. At the end of 10 weeks these rats develop foci and hepatic nodules. However, a diet with 1% orotic acid which showed significant tumor promotion eventually leading to cancer and metastasis did not show any significant mitoinhibition on the surrounding non-initiated hepatocytes following PH. It should be mentioned however, that such a treatment with dietary 1% OA resulted in a slight shift in the peak of DNA synthesis following PH. In addition, following 20 weeks of exposure to dietary 1% OA the livers of rats exhibited 17% decrease in total hepatic DNA content. Based on the experience with 2-AAF,
which inhibits DNA synthesis over 99% in normal hepatocytes, the observation that dietary OA could not inhibit DNA synthesis to any significant extent was rather puzzling. If OA promotes liver carcinogenesis by differential mitoinhibitory mode similar to the RH model, it should inhibit DNA synthesis in the surrounding liver over 90% as seen with 2-AAF. These results open up two avenues: either the original hypothesis that OA promotes by differential mitoinhibition is not correct; or the lack of inhibition could be a reflection of our experimental approach for determining the DNA synthesis. Before challenging the original hypothesis, I explored the latter possibility in some detail. Due to the nature of the dietary model, some of the biochemical effects of OA are transient. For example, the decrease in hepatic ATP levels and the increase in uridine nucleotides induced by OA are maximum in the early hours of the morning compared to late afternoon when the circulating dietary OA levels tend to decrease (Laconi 1988b). My experimental approach was to determine the effect of dietary OA on liver DNA synthesis following 2/3 PH. After surgery the rats tend to eat far less food in the first 24 to 48 hours. Because of this it could have been difficult to sustain the orotic acid induced imbalance in nucleotide pools, a condition necessary for orotic acid to exert its mitoinhibitory effects. In tumor promoting experiments these arguments are of less concern because of the chronic nature if the experiments.

Consequently, to sustain high levels of hepatic uridine nucleotides, a model using a 300 mg orotic acid tablet given i.p. at the time of PH was developed. These studies showed that orotic acid inhibited 90-100% of DNA synthesis for up to 3 days in normal hepatocytes and further, nodules generated by several tumor promoting models were resistant to the mitoinhibitory effects of orotic acid while the surrounding non-initiated hepatocytes were mitoinhibited when orotic acid was given as a i.p. tablet to rats. The tablet approach thus clearly establishes the criteria necessary for OA to promote liver carcinogenesis by the mitoinhibitory mode.
Having established these criteria, in the next series of experiments I examined whether a 300 mg tablet of OA would promote liver carcinogenesis. Accordingly, male Fischer 344 rats were initiated with 200 mg/kg diethylnitrosamine and two weeks thereafter animals received a 300 mg orotic acid tablet implanted i.p. coupled with a growth stimulus induced by 2/3 PH. Two weeks later microscopic analysis for glutathione S-transferase 7-7 (GST 7-7) positive foci was carried out. Surprisingly, the results showed no significant differences measured as size and number of foci between the orotic acid and control groups. Several possibilities were considered and tested to account for the lack of tumor promotion. For example, it was argued that the inhibition induced by a 300 mg tablet only lasted for up to 3 days and perhaps by prolonging this inhibitory effect promotion may be achieved. As a result, I developed a modified tablet model using binders and multiple tablets in various combinations to inhibit DNA synthesis for up to 7 days. Animals were initiated and promoted with the 7 day (500 mg) tablet. Under these conditions I was again unable to show any significant tumor promotion.

After exploring several other possibilities, I considered the possibility that levels of orotic acid mitoinhibitory to normal hepatocytes did not allow the amplification of the initiated hepatocytes probably because the initiated hepatocytes were not yet resistant to the mitoinhibitory effects of orotic acid. This consideration raises several important questions let alone challenging the dogma that the "initiated cell is a resistant phenotype". In my thesis I have attempted to examine this question in some detail.
1.6 Objectives of the Thesis

The overall objectives of this thesis therefore are:

(i) to determine whether the initiated hepatocyte is inherently a resistant phenotype or whether resistance is acquired during the exposure to tumor promoting regimens. Resistance defined as being resistant to the mitoinhibitory effects of orotic acid.

(ii) to characterize the mechanism(s) of resistance of the hepatic nodules to the mitoinhibitory effects of orotic acid.
CHAPTER 2

ACQUISITION OF RESISTANCE BY INITIATED HEPATOCYTES TO THE MITOINHIBITORY EFFECTS OF OROTIC ACID AND 2-ACETYLAMINOFLUORENE.

The contents of this chapter has been modified from two separate articles:


2.1 Introduction

One of the challenges in understanding the carcinogenic process in general and tumor promotion in particular has been to elucidate the mechanism by which initiated cells proliferate in an otherwise cytotoxic and mitoinhibitory environment created by carcinogens and tumor promoters. Haddow (Haddow 1938) proposed that the neoplastic cell is a resistant phenotype based on the observation that the neoplastic cell was able to proliferate in a cytotoxic environment created by the carcinogens. Farber and his associates and several other investigators characterized the biochemistry of the hepatic nodules, a precursor population for hepatocellular carcinoma and observed that the alterations seen in the biochemical machinery in the nodules are compatible with the concept that neoplastic cell is a resistant phenotype (Farber et al. 1976; Feo et al. 1978; Pitot and Sirica 1980). Solt, Farber and coworkers further argued that the resistance seen in the hepatic nodules and hepatocellular carcinomas might have been acquired at the initiated cell stage itself and accordingly hypothesized that the 'initiated cell is a resistant phenotype' (Solt and Farber 1976; Solt et al. 1977). This hypothesis has been very useful and provides a fundamental approach to selectively amplify the initiated hepatocytes taking advantage of their differential resistance to the cytotoxic, mitoinhibitory or negative growth regulatory effects of the tumor promoters. Resistant hepatocyte (RH) model of liver tumor promotion was in fact based on this concept (Solt and Farber 1976). Interestingly, several liver tumor promoters are indeed mitoinhibitors to normal hepatocytes (Laconi et al. 1988; Pichiri-Coni et al. 1990; Jirtle and Meyer 1991; Tsai et al. 1991; Manjeshwar et al., 1992a; Yager et al. 1992; Nagai 1993) and in some instances, where tested, hepatic nodules are resistant to the mitoinhibitory effects of the liver tumor promoters (Sheikh et al. 1993b; Backway et al. 1994). Nonetheless, the concept that initiated cell is a resistant phenotype is open to discussion, largely because, there are no accurate criteria to tag initiated hepatocytes. Therefore, many of the biochemical studies
were carried out using early and late nodules and hepatocellular carcinomas and the conclusions drawn from these studies were extrapolated back to the initiated hepatocyte. More importantly, during the selective amplification of initiated hepatocytes, animals were exposed to liver tumor promoting agents and it is likely that some of these agents might have introduced in the initiated hepatocytes further modifications that are relatable to resistance either directly or indirectly. Further, neoplastic cell is a comprehensive multiresistant phenotype expressing resistance to cytotoxicity, mitoinhibition, negative growth regulation and cell death (see Table 1.1 in Chapter 1). It is important to determine how many of these properties relatable to this multiresistant phenotype are acquired at the initiated hepatocyte stage and how many are acquired during the subsequent stages of cancer development.

The present study was therefore designed to determine specifically whether the resistance to the mitoinhibitory effects of orotic acid and of 2-acetylamino-fluorene (2-AAF) is acquired at the initiated hepatocyte stage itself or upon exposure to the tumor promoting regimen.

2.2 Experimental approach and rationale to study development of resistance
In this study, expression of the resistant phenotype was monitored by determining the resistance to the mitoinhibitory effects of orotic acid or 2-AAF. Male Fischer 344 rats were initiated with a single administration of diethylnitrosamine (DENA) and later exposed to the RH model of liver tumor promotion (2-AAF coupled with CCl₄), or to no further treatment. Several weeks later the rats were subjected to 2/3 partial hepatectomy (PH) in the presence of mitoinhibitory levels of orotic acid or 2-AAF. Proliferating hepatocytes were labelled with tritiated thymidine. We arbitrarily defined the resistant focus as the focus that had a labelling index higher than the highest labelling index in the surrounding liver. If the foci generated by DENA alone (in the absence of an exposure to RH protocol)
are resistant to the mitoinhibitory effects of orotic acid or 2-AAF then it will be reasoned that the resistance to the mitoinhibitory effects of orotic acid or 2-AAF is acquired at the initiated hepatocyte stage itself. On the other hand, if the foci become resistant upon exposure to the RH protocol then it will be considered that initiated hepatocyte may not be a resistant phenotype but it has 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 orotic acid or 2-AAF.

2.3 Part 1 - Experiments on the Development of Resistance to Orotic Acid

2.3.1 Methods

2.3.1.1 To determine whether initiated hepatocytes are resistant to the mitoinhibitory effects of orotic acid.

All the protocols involving experimental animals were approved by the University of Toronto Animal Care Committee and animals were handled in accordance with the Canadian Council on Animal Care guidelines. The animals were acclimatized for one week before they were used for experiments. They were kept in plastic cages with food and water ad libitum. Male Fischer 344 rats (Charles Rivers breeding laboratories, St. Constant, Quebec) weighing 120-130 grams were initiated with a single necrogenic dose of DENA (Sigma Chemicals; 200 mg/kg i.p.). Two weeks later the rats were divided into two groups. Rats in Group 1 were subjected to the RH model of liver tumor promotion (2-AAF; 20 mg/kg i.g., daily for 3 days and on day 4, CCl₄; 2 ml/kg i.g., in a 1:1 with corn oil). The rats in the second group (Group 2) were not subjected to any further treatment. Six weeks later all animals were subjected to 2/3 PH in the presence of a 300 mg tablet of orotic acid implanted at the time of PH. A 300 mg tablet of orotic acid inhibits DNA synthesis nearly 100% in normal hepatocytes (Sheikh et al. 1993b). Multiple injections of tritiated thymidine (50 μCi each) were administered every four hours beginning 16 hours
after PH to determine the labelling index in the surrounding liver and foci. At 48 hours post PH, all animals were killed and their livers were sectioned and fixed in cold acetone for immunohistochemical staining for glutathione-S-transferase 7-7 (GST 7-7). The stained sections were then processed for autoradiography (Sheikh et al. 1993b).

2.3.1.2 To determine whether components of the Resistant Hepatocyte model induce resistance to the mitoinhibitory effects of orotic acid in initiated hepatocytes.
Male Fischer 344 rats were initiated with DENA as described above and were then divided into two groups. Two weeks after initiation rats in Group 1 received only 2-AAF (20 mg/kg., i.g., daily for 3 days), while the rats in Group 2 received only CCl4 (2 ml/kg., i.g., in a 1:1 with corn oil). Six weeks later all the rats were subjected to 2/3 PH in the presence of a 300 mg. tablet of orotic acid. Resistance of the foci was determined as described above. Rats initiated with DENA alone without any further treatment (Group 1 in the previous experiment) were used as control in this experiment.

2.3.1.3 To determine whether acquisition of resistance would permit initiated hepatocytes to be selectively amplified by 2/3 partial heptectomy in the presence of mitoinhibitory levels of orotic acid.
Male Fischer 344 rats (140-150 grams) maintained on a semisynthetic basal diet were initiated with DENA (200 mg/kg i.p); two weeks later they were divided into four groups. The first group (Group 1) received one dose of CCl4 (2 ml /kg., i.g., in a 1:1 with corn oil) to induce resistance and two weeks later the rats were given 2/3 PH in the presence or absence of a 500 mg orotic acid tablet. (In this experiment a 500 mg tablet was used because orotic acid lasts for a week). This group was compared to rats that did not receive an inducing dose of CCl4 (Group 2). Groups 3 (no orotic acid) and 4 (no CCl4 and no orotic acid) were included as controls. Two weeks after PH, the animals were sacrificed.
and acetone fixed sections were prepared and stained for GST 7-7 +ve foci. The number of foci per square centimeter and the percent area occupied by the foci was determined using image analysis.

2.3.2 Results

2.3.2.1 Resistance of foci/nodules to the mitoinhibitory effects of orotic acid
The results presented in Figure 2.1 and Table 2.1 indicate that the majority (95%) of the foci generated by DENA alone in the absence of an exogenous liver tumor promoting

![Graph A](image)

![Graph B](image)

Figure 2.1 Percent labelling index of hepatocytes in foci and surrounding non-nodular livers in rats initiated with DENA alone (A) and in the livers of rats initiated with DENA and promoted with the RH protocol (B). Every focus in the section, a total of 102 and 30 random fields in the surrounding liver in (A) and a total of 20 foci and 20 random fields in the surrounding liver in (B) were counted. Each triangle represents one focus or one field from the surrounding liver. The focus above the dashed line represents focus resistant to the mitoinhibitory effects of orotic acid. Further details are given in the text.
Figure 2.2 Percent labelling index of hepatocytes in foci and surrounding non-nodular livers in animals initiated with DENA and treated with 2-AAF (A) and with CCl₄ (B). Every focus in the section, a total of 20 foci and 20 random fields in the surrounding liver in (A) and a total of 33 foci and 30 random fields in the surrounding liver in (B) were counted. Each triangle represents one focus or one field from the surrounding liver. The focus above the dashed line represents focus resistant to the mitoinhibitory effects of orotic acid. Further details are given in the text.

regimen are not resistant to the mitoinhibitory effects of orotic acid. In contrast, majority (80%) of the foci initiated by DENA and promoted by the RH model are resistant to the mitoinhibitory effects of orotic acid. The resistance or the lack of it did not depend on the
size and zonal distribution of foci (this study; Sheikh et al. 1993b)). These results suggest that the initiated hepatocytes are inherently not resistant and become resistant upon exposure to the promoting regimen; or RH protocol selectively amplified those initiated hepatocytes that are inherently resistant.

Table 2.1 Percent of GST-7,7 foci resistant to the mitoinhibitory effects of orotic acid in the livers of rats exposed to different treatment protocols. Experimental details are given in the legends to Figures 2.1 and 2.2 The results presented in the table are compiled from the values given in the Figures 2.1 and 2.2.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Resistant Foci</th>
</tr>
</thead>
<tbody>
<tr>
<td>DENA+ RH Protocol</td>
<td>80</td>
</tr>
<tr>
<td>DENA Alone</td>
<td>5</td>
</tr>
<tr>
<td>DENA + 2AAF</td>
<td>60</td>
</tr>
<tr>
<td>DENA + CCl₄</td>
<td>48</td>
</tr>
</tbody>
</table>

The latter possibility is consistent with the concept that different tumor promoters promote different initiated hepatocytes (Columbano et al. 1982b; Dragan and Pitot 1992) and those promoted by the RH protocol happened to be resistant to the mitoinhibitory effects of orotic acid. In order to explore further the former possibility, in the next experiment rats initiated with DENA were exposed to either 2-AAF or CCl₄. It may be noted that 2-AAF and CCl₄ given separately does not result in tumor promotion as assessed by an increase in size and number of foci compared to those in DENA alone group ((Solt and Farber 1976); this study). The results presented in Figure 2.2 and in Table 2.1 clearly indicate that upon exposure to 2-AAF or to CCl₄ under non-tumor
promoting conditions, a large number of foci (60% and 48% respectively) exhibited resistance to the mitoinhibitory effects of orotic acid. These results are interpreted to indicate that initiated hepatocyte is not a resistant phenotype; however, it is so altered that it has acquired the ability to express resistance upon exposure to either the promoting regimen or to 2-AAF or CCl₄.

2.3.2.2 Effect of induction of resistance on selection of initiated hepatocytes.

Having observed that CCl₄ can confer resistance in the initiated hepatocytes, it became of interest to determine whether those initiated hepatocytes that acquired resistance to the mitoinhibitory effects of orotic acid can be selectively amplified with 2/3 PH in the presence of mitoinhibitory levels of orotic acid to normal hepatocytes. Accordingly, male Fischer 344 rats (140-150 g) were initiated with DENA; two weeks later they were divided into four groups (Figure 2.3). Rats in Group 1 received one dose of CCl₄ to induce resistance, while those in the Group 2 did not receive CCl₄. Group 3 (no orotic acid) and Group 4 (no CCl₄ and no orotic acid) served as controls. Two weeks later all the rats were subjected to 2/3 PH in the absence or in the presence of mitoinhibitory levels of orotic acid as indicated in the Figure 2.3. Two weeks thereafter the rats were killed and the livers were processed for GST 7-7 +ve foci. The results presented in the Table 2.2 indicate that the number of foci and percent area occupied by them were significantly higher in Group 1 compared to those in Group 2. Further, there was no significant inter group differences in the number and the percent area occupied by the foci in Groups 2, 3 and 4. These results suggest that induction of resistance by CCl₄ in the initiated hepatocytes permit them to be selectively amplified by 2/3 PH in the presence of a mitoinhibitory level of orotic acid.
Figure 2.3. Experimental protocol to determine whether acquisition of resistance would permit the initiated hepatocytes to respond to 2/3 PH and amplify in the presence of mitoinhibitory levels of orotic acid.
Table 2.2  Effect of induction of resistance on the ability of initiated hepatocytes to be selectively amplified in response to 2/3 PH in the presence of mitoinhibitory levels of orotic acid.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incidence of GST 7-7 Positive Foci</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. foci/cm²</td>
<td>Av. Foci Area (mm²)</td>
</tr>
<tr>
<td>Induction of Resistance</td>
<td>Tumor Promotion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCl₄</td>
<td>OA+PH</td>
<td>36±10.65</td>
<td>0.0437±0.0081</td>
</tr>
<tr>
<td>Group 2</td>
<td></td>
<td>19±5.99</td>
<td>0.0290±0.0068</td>
</tr>
<tr>
<td>Group 3</td>
<td></td>
<td>20±1.43</td>
<td>0.0448±0.0165</td>
</tr>
<tr>
<td>Group 4</td>
<td></td>
<td>16±2.09</td>
<td>0.0238±0.0066</td>
</tr>
</tbody>
</table>

All animals were initiated with DENA 200 mg/kg i.p.  
Values are mean ± S.D. of 5 to 6 rats  

<table>
<thead>
<tr>
<th></th>
<th>Group</th>
<th>Group</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p≤0.375</td>
<td>2 vs 4</td>
<td>p≤0.025</td>
</tr>
<tr>
<td></td>
<td>p≤0.010</td>
<td>1 vs 2</td>
<td>p≤0.010</td>
</tr>
<tr>
<td></td>
<td>p≤0.025</td>
<td>1 vs 3</td>
<td>p&gt;0.400</td>
</tr>
<tr>
<td></td>
<td>p≤0.025</td>
<td>3 vs 4</td>
<td>p≤0.050</td>
</tr>
<tr>
<td></td>
<td>p≤0.050</td>
<td>1 vs 3</td>
<td>p≤0.005</td>
</tr>
<tr>
<td></td>
<td>p≤0.010</td>
<td>1 vs 3</td>
<td>p≤0.010</td>
</tr>
</tbody>
</table>
2.4. PART 2 - EXPERIMENTS ON THE DEVELOPMENT OF RESISTANCE TO 2-ACETYLMINOFUORENE

The results of the previous study suggested that initiated hepatocytes are not inherently resistant to the mitoinhibitory effects of orotic acid, but have acquired the ability to express resistance upon exposure to the RH tumor promoting regimen or to 2-AAF or CCl₄. In order to determine whether this phenomenon is unique to orotic acid, a similar study was carried out using 2-AAF another mitoinhibitor to monitor the resistance of the foci/nodules.

2.4.1 Results

2.4.1.1 To determine whether the initiated hepatocytes are resistant to the mitoinhibitory effects of 2-acetylaminofluorene

Experimental details are identical to those described in the section on studies to determine whether the initiated hepatocyte is resistant to the mitoinhibitory effects of orotic acid, except that instead of orotic acid, 2-AAF was used to monitor the resistance. A single administration of 2-AAF (5 mg/kg) was given immediately following 2/3 PH. This dose of 2-AAF inhibits DNA synthesis in normal hepatocytes following 2/3 PH by upto 100%.

The results presented in Figure 2.4 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
2.4). The latter observation is in agreement with the conclusion drawn from the RH model of liver tumor promotion (Solt and Farber 1976).

![Figure 2.4](image-url)

**Figure 2.4** Percent labeling index of hepatocytes exposed to a mitoinhibitory dose of 2-AAF (5mg/kg) in foci/nodules and surrounding non-nodular liver in animals initiated with necrogenic dose of DENA and exposed to no further treatment (A); and to a complete RH protocol of liver tumor promotion (B). Every focus/nodule in the section and 30 random fields in the surrounding liver in Group A and 60 random fields in the surrounding liver in Group B were counted. Each triangle represents one focus or one field from the surrounding liver. Data shown are for 3 animals in Group A and 6 animals in Group B. Dashed line represents the highest labeling index in the surrounding liver. Experimental details are as those described for experiments with orotic acid in part 1.
2.4.1.2 To determine whether components of the Resistant Hepatocyte model induce resistance to the mitoinhibitory effects of 2-acetylaminofluorene in initiated hepatocytes.

In the previous study it became obvious that 2-AAF and CCl₄, the components of RH model by themselves were capable of inducing resistance in the initiated hepatocytes to the mitoinhibitory effects of orotic acid. Logically therefore, it became of interest to determine whether 2-AAF and CCl₄ by themselves are capable of inducing resistance in the initiated hepatocytes to the mitoinhibitory effects of 2-AAF also. Accordingly, rats initiated with DENA were exposed to either 2-AAF or to CCl₄ and the percent of foci/nodules resistant to the mitoinhibitory effects of 2-AAF was determined as described above.

The results presented in Figure 2.5 indicate that a large number of foci (71%) initiated with DENA and exposed to either 2-AAF or CCl₄ exhibited resistance to the mitoinhibitory effects of 2-AAF. The resistance or the lack of it did not appear to depend on the size and zonal distribution of foci.

2.5 DISCUSSION

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 CCl₄. Since 2-AAF and CCl₄ are genotoxic, it should be interesting to determine whether the second exposure has to be a genotoxic insult for the initiated hepatocyte to express the resistant phenotype. The fact that the surrounding non-initiated hepatocytes under identical conditions did not express resistance to either
Figure 2.5  Percent labeling index of hepatocytes exposed to a mitoinhibitory dose of 2-AAF (5mg/kg) in foci/nodules and surrounding non-nodular liver in animals initiated with DENA (200 mg/kg) and exposed either only to 2-AAF (A); and only to CCl₄ (B). Every focus in the section, and 50 random fields in the surrounding liver were counted. Each triangle represents one focus or one field from the surrounding liver. Data shown are for 5 animals. Dashed line represents the highest labeling index in the surrounding liver. Experimental details are as those described for experiments with orotic acid in part 1.

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 (Rao et al. 1986b; Pichiri-Coni et al. 1990). Conversely, 2-AAF needs to be hydroxylated and esterified for it to exert its mitoinhibitory effects (Weisburger and Williams 1982). 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.

The results of the present study raise several questions. For example, what is the significance of the two populations of the lesions, i.e., the foci that express resistance and those that express resistance upon exposure to the tumor promoting regimen in terms of carcinogenesis? 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 fate of these preneoplastic lesions in the pathogenesis of the carcinogenic process. These considerations also will 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 Solt and Farber clearly point out that acquisition of resistance is one mechanism for the initiated cell to progress through the carcinogenic process (Solt and Farber 1976; Solt et al. 1977). For example, the 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 developed an incidence of 70% and 45% hepatocellular carcinoma respectively within 8 months (Solt et al., 1983). 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 the growth and survival advantage in an otherwise mitoinhibitory and cytotoxic environment created by tumor promoters and carcinogens.

The results of this study also suggest that certain tumor promoters, especially those that are mitoinhibitory to normal hepatocytes, may promote by inducing resistance in the initiated hepatocytes and selectively amplify those that acquire resistance to the mitoinhibitory effects of that promoter. This consideration provides an opportunity to identify another group of agents which are not necessarily initiators or promoters but may still participate in carcinogenic process by virtue of their ability to induce resistance in the initiated hepatocytes (Figure 2.6). These results also suggest that different biochemical properties relatable to resistance are acquired during the carcinogenic process beginning with the initiated cell. This would mean that the initiated cell is altered in such a fashion that it has acquired the potential to express the resistance upon exposure to certain tumor promoters and/ or certain genotoxic agents. Obviously, it will be of great interest to identify these alterations at genetic level and determine using molecular approaches whether one can induce such a resistant phenotype with the potential to progress to cancer.
Neoplastic cell has long been recognized as a multiresistant phenotype. However, it is not known whether different properties relatable to resistance were acquired at the initiated cell stage itself or acquired at various stages during the carcinogenic process. This question was explored using resistance to the mitoinhibitory effects of orotic acid, a liver tumor promoter and to 2-AAF. Accordingly, male Fischer 344 rats were initiated with a single necrogenic dose of DEHA(200mg/kg; i.p.) and later exposed to the RH model of liver tumor promotion or to no further treatment. Six weeks later the rats were subjected to 2/3 PH in the presence of orotic acid (300mg tablet transplanted i.p. at the time of PH) at levels which are mitoinhibitory to the normal hepatocytes. Proliferating hepatocytes
were labelled for 48 hours with tritiated thymidine. Any focus that had a labelling index higher than the highest labelling index in the surrounding liver was considered as a resistant focus. The results indicated that the majority (95%) of the foci generated by DENA alone in the absence of an additional exogenous treatment are not resistant to the mitoinhibitory effects of orotic acid. In contrast, a large number (80%) of the foci initiated by DENA and promoted by the RH model are resistant to the mitoinhibitory effects of orotic acid. Further, rats initiated with DENA and exposed to the components of the RH model i.e., 2-AAF or CCl₄ under non-tumor promoting conditions also exhibited foci many of which (60 and 48% respectively) are resistant to the mitoinhibitory effects of orotic acid. These results are interpreted to indicate that initiated hepatocyte is not resistant to the mitoinhibitory effects of orotic acid but acquires resistance upon exposure to either the promoting regimen or to 2-AAF or CCl₄. Similar results were observed when resistance to the mitoinhibitory effects of 2-AAF was determined. In order to assess the functional significance of this acquired resistance the next series of experiments were designed to determine whether the initiated hepatocytes that acquired resistance to the mitoinhibitory effects of orotic acid can be selectively amplified with 2/3 PH in the presence of levels of orotic acid that are mitoinhibitory to normal hepatocytes. Accordingly, rats were initiated with DENA (200mg/kg;i.p.) and a group of them were exposed to CCl₄ to induce resistance. The initiated hepatocytes that acquired resistance were amplified with 2/3 PH in the presence of mitoinhibitory levels (500 mg tablet implanted i.p.) of orotic acid. The rats were killed at the end of two weeks and the livers processed for glutathione-S-transferase 7,7 positive foci. The results indicated that the number and the percent area occupied by the foci were significantly higher in initiated rats that were exposed to CCl₄ than in those initiated rats not exposed to CCl₄. These results suggest that induction of resistance by CCl₄ in the initiated hepatocytes permits them to be selectively amplified by 2/3 PH in the presence of a mitoinhibitory level of orotic acid. Taken together the results of the present study suggest that initiated
hepatocyte is not a resistant phenotype (resistance defined as resistance to the mitoinhibitory effects of orotic acid and 2-AAF), but it has acquired the ability to express resistance upon exposure to either the promoting regimen (the RH model) or to 2-AAF or CCl₄. Further, using orotic acid model of liver tumor promotion it was demonstrated that the initiated hepatocytes that acquired resistance to the mitoinhibitory effects of orotic acid can be selectively amplified with 2/3 PH in the presence of levels of orotic acid that are mitoinhibitory to normal hepatocytes.
CHAPTER 3

THE EFFECT OF 1/3 PARTIAL HEPATECTOMY ON THE GROWTH OF GLUTATHIONE-S-TRANSFERASE POSITIVE FOCI

The contents of this chapter has been modified from:

3.1 Introduction

Initiated hepatocytes are characterized by their ability to form focal proliferations when exposed to tumor promoting regimens (Pitot and Sirica 1980; Farber and Sarma 1987). The results presented in the previous chapter indicated that one phenotypic attribute of biological relevance to initiated cells is their ability to acquire resistance to the mitoinhibitory effects of certain tumor promoters especially those that promote by differential mitoinhibitory mode. Such a resistance can provide the basis for their selective growth under conditions that are non-permissive for the surrounding, non-initiated cells. However, growth of initiated hepatocytes also occurs, albeit at low level, in initiated rat liver without exposure to any exogenous promoting regimen. Thus, there may be other phenotypic properties, in addition to "resistance", which may explain how focal growth takes place during the carcinogenic process in the absence of exogenous tumor promoter. In fact there have been reports indicating an enhanced proliferative response to direct mitogenic agents such as phenobarbital, alpha-hexachlorocyclohexane, cyproterone acetate, and nafenopin in enzyme altered foci appearing early during rat liver carcinogenesis (Schulte-Hermann et al. 1981; Schulte-Hermann et al., 1982). Although these studies generated important information, the possibility that the increased response of enzyme altered hepatocytes could be related to differences in the metabolism of the mitogenic chemicals was not ruled out. To address this issue, Laconi et al (Laconi et al., 1994) examined the kinetics of the response of enzyme altered foci following 2/3 partial hepatectomy (PH). The results obtained by these investigators as well as those of others (Ogawa et al., 1985) indicated that enzyme altered foci have an earlier response towards the growth stimulus induced by 2/3 PH, compared to surrounding parenchyma. Since 2/3 PH induces intense liver cell proliferative stimulus the magnitude of the growth advantage of the initiated hepatocytes over that of the non-initiated or normal hepatocytes might not have been fully appreciated.
The present study was therefore designed to provide further evidence that initiated hepatocytes compared to non-initiated hepatocytes have a lower threshold for growth stimuli. Accordingly, the effect of 1/3 PH on the size of foci in initiated rats was determined. The rationale for using a 1/3 PH is that since only 50% of the remaining 2/3 of hepatocytes need to proliferate to compensate for the loss, the initiated hepatocytes do not form foci unless they have a growth advantage over the non-initiated hepatocytes.

3.2 Materials and Methods
General materials and methods are not presented here because they are similar to those described in the Chapter 2. Specific experimental details however, are given in the legends to the individual figures and tables.

3.3 Results and Discussion

3.3.1 The kinetics of DNA synthesis following 1/3 vs 2/3 partial hepatectomy
I first examined the kinetics of liver regeneration following 1/3 or 2/3 PH under the experimental conditions of the present study. Two-month old male Fischer 344 rats were subjected to either a 1/3 (median lobe removed) or 2/3 (median and left lobes removed) PH and killed at different time points thereafter. Tritiated thymidine was given to each animal two hours before killing. Liver samples were processed for standard histological analysis and for autoradiography. Results are reported in Figure 3.1. As expected, rats given a 2/3 PH exhibited a peak labeling index at 24 hours (40±2), declining rapidly within 48 hrs (7±1) and returning close to baseline levels by 4 days (1±0.5). By contrast, the proliferative response to 1/3 PH was undetectable at 24 hrs, was still low at 48 hrs (11±3), peaked at day 3 (19±6) and remained well above baseline for at least 5 days (15±4). However, the peak labeling index in response to 1/3 PH was less than 50% of that seen with 2/3 PH. These data are in agreement with previous reports in the literature.
They clearly indicate that the kinetics of response to either 1/3 or 2/3 PH are remarkably different with respect to the time of maximum hepatocyte DNA synthesis, the level of the peak response and the duration of the proliferative stimulus.

Figure 3.1  The kinetics of liver regeneration following 1/3 or 2/3 PH. Two-month old male Fischer 344 rats (110-120 g) were subjected to either a 1/3 (median lobe removed) or 2/3 (median and left lobes removed) PH and killed at different time points. A single injection of $^3$H-labeled thymidine (100 μCi/rat, specific activity 80.9 Ci/mmol, New England Nuclear, Montreal, Quebec) was given to each animal 2 hours before killing. Rats were killed under mild anesthesia. Liver samples were processed for standard histological analysis and for autoradiography (Sheikh et al. 1993b). Labeled nuclei were counted and expressed as percent of the total hepatocytes. At least 3000 nuclei were scored for each sample. Mean ± S.D. of 5 rats.
3.3.2 The effect of 1/3 vs 2/3 partial hepatectomy on the growth of enzyme altered foci

The next study was performed to compare the effect of 1/3 and 2/3 PH on the growth of enzyme altered foci in initiated liver. Male Fischer 344 rats were injected with diethylnitrosamine (DENA, 200 mg/kg; i.p.) and three weeks later they were subjected to sham hepatectomy (SH), 1/3 (median lobe removed) or 2/3 (median and left lobes removed) PH; an additional group of animals underwent removal of the median lobe first and 3 weeks later, the left lobe was removed (1/3+1/3 PH). All rats were killed eight weeks after DENA administration. Liver samples were processed for glutathione-S-transferase (GST) 7-7 positive foci and for DNA content (Sheikh et al. 1993b).

The results presented in Table 3.1 show that there was no difference in the number foci/cm² among the different groups. However, the mean foci area was significantly higher in all groups of animals receiving PH as compared to sham operation. This pattern was further substantiated by the results on percent area occupied by enzyme altered foci (Table 3.1). Of greater significance was the finding that the percent area occupied by the foci was very similar in the 1/3 and 2/3 PH groups, although the magnitude of the total growth stimulus is clearly different in these two cases. Moreover, rats undergoing sequential removal of the median and the left lobes showed the highest value in percent area occupied by foci, and yet the magnitude of the total stimulus was roughly comparable to that of 2/3 PH. Further, the observation that 1/3 PH promoted the growth of initiated hepatocytes is in itself interesting in view of the fact that only half of the remaining 2/3 of hepatocytes need to respond to proliferate following 1/3 PH.
Table 3.1  The Effect of 1/3, 2/3 or 1/3+1/3 PH on number and mean foci area eight weeks post initiation. Male Fischer 344 rats (110-120 g) were injected with DENA (200 mg/kg; i.p; Sigma Chemicals Co. St. Louis, MO.) and three weeks later they were subjected to sham hepatectomy (SH), 1/3 (median lobe removed) or 2/3 (median and left lobes removed) PH. An additional group of animals underwent removal of the median lobe first and 3 weeks later, the left lobe (1/3+1/3 PH). All rats were killed eight weeks after DENA administration under mild anesthesia. Liver samples were taken, fixed in cold acetone and processed for immunohistochemical detection of GST 7-7 (Sheikh et al. 1993b). Enzyme altered foci were quantitated with the help of a computer assisted image analyzer.

<table>
<thead>
<tr>
<th>Partial Hepatectomy</th>
<th>GST 7-7 Positive Foci</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No/cm²</td>
</tr>
<tr>
<td>SH</td>
<td>5±1.19</td>
</tr>
<tr>
<td>2/3 PH</td>
<td>5±1.16</td>
</tr>
<tr>
<td>1/3</td>
<td>6±1.23</td>
</tr>
<tr>
<td>1/3 + 1/3</td>
<td>8±2.79</td>
</tr>
</tbody>
</table>

Mean ±S.D. of 3-5 animals

* p≤ 0.005, all groups vs SH;  ** p≤ 0.007, 1/3+1/3 PH vs 1/3 PH

* p≤ 0.01, all groups vs SH;  ** p≤ 0.02, 1/3+1/3 PH vs 1/3 PH;

* p≤ 0.03, 1/3+1/3 PH vs 2/3 PH

Levels of total liver DNA in different groups and the amount of liver DNA per 100 g body weight at the end of 8 weeks are reported in Table 3.2. No significant decrease in this parameter was observed between the 1/3 (6.69±0.47) and 2/3 (6.64±0.46) PH groups and the SH controls (6.96±0.42), while liver DNA content was significantly lower in animals which had the median and left lobes removed three weeks apart (6.05±0.30) compared to SH group (p≤0.002). These results indicate that liver regeneration is largely complete in all experimental groups at the time point considered, although a slight delay
in the response seems to be present and being more accentuated in the group having the median and left lobes removed sequentially.

Table 3.2 The effect of 1/3, 2/3 or 1/3+1/3 PH on liver DNA content eight weeks post initiation. Liver samples derived from the experiment described in Table 3.1 were used for the measurement of liver DNA. Total DNA content was measured in the PCA precipitable fraction of the liver homogenate (Sheikh et al. 1993b).

<table>
<thead>
<tr>
<th>Group</th>
<th>mg DNA/g liver</th>
<th>mg DNA/liver</th>
<th>mg DNA/liver/100g bwt</th>
</tr>
</thead>
<tbody>
<tr>
<td>SH</td>
<td>2.13±0.10</td>
<td>21.49±1.39</td>
<td>6.96±0.42</td>
</tr>
<tr>
<td>2/3 PH</td>
<td>2.28±0.15</td>
<td>19.99±1.72</td>
<td>6.64±0.46</td>
</tr>
<tr>
<td>1/3 PH</td>
<td>2.25±0.13</td>
<td>19.97±2.14</td>
<td>6.69±0.47</td>
</tr>
<tr>
<td>1/3 + 1/3 PH</td>
<td>2.29±0.13*</td>
<td>17.19±1.78**</td>
<td>6.05±0.30***</td>
</tr>
</tbody>
</table>

Mean ±S.D. of 3-5 animals

*p≤0.05, 1/3 +1/3 vs SH; **p≤0.001, 1/3 +1/3 vs SH; ***p≤0.002, 1/3 +1/3 vs SH

The results of this study raise two important considerations. The first consideration is that initiated hepatocytes compared to carcinogen treated non-initiated hepatocytes have a lower threshold for growth stimuli. This is based on the observation that initiated hepatocytes responded to 1/3 PH and formed foci. As pointed above, the logic has been with 1/3 PH only 50% of the remaining 2/3 of hepatocytes need to proliferate to compensate for the loss, the initiated hepatocytes do not form foci unless they have a growth advantage over the non-initiated hepatocytes. The second consideration is that the duration more than the intensity of a proliferative stimulus may be an important
determinant for its overall effect on the selective growth of focal lesions. This arises from the observation that the percent area occupied by the foci was very similar in the 1/3 and 2/3 PH groups, although the magnitude of the proliferative stimulus is less intense but more prolonged with 1/3 PH compared to 2/3 PH. Moreover, rats undergoing sequential removal of the median and the left lobes showed the highest value in percent area occupied by foci, and yet the magnitude of the total stimulus was roughly comparable to that of 2/3 PH. This conclusion is also consistent with the findings indicating that initiated hepatocytes are more efficient in their response to various growth stimuli, including the one(s) elicited by PH (Schulte-Hermann et al. 1981; Schulte-Hermann et al. 1982; Ogawa et al. 1985; Laconi et al. 1994). This could be attributed to at least two reasons: (i) an inherent increased responsiveness of initiated cells to proliferative signals (Schulte-Hermann et al. 1981; Schulte-Hermann et al. 1982; Ogawa et al. 1985; Laconi et al. 1994) and/or (ii) a primary defect in the surrounding non-initiated cells, such that their response to growth stimuli is delayed. Such a possibility is not unreasonable since most carcinogens, including DENA, are known to inhibit cell proliferation (Pentecost and Craddock 1983; Novicki et al., 1985).

Taken together, the results presented in Chapters 2 and 3 indicate that initiated hepatocytes have inherent growth advantage compared to the non-initiated hepatocytes and grow albeit slowly. However, upon exposure to certain tumor promoting regimen the initiated hepatocytes acquire resistance and grow in the presence of mitoinhibitory environment created by the tumor promoters especially those that appear to promote by the differential mitoinhibitory mode. Literature evidence indicates that the initiated hepatocytes that did not acquire resistance although grow to form foci and nodules, they do so very slowly and further, they do not progress to form hepatocellular carcinomas at least within in one year (Solt and Farber 1976). However, those foci that acquired resistance appear to grow rapidly to form nodules and ultimately hepatocellular
carcinomas with a 70 –100% incidence in one year (Solt and Farber 1976; Laconi et al., 1993b).

The above considerations suggest that acquisition of resistance plays an important role in the pathogenesis of carcinogenic process. It therefore became of interest to examine the molecular mechanisms of resistance. Accordingly, in the next series of experiments, reported in the next two chapters I have examined the mechanism of resistance acquired by the hepatic nodules to the mitoinhibitory effects of orotic acid, a liver tumor promoter which appears to promote by differential mitoinhibitory mode.

3.4 Summary
The present study was designed to determine whether GST 7-7 positive foci induced after initiation and not exposed to any exogenous tumor promoters have a lower threshold towards a proliferative stimulus. Accordingly, the effect of 1/3 PH on the incidence and growth of foci in initiated rat liver was determined. The rationale for using a 1/3 PH is that since only 50% of the remaining 2/3 of hepatocytes need to proliferate to compensate for the loss, the initiated hepatocytes do not form foci unless they have a growth advantage over the non initiated hepatocytes. Accordingly, male Fischer 344 rats (110-120 g) were initiated with DENA (200 mg/kg, i.p.). Three weeks later 1/3 PH (median lobe), 2/3 PH (median and left lobes) or sham operation (SH) were performed. An additional group of initiated animals had the median lobe and the left lobe of the liver removed sequentially (1/3 + 1/3 PH), 3 weeks apart. All rats were killed 8 weeks after carcinogen administration. The results indicated that the number of GST 7-7 positive foci was similar in all groups; however, percent area occupied by foci was increased in rats receiving 2/3 PH compared to SH (0.21±0.08 vs 0.09±0.03). Interestingly, 1/3 PH was nearly as effective as 2/3 PH in stimulating the growth of foci (percent area 0.18±0.06 vs 0.21±0.08), although the magnitude of the stimulus is only half for the former group compared to the latter; peak
labeling index was 19±6 with 1/3 PH compared to 40±2 with 2/3 PH. Moreover, the maximum increase in the size of foci (percent area 0.37±0.12) was achieved when the median and left lobes were removed sequentially, three weeks apart. These results indicate that initiated hepatocytes compared to the non-initiated hepatocytes have a lower threshold for growth stimuli and that persistent growth stimuli of low intensity can be very effective in promoting the growth of focal lesions.
CHAPTER 4

DEVELOPMENT OF RESISTANCE IN HEPATIC NODULES TO THE MITOINHIBITORY EFFECTS OF OROTIC ACID: UPREGULATION OF URIDINE PHOSPHORYLASE IN HEPATIC NODULES

The contents of this chapter has been modified and submitted for publication

4.1 Introduction

The present study was designed to determine the molecular mechanisms of resistance of hepatic nodules to the mitoinhibitory effects of orotic acid (OA). Orotic acid was chosen to measure resistance largely because our laboratory has been working on liver tumor promotion by OA, a precursor for de novo synthesis of pyrimidine nucleotides. Our laboratory and subsequently others have shown that OA promotes carcinogenesis in other organs as well as in different species (Rao et al. 1983; Laurier et al. 1984; Rao et al., 1989; Kokkinakis et al. 1991; Bradley et al. 1992; Visek 1992; Laconi et al., 1993a; Kokkinakis and Albores-Saavedra 1994; Vasudevan et al., 1994). In addition, there are metabolic and genetic disorders that pertain to impairment of the urea cycle, which are associated with high levels of OA (Milner and Visek 1973; Kelley 1983; Gerrits et al., 1993; Vasudevan et al., 1995). Interestingly, some of these disorders pose increased risk of hepatocellular carcinoma (Bjorneboe et al., 1985; Jeffers et al. 1988; Nakayama et al. 1990).

It was postulated that OA promotes liver carcinogenesis by a differential mitoinhibitory mode, similar to that postulated for the Resistant Hepatocyte (RH) model of liver tumor promotion (Solt and Farber 1976). This postulate was based on the observations that OA inhibits DNA synthesis in normal hepatocytes (Laconi et al. 1988; Sheikh et al. 1993b), while the hepatocytes from nodules are relatively resistant to the mitoinhibitory effects of OA (Laconi et al., 1991; Manjeshwar et al. 1993a; Sheikh et al. 1993b) Both in vivo and in vitro studies have shown that one of the mechanisms contributing to the resistance of hepatic nodules to OA induced mitoinhibition is their inability to accumulate uridine nucleotides upon exposure to OA (Manjeshwar et al. 1992b; Backway et al. 1994), a condition necessary for OA to exert its mitoinhibitory effects. The present study identifies that increased activity of uridine phosphorylase (URPase, EC 2.4.2.3), an enzyme which degrades uridine and in hepatic nodules could be one of the contributing factors for the failure of hepatocytes in nodules to accumulate OA induced uridine nucleotides.
4.2 Materials And Methods

4.2.1 Generation and harvesting of hepatic nodules:
Unless otherwise stated hepatic nodules were generated by the OA model of liver tumor promotion. In brief, male Fischer 344 rats (Charles Rivers breeding laboratories, St. Constant, Quebec) weighing 120-130 g were initiated with a single necrogenic dose of diethylnitrosamine (DENA; Sigma Chemicals; 200 mg/kg i.p.) and promoted with dietary 1% orotic acid for 30 weeks. At the end of 30 weeks some of these rats were transferred to basal diet for an additional 20 weeks for the development of hepatocellular carcinomas. The animals were taken off the orotic acid diet for 2 weeks before they were sacrificed. Nodules and hepatocellular carcinomas were scooped free from the surrounding tissue and non-nodular surrounding liver were taken and snap frozen in liquid nitrogen and stored at -70°C.

4.2.2 Assay for uridine phosphorylase activity
The nodules, the non-nodular surrounding liver or the normal liver was homogenized in 3 volumes of 5 mM phosphate buffer pH 7.4 and centrifuged for 15 minutes at 14,000g. The post-mitochondrial supernatant was removed and subjected to ultracentrifugation at 100,000g for 90 minutes. The post microsomal supernatant was dialyzed in three changes (1 hour each) of 5 mM phosphate buffer pH 7.4 containing 2 mM dithiothreotal. Protein concentration in the dialyzed preparation was determined using BCA reagent (Smith et al., 1985). URPass assay was optimized with respect to concentration of uridine, the time of incubation and enzyme protein concentration. A typical incubation mixture for assaying URPass activity consisted of the following in a total volume of 250 μl: 5 mM phosphate buffer pH 7.4, 2 mM dithiothreotal, 6 mM magnesium chloride, the enzyme preparation (0.25 - 1 mg protein) and different concentrations of uridine as the substrate.
Following incubation at 37°C for 20 minutes the reaction was arrested by keeping the tubes in a boiling water bath for 3 minutes. The samples were then cooled and centrifuged at 15,000 rpm for 20 minutes in a microcentrifuge. The supernatant was filtered and subjected to HPLC analysis (Rao et al. 1987). As a routine, stoichiometry between the degradation of uridine and the appearance of uracil was determined.

### 4.2.3 Determination of mRNA transcripts for URPase:
Total RNA was prepared from nodules of equal size (~1 g tissue) from three different animals by the method of Chomczynski et al. (Chomczynski and Sacchi 1987). Polyadenylated RNA was obtained from this by oligo (dT)-cellulose chromatography (Aviv et al., 1972). Electrophoresis in 0.8% agarose formaldehyde gel and transfer to nitrocellulose were done as described by Maniatis et al. (Maniatis et al., 1982). As controls polyA mRNA from surrounding non-nodular liver was used. A cDNA probe for mouse URPase (Watanabe et al., 1995) consisting of 1327 base pairs that contain a 5' untranslated region, a coding region of 933 base pairs and a 3' nontranslated region with a polyadenylated tail inserted in the EcoR1 sites of a pSG5 vector was used as a probe. The URPase probe was labeled with [32P]dCTP (Amersham, sp. Act. 6000 Ci/mmol) using random primed DNA labeling kit (Amersham) and hybridized for 48 hours at 42°C. The filters were washed as described by Maniatis et al. (Maniatis et al. 1982) and kept for autoradiography at -70°C for 3 days.

### 4.2.4 Determination of uridine phosphorylase protein by western analysis:
SDS-PAGE and western blotting was performed according to standard protocols (Laemmli 1970; Towbin et al., 1979). Briefly, 40 μg of post mitochondrial supernatant protein was mixed with Laemmli sample buffer and boiled for 3 minutes and then cooled. Sample was loaded onto a 8% SDS polyacrylamide gel. After electrophoresis the samples were
transferred onto nitrocellulose and blocked overnight in 4% skim milk and PBS at 4°C. The BAU bound polyclonal URPase antibody raised against URPase from murine cancer cells (1:1000 dilution, 1µg/µl) in 4% skim milk, 0.1% Tween 20 and 1x PBS was applied for 2 hours at room temperature. Filters were rinsed three times in 4% skim milk, 0.1% Tween 20 and 1x PBS then the secondary antibody was applied (anti-rabbit Ig horseradish-peroxidase conjugated) for 1 hour at room temperature then washed again twice and then three times in 0.1% Tween 20 and 1xPBS. Bands were then detected using enhanced chemiluminescence (Amersham).

4.2.5 Immunohistochemical determination of URPase:
Immunohistochemical staining for URPase was performed on sections fixed in cold acetone. After rinsing with PBS, endogenous peroxidase activity was blocked. The vector ABC reagents (Vector Laboratories, Burlingame, CA) were used for immunohistochemical detection of URPase (1:1000 dilution of 1µg/µl antibody). Biotinylated goat anti-rabbit antibody was used as the secondary antibody. A streptavidin-horseradish peroxidase conjugate followed by reaction with diaminobenzidine (Sigma) and hydrogen peroxide were used to localize the biotinylated label.

4.3 Results

4.3.1 Uridine phosphorylase activity and the levels of mRNA transcripts of uridine phosphorylase in hepatic nodules and non-nodular surrounding hepatic tissue
The results presented in Figure 4.1 show that URPase activity is increased significantly in the nodules compared to the surrounding non-nodular liver tissue. This increase in URPase activity is paralleled with an increase in the levels of mRNA transcripts (Figure 4.2). The increased activity of URPase in the nodules may degrade the increased uridine formed as a result of exposure to OA and can thus account for the lack of OA induced accumulation of uridine nucleotides, a condition necessary for OA induced mitoinhibition.

![URPase Activity](image)

**Figure 4.1** URPase activity in the cytosolic fractions from nodules and non-nodular surrounding liver tissue. Enzyme reaction was carried out with 1 mg of cytosolic protein and for 20 minutes. The results presented are from a typical experiment and the experiment was repeated several times with similar pattern of results. Details of URPase assay are given in Materials and Methods Section.
Figure 4.2  Levels of polyA mRNA transcripts in resting liver (R), surrounding non-nodular liver (S) and hepatic nodules (N). Ten micrograms of polyA mRNA was loaded in each lane and hybridized to the cDNA probe for URPase. Similar pattern of results were obtained when the experiment was repeated with different nodule samples from rats initiated with DENA and promoted with OA. Further details are given in Materials and Methods section.

4.3.2 Uridine phosphorylase activity and the level of mRNA transcripts in Kupffer cells

An observation of significance in this context is that in the normal liver, URPase is present predominantly in the Kupffer cells (Holstege et al., 1985; Liu et al., 1998b). The activity of URPase in the Kupffer cell enriched fraction of normal liver is approximately 6 to 7 fold higher than in the hepatocyte fraction and further, polyA mRNA expression is elevated in Kupffer and endothelial cells as compared to normal isolated hepatocytes (Figure 4.3). Both in vivo (Figure 4.4A) and in vitro studies (Figure 4.4B) have shown that concentrations of OA which inhibit DNA synthesis over 90% in normal hepatocytes (low URPase activity) have no inhibitory effect on DNA synthesis in Kupffer cells (high URPase activity). These results support the hypothesis that increased URPase activity plays an
important role in resistance to the mitoinhibitory effects of OA. Given the fact that hepatic nodules contain a relatively fewer percentage of Kupffer cells compared to the normal liver (Janossy et al., 1986) and yet exhibit higher levels of URPase activity compared to normal and non-nodular surrounding liver, the question of its regulation in nodule hepatocytes assumes importance.

![Image of Figure 4.3](image_url)

Figure 4.3  Levels of polyA mRNA transcripts in hepatocytes (HC), and Kupffer cells (KC) and endothelial cells (EC) enriched fractions. Purified hepatocytes and Kupffer cells and endothelial cells enriched fractions were prepared as described (Holloway and Sellar 1997). Ten micrograms of polyA mRNA was loaded in each lane and hybridized to the cDNA probe for URPase. Similar pattern of results were obtained when the experiment was repeated with different samples.
Figure 4.4 In vivo (A) and in vitro (B) mitoinhibitory effects of orotic acid on hepatocytes and Kupffer cells. In vivo mitoinhibitory effect of OA was determined by implanting a 300 mg tablet of orotic acid methyl ester i.p. at the time of two-thirds partial hepatectomy (PH) (Sheikh et al. 1993b). Control rats were subjected to PH in the absence of OA tablet. Sixteen hours after PH the rats were repeatedly injected with tritiated thymidine (100μCi) every 8 hours until 48 hours after PH and then killed. The livers were processed for autoradiography. Note the labelled non-parenchymal cells (NPC) compared to unlabelled hepatocytes. To determine the effect of OA on DNA synthesis in primary hepatocytes and Kupffer cells the isolated hepatocytes and fraction enriched with Kupffer cells were incubated in the minimum essential medium (MEM) in the absence of added growth factors. After a 2 hour attachment period, the medium was washed and replaced with MEM containing orotic acid methylester (120 μM) and tritiated thymidine (5μCi per 2.5 cm dish). After a 48 hour period of incubation, the cells were processed for acid precipitable radioactivity (Kupffer cells) as described earlier (Manjeshwar et al. 1994). The experiment was repeated three times with a similar pattern of results. Each value is the mean of 4 samples ± S.D.
4.3.3 Analysis of the uridine phosphorylase protein by immunohistochemistry and western blot

The increased levels of mRNA transcripts in the nodules do not appear to be either due to hypomethylation or due to amplification of the URPass gene (data not presented). The next series of experiments were designed to determine whether the increased enzyme activity is paralleled with increased levels of URPass protein. Immunohistochemical analysis of the nodules as well as western analysis of the cytosolic proteins isolated from the nodules and the surrounding non-nodular tissue were performed using an antibody raised against URPass from murine cancer cells (gift from Drs. Pizzomo and R. E. Handshumacher, Yale University, New Haven). Similar to that reported by Liu et al. (Liu et al., 1998a, our unpublished observations), I have also observed that this antibody cross reacted with hepatic URPass from mouse, rat and humans (data not presented).

Figure 4.5  Pictomicrograph of a liver section with a nodule and surrounding non-nodular tissue stained with a polyclonal antibody for URPass. Note the negative staining nodule and the positively staining surrounding non-nodular cells. Liver sections were taken from animals initiated with DENA and promoted with a 1% orotic acid diet for 30 weeks. Similar pattern of results were obtained with several other nodule preparations.
Interestingly, the results show that although the antibody cross reacted with the normal liver and the surrounding non-nodular tissue, it failed to interact strongly with either the nodules (Figure 4.5) or the cytosolic fraction of the nodules and

![Western analysis (A) and enzyme activity (B) of cytosolic preparations of normal/surrounding rat liver, hepatic nodule and hepatocellular carcinoma initiated with DENA and promoted by the orotic acid promotion protocol. For western analysis, 40μg of protein was loaded in each lane and an antibody to URPase was used to determine cross reactivity. Arrow indicates URPase protein. URPase activity was determined using 0.5mg of protein and 0.1mM uridine as the substrate. The incubation was carried out for 20 minutes. The results presented are from a typical experiment and the experiment was repeated with several preparations of nodules and hepatocellular carcinomas with similar pattern of results. Further details are given in the text.](image-url)
hepatocellular carcinomas in the western blot (Figure 4.6A) despite the fact that nodules
and hepatocellular carcinomas exhibited increased enzyme activity (Figure 4.6B).
Furthermore, benzylacyclouridine (BAU), an inhibitor for URPase (Niedzwicki et al., 1982),
at concentrations that inhibit near completely the URPase activity from normal hepatocytes could not completely inhibit the URPase activity from hepatic nodules (Figure 4.7). These results suggest that perhaps nodules may have two forms of URPase, one a normal form and the other a variant form. The latter result is in agreement with the one recently reported by Liu et al. (Liu et al. 1998a) that a significant fraction (40-60%) of URPase in breast, head and neck were resistant to the inhibitory effects of BAU. The term variant is used to denote that the URPase from nodules is different compared to that from normal or surrounding non-nodular liver.

Figure 4.7 Effect of BAU on URPase activity in cytosolic fractions of normal rat liver, and hepatic nodule and hepatocellular carcinoma generated by orotic acid promotion protocol. URPase activity was assayed using 0.1 mM uridine and 1 mg of total protein in the presence or in the absence of BAU (10 μM). The reaction was carried out for 20 minutes and the URPase activity was determined as described in the text. The experiment was repeated three times and typical results are presented. The experiment was also repeated with different concentrations of BAU. At each concentration, the percent inhibition by BAU in hepatic nodules and in hepatocellular carcinomas is less compared to that in the control liver samples (data not presented).
4.3.4 Uridine phosphorylase activity in the Resistant Hepatocyte model and in hepatocellular carcinoma generated by the orotic acid model

Many of the biochemical functions expressed by hepatic nodules are independent of the initiators and/or promoters employed to generate them (Roomi et al. 1985; Sarma et al. 1986; Farber and Sarma 1987). It therefore became of interest to determine whether the increased activity of URPase exhibited by hepatic nodules promoted by OA is also seen in nodules promoted by other models of liver tumor promotion. The results presented in Figure 4.8A indicate that hepatic nodules initiated with DENA and promoted by the resistant hepatocyte model (RH) model (Semple-Roberts et al., 1987) which does not involve OA also exhibited increased activity of URPase. Further, URPase activity appears to increase with progression of the nodules to hepatocellular carcinoma (Figure 4.6B). Interestingly, one human hepatocellular carcinoma examined also exhibited an increase in URPase activity compared to its surrounding non-nodular liver (Figure 4.8B). These results suggest that increased URPase activity may have greater significance than conferring resistance to OA induced mitoinhibition.

4.3.5 Pyrimidine nucleoside phosphorylase activity in hepatic nodules and hepatocellular carcinoma using 2-deoxyuridine and thymidine as substrates

The recent observation that 2-deoxy-D-ribose can act as an angiogenic factor (Haraguchi et al., 1994; Brown and Bicknell 1998; Stevenson et al., 1998) is of considerable significance in the present context, because URPase and thymidine phosphorylase, the other pyrimidine nucleoside phosphorylase act on 2-deoxyuridine and thymidine respectively to generate 2-deoxy-D-ribose. The results presented in Figures 4.9A and 4.9B show that hepatic nodules and hepatocellular carcinomas compared to surrounding non-nodular liver exhibited increased activities when 2-deoxyuridine and thymidine were used as substrates.
Figure 4.8 URPase activity in surrounding non-nodular liver (normal hepatocytes), in nodules (4.8A) and in human hepatocellular carcinoma initiated with DENA (200 mg/kg) and promoted with the RH model, (Semple-Roberts et al. 1987). The experiment was repeated three times with similar results. Total protein of 0.25 mg was incubated for 30 minutes using 0.1 mM of uridine as the substrate. In Figure 4.8B the URPase activity in one human hepatocellular carcinoma (human HCC) was presented. This experiment was repeated three times with similar result. Total protein of 0.5 mg was incubated for 20 minutes using 0.1 mM of uridine as the substrate.
Figure 4.9  Pyrimidine nucleoside phosphorylase activity in non-nodular surrounding (normal) liver, hepatic nodules and hepatocellular carcinomas using 2-deoxyuridine (A) and thymidine (B) as the substrates. In experiment A the reaction was carried out for 20 minutes with 0.1mM of 2-deoxyuridine as the substrate and total protein of 0.5 mg, while in experiment B, the reaction was carried out for 30 minutes with 0.1mM thymidine as the substrate and 0.25 mg of total protein as the enzyme source. This experiment was repeated three times with similar results.
4.4 Discussion
Our earlier studies demonstrated that the incidence of hepatocellular carcinoma in rats initiated by several carcinogens and promoted by OA was nearly 100% and associated with 40-70% lung metastases (Laurier et al. 1984; Bradley et al. 1992; Laconi et al. 1993a; Laconi et al. 1993b; Denda et al., 1994). OA induced promotion was dependent on the rapid metabolism of OA to uridine nucleotides and their accumulation (Rao et al. 1986b). This kind of an imbalance in the pool sizes of nucleotides affected ribonucleotide reductase causing an inhibition of DNA synthesis (Manjeshwar et al. 1993b; Manjeshwar et al. 1999). The response of initiated cells to such a mitoinhibitory environment challenging its growth and survival is to develop into a phenotype that overcomes the difficulty encountered for its survival and growth. Therefore, it evolves into a resistant phenotype with reduced capacity for a net uptake of OA (Backway et al. 1994) and increased capacity to degrade uridine nucleotides. The results of the present study clearly indicate that hepatic nodules exhibit increased activity of URPase which degrades uridine. It was also observed that post mitochondrial supernatant from hepatic nodules exhibited increased activity to degrade uridine-mono-phosphate (UMP) to uridine compared to that from non-nodular surrounding liver (Backway and Sarma, unpublished observation). Taken together, these results could account for the resistance of hepatic nodules to the OA induced accumulation of uridine nucleotides and the consequent mitoinhibition. The association between increased URPase activity and resistance to mitoinhibition by OA is also borne out from the fact that Kupffer cells which exhibit higher activities of URPase compared to normal hepatocytes, are relatively resistant to the mitoinhibitory effects of OA. The observations that hepatic nodules generated by the RH model of liver tumor promotion which have not been exposed to OA also exhibit increased activity of URPase and that the URPase activity in hepatic nodules increases with the progression of hepatic nodules to hepatocellular carcinomas suggest that URPase may have a role in the
carcinogenic process besides overcoming the mitoinhibitory effect of OA when used as a promoter.

The observation that a majority of the URPase from nodules and hepatocellular carcinoma could not react with the antibody inspite of the fact that these livers exhibited increased enzyme activity and increased levels of mRNA transcripts is of significance. One of the possibilities for this apparent discrepancy is that the URPase used was the fraction that was absorbable to a BAU column (Liu et al. 1998a). Since URPase from normal liver is nearly 100% sensitive to BAU, while that from nodules and hepatocellular carcinoma is not (Figure 4.7), it may be argued that the URPase expressed in the neoplastic lesions is a different one. Furthermore, the data indicate that as the lesions progress, the variant form increases as compared to normal. More information on the nature of the variant can be obtained by purifying the BAU sensitive and resistant forms and using antibodies against the BAU sensitive and resistant forms of URPase.

URPase and thymidine phosphorylase are the two key enzymes in the degradation pathway of pyrimidine nucleotides and may play an important role in the maintenance of nucleotide levels in the cell, for an unbalanced increase in any one nucleotide pool may interfere with DNA synthesis. The recent observation that 2-deoxy-D-ribose can function in angiogenesis indicates a new role for pyrimidine nucleoside phosphorylases in angiogenesis. In this connection it is worthy to mention that the angiogenic property of thymidine phosphorylase was attributed to 2-deoxy-D ribose, one of the degradation products of this enzyme (Haraguchi et al. 1994; Brown and Bicknell 1998; Stevenson et al. 1998). URPase can also generate 2-deoxy-D-ribose by acting on 2-deoxyuridine. Thus, the increased activity of URPase may perhaps be related to angiogenesis, a process which helps growth, progression and metastasis phases of the carcinogenic process. Increased activity of URPase and thymidine phosphorylase exhibited by several types of cancers
further support this consideration (Blair and Hall 1969; Higley et al., 1982; Leyva et al., 1983; Eda et al., 1993a; Eda et al., 1993b; Luccioni et al., 1994; Sadanand et al., 1998). On the other hand, increased activities of URPase and thymidine phosphorylase in cancer cells appear counter productive because these enzymes degrade pyrimidine nucleosides which are important for the proliferating cells. However, if the pyrimidine nucleoside phosphorylases in the cancer cells are a variant, in that they exhibit for example higher Km for the substrates uridine, thymidine and 2-deoxyuridine, then one can reason that the enzymes will degrade the nucleosides when their concentrations go beyond a certain level. This way there will be enough concentration of nucleosides required for the growth of the cancer cells, and anything in excess will be degraded for the generation of 2-deoxy-D-ribose, an angiogenic factor. Alternatively, the variant and the normal forms of URPase are located in different subcellular compartments and act on different pools of deoxyuridine and uridine. There may be one pool for nucleic acid synthesis, while the other may be a source for deoxyribose. Further studies are needed to separate the variant form from the normal one and assess the functional significance of the variant form of URPase exhibited by the hepatic nodules and hepatocellular carcinomas.

4.5 Summary:
The present study was designed to determine the mechanism of resistance of hepatic nodules to the mitoinhibitory effects of OA. Hepatic nodules initiated with DENA and promoted by OA failed to accumulate OA induced uridine nucleotides, a condition necessary for OA to exert its mitoinhibitory effects. The results of the present study show that the activity of uridine phosphorylase (URPase, EC 2.4.2.3), an enzyme that degrades uridine is increased significantly in the nodules compared to the surrounding non-nodular liver tissue. This increase in URPase activity is paralleled with an increase in the levels of mRNA transcripts of URPase. Interestingly, an antibody raised against URPase from murine cancer cells, although cross reacted with the normal liver and surrounding non-nodular
tissue, it failed to interact strongly either with the nodules immunohistochemically or with
the cytosolic fraction of the nodules on western analysis, inspite of the fact that nodules
exhibited increased enzyme activity. Furthermore, benzylacyclouridine, an inhibitor of
URPase, at concentrations that inhibit nearly 100% of the URPase activity from normal
hepatocytes could not completely inhibit the URPase activity from hepatic nodules and
hepatocellular carcinomas. These results suggest that perhaps the URPase expressed in the
nodular tissue and hepatocellular carcinoma could be a variant. Kupffer cells which show
a 6 to 7 fold increase in URPase activity compared to normal hepatocytes also are resistant
to the mitoinhibition by OA. These results are consistent with the suggestion that the
increased activity of URPase could account for the resistance of hepatic nodules to OA
induced accumulation of uridine nucleotides and its mitoinhibitory effects. Hepatic
nodules promoted by the resistance hepatocyte model, which have not been exposed to
OA also exhibited an increase in URPase activity. In addition, URPase activity appears to
increase with the progression of the hepatic nodules to hepatocellular carcinoma. These
results suggest that increased URPase activity may have greater significance other than
conferring resistance to OA induced mitoinhibition. The recent observation that 2-deoxy-
D-ribose can act as an angiogenic factor is of considerable significance in the present
context, because URPase and thymidine phosphorylase, the other pyrimidine nucleoside
phosphorylase act on 2-deoxyuridine and thymidine respectively to generate 2-deoxy-D-
ribose. Consistent with this suggestion is the observation that the hepatic nodules and
hepatocellular carcinomas compared to surrounding non-nodular liver exhibited increased
activities when 2-deoxyuridine and thymidine were used as substrates.

The results of the present study thus indicate that increased expression of URPase could
not only confer resistance in hepatic nodules to the mitoinhibitory effects of OA, but may
also contribute to the carcinogenic process by generating 2- deoxy-D-ribose, an
angiogenic factor.
4.6 Acknowledgements

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CHAPTER 5

DEVELOPMENT OF RESISTANCE IN HEPATIC NODULES TO THE MITOINHIBITORY EFFECTS OF OROTIC ACID: A POSSIBLE INVOLVEMENT OF P-GLYCOPROTEIN/P-GLYCOPROTEIN-LIKE PROTEIN(S) IN THE EFFLUX OF OROTIC ACID

The contents of this chapter are being written for publication

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5.1 Introduction:
Orotic acid, a pyrimidine 2,4 diketo-6-carboxylic acid is a pyrimidine precursor and in excess has been shown to promote carcinogenesis in the liver (Columbano et al. 1982a; Rao et al. 1983; Laurier et al. 1984; Laconi et al. 1993a) as well as in several other organs (Rao et al. 1983; Rao et al. 1987; Rao et al. 1989; Kokkinakis et al. 1991; Kokkinakis and Albores-Saavedra 1994). Based on the observations that it inhibits DNA synthesis in normal hepatocytes (Sheikh et al. 1993b) but not in the hepatic nodules, (Manjeshwar et al. 1993a; Sheikh et al. 1993b) it was suggested that orotic acid promotes liver carcinogenesis by a differential mitoinhibitory mode (Rao et al. 1983; Laurier et al. 1984; Laconi et al. 1993b) similar to that proposed for the resistant hepatocyte model of liver tumor promotion (Farber and Sarma 1987). Several lines of evidence suggested that hepatic nodules have a defect in the accumulation of uridine nucleotides following exposure to orotic acid, a condition which is crucial for orotic acid to exert its mitoinhibitory and tumor promoting effects (Rao et al. 1987; Pichiri-Coni et al. 1990; Manjeshwar et al. 1992b; Sheikh et al. 1993b). One of the mechanisms for the lack of orotic acid induced accumulation of uridine nucleotides in hepatic nodules is a defect in the net uptake of orotic acid (Backway et al. 1994) (the term “net uptake” was used to indicate that the defect could be either at the level of influx or at the level of efflux). Unfortunately, very little is known about the mechanism of net uptake of orotic acid in normal hepatocytes and virtually nothing is known in hepatic nodules. The present study examined whether p-glycoprotein (P-gp) or P-gp-like molecules are involved in the net uptake of orotic acid in normal hepatocytes. The rationale for this consideration stems from the observations that hepatic nodules which are defective in the net uptake of orotic acid exhibit an increased expression of P-gp (Bradley et al. 1992). Further, P-gp and P-gp-like molecules have recently been shown to mediate efflux across the plasma membrane of small molecules in addition to the classic large hydrophobic molecules (Gros et al., 1992; Martel et al., 1996; Awasthi and Zimniak 1997).
Presentation of the material and methods are given in previous chapters. Specific experimental details however are given in the legends to the individual figures.

**5.2 Results:**
The experimental approach was to administer inhibitors of P-gp, Cyclosporin A (CyA) or PSC 833 (gift from Novartis to Dr. J. Thiessen) and determine their effect on orotic acid induced accumulation of uridine nucleotides in the liver in vivo. Accordingly, CD1 male

![Graph showing the effect of CyA and PSC 833 on orotic acid (OA) induced hepatic uridine nucleotide pools. CD1 male mice (30g) were exposed to basal diet (BD); BD + OA 1% (OA); BD + OA+ inhibitors of P-gp, CyA or PSC 833 (17.5 mg%) or BD + inhibitors. At the end of one week the mice were killed. The liver samples were homogenized in 3 volumes of 0.5N perchloric acid. The acid soluble supernatant was treated with alkaline phosphatase to convert the ribonucleotides to ribonucleosides. Ribonucleosides were separated from deoxyribonucleosides by boronate affinity gel 601 column chromatography. The ribonucleoside fractions were subsequently analyzed by high performance liquid chromatography (HPLC) using µbondapak C18 column. The individual ribonucleosides were expressed as µg/g liver and the ratio of uridine to adenosine + inosine nucleosides determined (Rao et al. 1987). Values are the mean±S.D. of 6 animals per group. The experiment was repeated 3 times with similar pattern of results. p≤0.005 for OA vs all groups.]
mice were exposed to basal diet (BD); BD + OA 1% (OA); BD + OA+ inhibitors of P-gp, CyA or PSC 833 (17.5 mg%) or BD + inhibitors. At the end of one week the mice were killed and the livers were snap frozen in liquid nitrogen and processed for uridine nucleotides. The results presented in Figure 5.1 indicate that mice exposed to orotic acid exhibited an increase in hepatic uridine nucleotides measured as the ratio of uridine to adenosine + inosine. Such an increase induced by orotic acid was inhibited by PSC 833 and by CyA. The inhibitors however, had no effect on the basal levels of uridine nucleotides (data not presented). The decreased accumulation of uridine nucleotides by the inhibitors is not a reflection of increased degradation of uridine nucleotides or decreased synthesis of uridine nucleotides, because the inhibitors did not exert any effect on hepatic uridine phosphorylase, the enzyme that degrades uridine or on the machinery involved in the synthesis of uridine nucleotides from orotic acid (data not presented). Taken together, these results indicate that the net hepatic uptake of orotic acid is directly or indirectly dependent on PSC 833 and CyA modulatable processes.
In the next series of experiments the length of exposure to PSC 833 necessary to exert an inhibitory effect on the accumulation of orotic acid induced uridine nucleotides was determined. Accordingly, mice were pre-fed with BD or BD + PSC 833 (17.5 mg%) for different lengths of time. At the end of 2, 4, 7 and 13 days, the mice that were on BD were exposed to BD + 1% OA diet, while those that were on BD + PSC 833 were transferred to OA + PSC 833 diet. After 24 hours the mice were killed and the livers were processed for uridine nucleotide levels. The results presented in Figure 5.2 indicate that prefeeding for 2 days with PSC 833 was sufficient to inhibit the orotic acid

![Figure 5.2](image)

Figure 5.2 Minimum length of exposure to PSC 833 required for it to exert its inhibitory effect on orotic acid induced hepatic uridine nucleotide pools. CD1 male mice were pre-fed with BD or BD + PSC 833 (17.5 mg%) for different lengths of time. At the end of 2, 4, 7 and 13 days, the mice that were on BD were exposed to BD + 1% OA diet, while those that were on BD + PSC 833 were transferred to OA + PSC 833 diet. After 24 hours the mice were killed and the livers were processed for uridine nucleotide levels. Further experimental details are similar to those given in the legend to Figure 1. Values are the mean ±S.D. 6 animals per group. The experiment was repeated three times with similar pattern of results. p≤0.0001 at 2 days; p≤0.05 at days 4, 7 and 13 when OA+PSC groups were compared to their corresponding BD+OA groups.
Figure 5.3 Effect of PSC 833 on the expression of P-gp in mouse liver. Seven days liver samples from the experiment described in Figure 5.2 were used in the present study. Freshly excised livers were homogenized in cold 5mM phosphate buffer pH 7.4 containing a cocktail of proteolytic inhibitors (Boehringer Mannheim; 1 tablet per 10 ml of the homogenizing buffer). The homogenate was centrifuged at 1,000 g and the post-nuclear supernatant was aliquoted and further ultracentrifuged at 100,000 g for 1 hour. The pellet containing the membrane fraction was carefully resuspended in 50 mM mannitol and 20 mM HEPES Tris solution pH 7.5 containing a cocktail of proteolytic inhibitors. SDS-PAGE and western blotting were performed according to standard protocols (Laemilli 1970; Towbin et al. 1979). Briefly, 20 μg of protein was mixed with Laemilli sample buffer and incubated at 37°C for 20 minutes. Sample was loaded onto a 8% SDS polyacrylamide gel. After electrophoresis the samples were transferred onto nitrocellulose and blocked overnight in 4% skim milk/PBS at 4°C. The C219, a monoclonal antibody raised against P-gp (1:2000 dilution, 1μg/μl) in 4% skim milk/0.1% Tween 20/ 1x PBS was applied for 2 hours at room temperature. Filters were rinsed three times in 4% skim milk/0.1% Tween 20/ 1x PBS then the secondary antibody was applied (anti-mouse Ig horseradish-peroxidase conjugated) for 1 hour at room temperature then washed again twice and then three times in 0.1% Tween 20/1×PBS. Bands were then analyzed using enhanced chemiluminescence (Amersham). Lanes 1-3, 8D+OA and lanes 4-6, OA+PSC 833. The experiment was repeated three times with similar pattern of results.

induced hepatic accumulation of uridine nucleotides. Seven days liver samples were processed for the levels of P-gp using C219, a monoclonal P-gp antibody (gift from Drs. V. Ling and D. Rose). The results presented in Figure 5.3 indicate that exposure to PSC 833 resulted in an increased expression of a protein band in the range of 150-170 kDa where P-glycoprotein would band. A similar increase was observed in weanling Fischer 344 rats treated with PSC 833 for one week (data not presented). Similar increase in C219 cross reactable band with a ~170 kDa range was observed upon exposure to PSC 833
under slightly different experimental conditions (Jette et al., 1998). These results are interpreted to indicate that exposure to PSC 833 results in increased levels of P-gp. The increased levels of P-gp perhaps may be responsible for the decreased net uptake of orotic acid and the consequent decreased levels of orotic acid induced accumulation of uridine nucleotides.

Figure 5.4 Effect of PSC 833 on hepatic uridine nucleotide pools in spf mutant mice. Male spf mice (25 to 30g) were exposed to either the mouse chow or the mouse chow containing PSC 833 (17.5 mg%) at the end of eight days the mice were killed and the livers were processed for uridine nucleotide levels as described in figure 5.1. Values are the mean±S.D. of 5 animals. Experiment was repeated twice with a similar pattern of results *p≤0.03 compared to the control group.
In the above described experiments orotic acid was given exogenously and therefore, it was difficult to determine whether the primary effect of PSC 833 is at the intestine level or at the level of the liver. To overcome this problem, a system with high levels of endogenous orotic acid was used. For this purpose, sparse fur mutant (spf) mice were used. These mice which are over 90% deficient in ornithinetranscarbomylase synthesize large amounts of hepatic orotic acid and uridine nucleotides and are also associated with high concentrations of urinary orotic acid (Qureshi et al. 1979; Vasudevan et al., 1992). It is argued that if spf mice exposed to PSC 833 exhibit decreased hepatic uridine nucleotides and increased urinary orotic acid, it will be interpreted to indicate that PSC 833 modulatable process and by extrapolation, P-gp or P-gp-like molecules may be involved either directly or indirectly with the efflux of orotic acid from the liver. Accordingly, male spf mice were exposed to either basal diet or basal diet containing PSC 833 (17.5 mg%). Eight days later 24 hour urinary orotic acid excretion and hepatic uridine nucleotides were measured. The results presented in Figures 5.4 and 5.5 show that spf mice exposed to PSC 833 not only exhibited decreased hepatic levels of uridine nucleotides but also exhibited increased excretion of urinary orotic acid. Further, these effects of PSC 833 are not a reflection of increased renal clearance because orotic acid normally is cleared rapidly (Webster et al., 1981). These results are interpreted to indicate that PSC 833 by inducing P-gp/P-gp-like molecules increased the efflux of endogenously synthesized orotic acid from the liver, which in turn resulted in an increased urinary excretion of orotic acid with a consequent decrease in hepatic uridine nucleotides.
Figure 5.5 Effect of PSC 833 on urinary excretion of endogenous orotic acid in spf mutant mice. The mice used in the experiment described in Figure 5.4 were used for this study. On 7th day a 24 hour urine was collected and analyzed for orotic acid. Values are the mean±S.D. of 5 mice. The experiment was repeated twice with similar pattern of results. *p≤0.001 compared to the control group.

5.3 Discussion

Taken together, the data presented suggest that P-gp or P-gp-like molecules may be involved in the efflux of orotic acid from the hepatocytes. Although P-gp and P-gp like molecules have been considered to mediate efflux across the plasma membrane of large hydrophobic molecules, recently P-gp and P-gp-like molecules have been shown to mediate the efflux of small molecules also (Gros et al. 1992; Martel et al. 1996; Awasthi and Zimniak 1997). In addition, some of these also appear to be involved in flipping molecules inside-out (Higgins and Gottesman 1992). Therefore, it is not unreasonable to visualize that they may be involved in the efflux of orotic acid. In the present study no attempt has been made to identify the exact mechanism of efflux of orotic acid.
Nonetheless, the observation that agents that can modulate the levels of P-gp could increase the urinary excretion of orotic acid with an associated decrease in the accumulation of hepatic uridine nucleotides is significant because for the first time it provides a direction for further exploration to an understanding of the transport of orotic acid in and out of the hepatocyte.

Further, by extrapolation the results presented in this chapter also suggest that the increased expression of P-gp in hepatic nodules could explain the decreased net uptake of orotic acid by hepatic nodules reported earlier (Backway et al. 1994). In this context it should be interesting to speculate on the mechanism of resistance of hepatic nodules to the mitoinhibitory effects of 2-acetylaminofluorine (2-AAF) used in the resistant hepatocyte model of liver tumor promotion. One of the mechanisms for such resistance was attributed to the decreased macromolecular adducts of 2-AAF in hepatic nodules (Gupta et al., 1988b; Huitfeldt et al., 1988). This decrease was attributed to an inability of nodules to metabolize 2-AAF to its hydroxylated derivatives (Cameron et al. 1976; Farber 1984a). However recently, it was reported that cytochrome P450 1A2, the isozyme implicated in the metabolism of 2-AAF was in fact not decreased in hepatic nodules (Ghoshal 1992). Since hepatic nodules have been shown to overexpress P-gp, it is very likely that the increased levels of P-gp, may mediate the efflux 2-AAF and/or its metabolites and thereby confer resistance in hepatic nodules to the mitoinhibitory effects of 2-AAF in hepatic nodules.

It has been suggested that both the resistant hepatocyte model and the orotic acid model promote liver carcinogenesis by the differential mitoinhibitory mode; i.e., the initiated hepatocytes by being resistant to the mitoinhibitory effects of 2-AAF in the resistant hepatocyte model and to the mitoinhibitory effects of orotic acid in the orotic acid model grow in the mitoinhibitory environment created by these two agents. It is interesting to
note that in both these models hepatic nodules exhibit increased expression of P-gp, which has the potential to efflux 2-AAF and orotic acid and thereby confer resistance in the hepatic nodules. These considerations suggest the possibility that P-gp and P-gp-like molecules play an important role in the early stages of liver cancer development.

Thus in summary, the results presented in this chapter indicate that a process(s) modulatable by PSC 833 and CyA of P-gp is involved in the efflux of orotic acid. These observations were interpreted to indicate that P-gp or P-gp-like molecules may be involved in the efflux of orotic acid.

5.4 Summary
Orotic acid has been suggested to promote liver carcinogenesis by a differential mitoinhibitory mode. A prerequisite for this mode of tumor promotion is that initiated hepatocyte must be resistant to the mitoinhibitory effects of orotic acid. One of the mechanisms for such resistance is a decreased net uptake of orotic acid by hepatic nodules. Unfortunately, very little is known concerning influx and efflux of orotic acid in normal hepatocytes and virtually nothing is known about its net uptake in hepatic nodules. The present study was designed to determine whether P-glycoprotein (P-gp) or P-gp-like molecules are involved in the net uptake of orotic acid by normal hepatocytes. The experimental approach was to determine whether inhibitors of P-gp would influence the uptake of orotic acid monitored as an accumulation of hepatic uridine nucleotides, the immediate metabolic products of orotic acid and urinary excretion of orotic acid. Accordingly, mice were exposed to diets containing PSC 833 and Cyclosporin A, two known inhibitors of P-gp (17.5mg%), or similar diets together with 1% orotic acid. The results indicated that these two inhibitors of P-gp inhibited the accumulation of hepatic uridine nucleotides induced by orotic acid. Further, in the presence of PSC 833 there was an increase in the levels of protein(s) banding in the region of ~170 kDa, which cross
reacted with C219, a monoclonal antibody for P-gp. The decreased accumulation of uridine nucleotides by the inhibitors is not a reflection of increased degradation of uridine nucleotides or decreased synthesis of uridine nucleotides, because the inhibitors did not exert any effect on hepatic uridine phosphorylase, an enzyme that degrades uridine or on the machinery involved in the synthesis of uridine nucleotides from orotic acid. Since in these experiments orotic acid was given exogenously, it is difficult to determine whether the primary effect of the inhibitors is at the level of the intestine or the liver. To overcome this problem, sparse fur mutant (spf) mice which exhibit high levels of endogenous orotic acid were used. These mice which are over 90% deficient in ornithinetranscarbomylase synthesize large amounts of hepatic orotic acid. It is argued that if spf mice exposed to PSC 833 exhibit decreased hepatic uridine nucleotides and increased urinary orotic acid, it will be interpreted to indicate that P-gp may be involved with the efflux of orotic acid from the liver. Accordingly, male spf mice were exposed to either a basal diet or the basal diet containing PSC 833 (17.5 mg%). Eight days later 24 hour urinary orotic acid excretion and hepatic uridine nucleotides were measured. The results show that spf mice exposed to PSC 833 not only exhibited decreased levels of hepatic uridine nucleotides, but also exhibited increased excretion of urinary orotic acid. Taken together, these data suggest the possibility that P-gp or P-gp-like molecules may be involved in the efflux of orotic acid from the hepatocytes.
CHAPTER 6

GENERAL DISCUSSION AND SUMMARY
The overall aim of the thesis is to explore the mechanisms of selective amplification of initiated hepatocytes to form focal proliferations. Pioneering studies on the mouse skin carcinogenesis illustrated that agents which cause hyperplasia have the potential to amplify the initiated cells giving them a growth advantage in response to the stimuli over the non-initiated cells (Friedewald and Rous 1944). Later, in liver carcinogenesis models, similar observations were made with respect to the response of the initiated cells to a number of cell proliferative stimuli (Peraino et al. 1971; Shinozuka et al. 1979; Farber 1980; Shinozuka and Lombardi 1980; Schulte-Hermann et al. 1981; Schulte-Hermann et al. 1982; Laconi 1988a). However, none of these studies highlighted the principle underlying the focal proliferation of the initiated cell.

6.1 Response of initiated cells to cell proliferative stimuli

It is important to consider whether the response of the initiated cells to a growth stimuli is more efficient than normal cells. The growth stimuli used in earlier studies was so intense, for example TPA in mouse skin models (Berenblum and Shubik 1947) and 2/3 PH in the liver carcinogenesis models, the subtle regulatory mechanisms that could account for the selective growth advantage of initiated cells might have been overshadowed. For example, the initiated cell may respond to growth stimuli at levels where normal cells may not be able to commit to proliferate. Alternately, the initiated cell may be refractory to the regulation of growth inhibition such that they may go through more than one cell cycle while the normal cell can only go through one cycle in response to a cell proliferative stimulus. The results presented in Chapter 3 demonstrate that initiated hepatocytes form focal proliferations in response to 1/3 PH, suggesting that the initiated hepatocytes may have a lower threshold to growth stimuli such as PH compared to non-initiated hepatocytes. The novelty of using 1/3 PH was that only 50% of the remaining 2/3 hepatocytes need to proliferate to compensate for the loss. Under these conditions, initiated hepatocytes do not form foci unless they have some growth advantage.
6.2 Role of a chronic mitoinhibitory environment in the evolution of initiated cells

In the context of experimental carcinogenesis perhaps two emerging principles are of great significance in addition to the observed inherent growth advantage within initiated cells. These are:

(i) non-initiated cells are affected to some degree by carcinogen treatment such that they become mitoinhibited, perhaps chronically;

(ii) initiated cells have acquired the ability to grow in the presence of this chronically mitoinhibited environment induced by carcinogens.

These considerations give a new direction in our understanding of carcinogenesis and suggest that a chronic mitoinhibitory environment or a compromise in the functional integrity in general, may be an important component in the evolution of these rare initiated cells. Evidence for a role of a growth inhibitory environment during carcinogenesis has also emerged from in vitro studies. Rubin and coworkers have reported that the transformation frequency of NIH 3T3 fibroblasts in vitro increases upon exposure of the cells to growth constraint conditions (Yao et al., 1990; Rubin et al., 1995). Also, SV40-immortalized hepatocytes grown in a low choline medium have an increased tumorigenic potential when transplanted into nude mice, while passage-matched control hepatocytes are not (Zeisel et al., 1997). Similarly, immortalized human keratinocytes become tumorigenic in vivo when cultured in vitro in a minimal medium without serum, while control cells grown in presence of serum are not (Hill et al., 1991). Such in vitro findings suggest that the conditions associated with growth constraint may favour the evolution of these rare transformed cells. In a human situation, even though there may not be a deliberate exposure to a carcinogen, the aging process may exert a similar effect on the bulk of non-initiated cell population. Age is often considered to be a risk factor in human and experimental rodent neoplasia (Peto et al., 1975; Newell et al., 1989). One of
the characteristics of aging in animals is the slowdown of cell proliferation, which may be a reflection of free radical damage, accumulation of toxic metabolites and/or decreased functional integrity in general (Sohal and Weindruch 1996). Whatever the cause, the chronic decreased response to a mitogenic stimulus may set the stage for the emergence of the rare resistant cells which are better adapted to grow in this constrained environment. The hypothesis that aging may favour carcinogenesis via the effects induced on the bulk of the tissue as opposed to those on the rare altered cells, derives support from an observation that when transformed rat liver epithelial cells were injected into the liver of either young or old syngeneic rats, no cell growth was observed in the young rats, while tumors were seen in the livers of older recipients (McCullough et al., 1994; McCullough et al., 1997; McCullough et al., 1998). Immunologic mechanisms were considered less likely factors for this differential response. A significant feature of the carcinogenic process that should emerge from this brief discussion is that loss of functional integrity of the surrounding non-initiated tissue plays an important role in the evolution of the rare altered cells.

6.2.1 Stage at which resistance is acquired

The above studies further, indicate the possibility of the emergence of a resistant population on the backdrop of chronic toxicity and/or impaired growth capacity in the target organ. As pointed in the introduction (Chapter 1), cancers and a wide variety of precursor lesions are a multiresistant phenotype. The liver is unique in that it is primarily involved with the activation and/or detoxification of chemicals and unfortunately becomes the primary target for the activated reactive electrophilic species. Therefore, it is not surprising that the hepatic nodule, a resistant phenotype has evolved a biochemical machinery consistent with decreased activation and increased detoxification. However, one of the questions that was not addressed until recently, was at which stage is this
resistant property acquired? Is it at the initiated hepatocyte stage as postulated by the RH model or during promotion phase? Up front, this question appears esoteric because the initiated hepatocyte being a resistant phenotype by definition should not be a target for the mitoinhibitory and cytotoxic tumor promoting regimens. However, one can not ignore the fact that initiated hepatocytes are exposed for at least two to several weeks to the mitoinhibitory and cytotoxic tumor promoting regimens before any focal proliferations are seen (Peraino et al., 1973; Solt and Farber 1976; Sells et al. 1979; Shinozuka and Lombardi 1980; Colombano et al. 1982a; Rao et al. 1983; Laurier et al. 1984; Rao et al. 1984). It is likely that during this period initiated hepatocytes are further altered by these cytotoxic and mitoinhibitory tumor promoting regimens. Extension of these arguments suggest that perhaps the initiated hepatocyte is not a resistant phenotype but has acquired the ability to express resistance upon exposure to some of these cytotoxic and mitoinhibitory tumor promoters. The results presented in Chapter 2 indicate that the initiated hepatocyte may not be a resistant phenotype but can express resistance upon exposure to certain tumor promoters and certain genotoxic agents. As mentioned earlier I used the term “resistance” in a restricted sense in that resistance is defined as being resistant to the mitoinhibitory effects of orotic acid or 2-AAF. The concept that resistance can be induced in the initiated hepatocyte is novel and opens up new avenues in our understanding of the biology of carcinogenesis.
Figure 6.1  Schematic representation of the multi-step development of liver cancer (A) and induction of resistance (B) as a new step in the multi-step development of liver cancer in the rat initiated with genotoxic carcinogens.
6.2 A new step in the liver carcinogenic process

Based on the data presented in Chapter 2 an alternative pathway may be postulated for the evolution of hepatic foci. Once the initiated cell is generated the majority of these cells are not resistant to the mitoinhibitory effects of at least orotic acid and 2-AAF. For the initiated cells to progress onwards to focal proliferations agents which induce resistance are required to generate resistant hepatocytes. These resistant hepatocytes can then be amplified into focal lesions (figure 6.1).

Perhaps of practical significance is the prospect that this new concept will help in the identification of two groups of chemicals hitherto unidentified, which are not necessarily carcinogens or conventional tumor promoters but may still participate in carcinogenesis; (a) those that can induce resistance in the initiated hepatocytes, “inducers of resistance” and (b) those that can promote only the initiated hepatocytes that have acquired resistance. It should be interesting to identify the basic qualifications of the inducers of resistance. Similarly, it should be equally interesting to determine whether agents that inhibit DNA synthesis in normal hepatocytes can promote initiated hepatocytes that have acquired resistance. For example acetaminophen, a mitoinhibitor to normal hepatocytes has been shown not to be a liver tumor promoter (Kurata et al., 1985; Hasegawa et al., 1988; Maruyama et al., 1990). It remains to be determined whether it will promote when administered following induction of resistance.

6.3 Mechanism of resistance of hepatic nodules to the mitoinhibitory effects of orotic acid

The obvious next question is to determine the mechanism of induction of resistance. In order to approach this problem, it became important to first define the phenomenon of resistance and its mechanism. For this purpose, the mechanism of resistance to the mitoinhibitory effects of orotic acid was chosen to be further explored.
Attempts to understand the molecular mechanisms of resistance of hepatic nodules to the mitoinhibitory effects of orotic acid revealed that nodules do not accumulate uridine nucleotides upon exposure to orotic acid, a condition required for orotic acid to exert its mitoinhibitory effects (Manjeshwar et al. 1993a; Sheikh et al. 1993b; Backway et al. 1994). At least two defects were identified which can account for this lack of accumulation of uridine nucleotides in hepatic nodules:

(i) increased degradation of uridine nucleotides (Chapter 4)
(ii) decreased net uptake of orotic acid (Chapter 5)

6.3.1 Degradation of uridine nucleotides by hepatic nodules

The results presented in Chapter 4 show a several fold increase in URPase activity within the hepatic nodules compared to their corresponding surrounding non-nodular liver. Increased URPase as a mechanism by which nodules escape the mitoinhibitory effects of orotic acid is logical, however, many properties of the multiresistant nodules are independent of the initiator, promoter or carcinogen used. Interestingly, URPase is no different in this respect. Nodules and cancers generated by models in which no exposure to exogenous orotic acid was involved also show a significant increase in URPase activity (Chapter 4). Therefore, the role of URPase may be more capacious than initially thought. For example, there may be several possibilities as to why nodules upregulate URPase. It may be that uridine nucleotides beyond a certain level are inhibitory and/or potentially detrimental for the growth of nodules. It has been shown that an increase in deoxyuridine triphosphate is mutagenic upon its incorporation into DNA (Kunkel et al., 1982; Dong et al., 1993; James et al., 1997) and mitoinhibitory (Laconi et al. 1988; Pichiri-Coni et al. 1990; Manjeshwar et al., 1991; Sheikh et al. 1993b). It is also possible that the breakdown products of URPase are advantageous for the growth of the nodule and/or deleterious to the surrounding tissue. Interestingly, the downstream products of uridine breakdown i.e.
uracil and β-alanine were not inhibitory to normal hepatocytes following 2/3 partial hepatectomy (data not published). Whether these products assist in the growth of the nodule is not known. One of the products of the degradation of deoxyuridine by URPase is deoxyribose, an angiogenic factor. Furthermore, the results presented in Chapter 4 indicate that URPase activity paralleled with the progression of the carcinogenic process. In these studies, hepatocellular carcinomas always had a higher activity than the precursor nodular lesions. In a recent collaborative study we also have shown that in human astrocytoma cells lines there is a correlation between increased levels of URPase and malignancy which may be attributed to increased angiogenesis (Sadanand et al. 1998).

In addition to the role of pyrimidine nucleoside phosphorylases (PNPases) in cancer development, they also play a role in the resistance of neoplasia to certain cancer chemotherapeutic agents. For example, the levels of URPase influence the therapeutic effects (efficacy and selective toxicity) of several drugs such as 5-fluorouridine, 5-fluoro-2-deoxyuridine (Birnie et al., 1963; Ishitsuka et al., 1980; Woodman et al., 1980; Chu et al., 1984) and 3-azido-3-deoxythymine (AZT) (Sommadossi et al., 1988). In addition, host toxicities of the anti-cancer as well as anti-HIV drugs are antagonized by uridine (Sommadossi et al. 1988; van Groeningen et al., 1989), the availability and concentration of which are controlled by URPase (el Kouni et al., 1990; Pizzorno et al., 1998). Recent studies have shown that the expression of PNPase activity is upregulated by treatment with various cytokines such as interferon α, tumor necrosis factor α, interleukin-1α and interferon-γ in human (Tevaearai et al., 1992; Eda et al. 1993b) and in mouse tumor cells (Eda et al. 1993a). Thus, increased expression of URPase appears to play an important role in liver tumor promotion by orotic acid in particular, and it also appears to have a more broader role in carcinogenic process in general, as well as in development of a phenotype resistant to certain types of cancer chemotherapies.
6.3.2 Tumor uridine phosphorylase as a variant with different kinetic properties

Even though it can be argued that increased expression of PNPases is advantageous for the growth and survival of the cancers, such an increase is counterproductive for proliferative lesions like nodules and cancers, because they degrade pyrimidines required for the synthesis of RNA and DNA. Such an apparent conflict can be overcome if the PNPases in hepatic nodules are of a variant form with different kinetic properties and subcellular distribution. In the absence of purified enzyme, no definitive conclusions can be drawn.

Nonetheless, nodule URPase appears to be a variant by several criteria (Chapter 4). Interestingly, expression of a variant form of URPase was reported in other cancers as well. For example, like the URPase in hepatic nodules, the URPase in breast cancer and head and neck squamous carcinomas, only 40-60% of the URPase activity could be inhibited by BAU (Liu et al. 1998a). Purification of BAU sensitive and insensitive URPase will give insights into the nature of the URPase in tumors. This new information should be exploited in the design of better therapeutic interventions.

6.4 Decreased net uptake of Orotic acid by hepatic nodules

The observation that hepatic nodules exhibited decreased net uptake of orotic acid both in vitro and in vivo can logically explain at least in part the resistance of hepatic nodules to the accumulation of orotic acid induced uridine nucleotides. Unfortunately, the mechanism of such a defect could not be explored until recently because very little is known about the influx and efflux of orotic acid in hepatocytes and virtually nothing is known about its net uptake by hepatic nodules. The observation that hepatic nodules exhibit increased expression of P-gp and the fact that P-gp is involved in the efflux of several different types of endogenous as well as exogenous substances (Deuchars and Ling 1989; Endicott and Ling 1989; Gros et al. 1992; Ling 1995; Martel et al. 1996;
Awasthi and Zimniak 1997) prompted me to look into the possibility whether P-gp or P-gp-like molecules may be involved in the efflux of orotic acid as well.

Although no definitive conclusion can be drawn at present, the data presented in Chapter 5 suggest the possibility that P-gp or P-gp-like molecules may be involved in the efflux of orotic acid. This tentative conclusion is new and its significance is many fold. First of all, it gives us at least one direction to design experiments to prove or disprove this hypothesis. Of all the tissues, liver is unique and perhaps the major organ in using exogenous orotic acid and converting it uridine nucleotides. This appears to be a differentiated property of hepatocytes. Since the accumulation of uridine nucleotides inhibit DNA synthesis and have the potential to be mutagenic and mitoinhibitory, it seems logical that nodules exhibit decreased net uptake of orotic acid.

As was pointed above, the multiresistant properties exhibited by nodules are independent of the initiator, promoter or carcinogen used. It is not surprising therefore that nodules generated by not only orotic acid (Bradley et al. 1992) but also generated by the RH model express increase the levels of P-gp (Schrenk et al., 1994; Hill et al., 1996; Lecureur et al., 1996). Both orotic acid promoted nodules and those promoted by the RH model are resistant to the mitoinhibitory effects of orotic acid (Sheikh et al., 1993a). Interestingly, nodules generated by the phenobarbital model of tumor promotion are sensitive to the mitoinhibitory effects of orotic acid (Sheikh et al. 1993a; Manjeshwar et al. 1994), and there is no measurable increase in P-gp levels within these nodules (Thorgeirsson et al. 1987).

In addition to its possible role in conferring resistance to the mitoinhibitory effects of exogenous orotic acid, possibly to those of 2-AAF and to the cytotoxic effects of several cancer chemotherapeutic agents, P-gps may play an important role in cancer
development as well. Recently, P-gp has been implicated in the flipping out of ceramide (Cabot et al., 1998; Cabot et al., 1999; Come et al., 1999). This latter molecule has been shown to activate the caspase cascade, a critical pathway involved in apoptosis (Anjum et al., 1998; Spinedi et al., 1998; Yoshimura et al., 1998; Tepper et al., 1999). Therefore, it may be argued that P-gp is anti-apoptotic and thus favour tumor growth. Furthermore, P-gp has also been shown to be induced by reactive oxygen species suggesting that this molecule may protect the tumor cell from oxidative damage (Ziemann et al., 1999).

The results presented in this thesis are consistent with a possible role of URPase and P-gp in the development of resistance of hepatic nodules to the mitoinhibitory effects of orotic acid (Figure 6.2). Given that both URPase and P-gp are upregulated in hepatic nodules, it should be interesting to determine whether there is co-ordinated upregulation of these two molecules in nodules and cancer. Interestingly, various cytokines have been shown to induce both URPase (Tevaeearai et al. 1992; Eda et al. 1993a; Eda et al. 1993b) and P-gp (Scala et al., 1995; Savas et al., 1996; Hirsch-Ernst et al., 1998). Further, several tumor promoters (Gupta et al., 1988a; Jeon et al., 1997; Kitano et al., 1998), and the resistance inducing agents, 2-AAF (Danz and Brauer 1988; Stromblad et al., 1994) and CCl₄ (Roy et al., 1992; Bruccoleri et al., 1997; Jeon et al. 1997) have also been shown to upregulate cytokine activity. Therefore, it should be of considerable interest to explore the role of cytokines in the development of the resistant phenotype.
Figure 6.2   Schematic representation of the metabolic pathway for orotic acid. Highlighted is the influx and efflux of orotic acid and breakdown of uridine by URPase. OPRTase, ornithine phosphoribosyl transferase; ODCase, ornithine decarboxylase; UMP, uridine monophosphate; UDP, uridine diphosphate; dUMP, deoxyuridine monophosphate; dUDP, deoxyuridine diphosphate; RNR, ribonucleotide reductase; CP, carbamoylphosphate
Obviously, a lot more needs to be done to understand the mechanism of selective amplification of initiated cells to form focal proliferations. Nonetheless, the results presented in this thesis indicate that induction of resistance is one mechanism of selective amplification of initiated hepatocytes to form focal proliferations. Further, the initiated hepatocytes do not appear to be a resistant phenotype, but has acquired the ability to express resistance upon exposure to certain genotoxic agents. Increased expression of URPase and P-gp/P-gp-like molecules in hepatic nodules could account for the resistance of hepatic nodules to the mitoinhibitory effects of orotic acid by increased degradation of uridine nucleotides and increased efflux of orotic acid respectively. URPase by way of generating deoxyribose, an angiogenic factor and P-gp by inhibiting apoptosis and protecting against oxidative damage may play additional roles in cancer development.
6.5 Future directions

My studies have given several insights which need further exploration.

A. The development of a “resistance assay” suggested the possible existence of a new class of chemicals which by themselves may neither initiate nor promote carcinogenesis but instead induce resistance and thereby participate in carcinogenesis. Future studies in this area would include:

1. To determine the nature of the chemicals which induce resistance and classify them as either genotoxic, non-genotoxic, cell killing using the resistance assay. The approach will be to initiate rats with diethylnitrosamine and then expose them to various chemical agents and then determine whether these agents could confer in the hepatic foci/nodules resistance to the mitoinhibitory effects of either orotic acid or 2-acetylaminofluorene.

2. Using the resistance assay to identify a new class of tumor promoters that can promote only those initiated cells which have acquired resistance.

3. To use the resistance assay in developing better strategies for cancer interventions by identifying those cancer chemotherapeutic agents that have the ability to induce resistance.

4. Phenobarbital promoted nodules are not resistant to the mitoinhibitory effects of orotic acid. It should be interesting to determine the levels of both URPase and P-gp in these hepatic nodules. Furthermore, if resistance is induced in these nodules will they now become resistant to the mitoinhibitory effects of orotic acid and 2-acetylaminofluorene. Perhaps of greater significance is to determine whether acquisition of resistance influences the progression of the disease process.
B. An area which requires further exploration is the characterization and identification of the variant URPase within hepatic nodules and hepatocellular carcinoma.

The approach will be to isolate the URPase enzyme which is insensitive to benzylacyclouridine (BAU). This can be accomplished by passing the URPase from the nodular tissue through a BAU affinity column. The non-binding fraction should contain the variant URPase. This fraction can then be purified and antibodies to this form of URPase can be generated. Once the variant form of URPase is purified, studies on the molecular characteristics can be carried out. Further, knockout and transgenic mice can be developed, and the role of the normal as well as the variant form of URPase in carcinogenesis and angiogenesis can then be determined.

C. Another area which requires further exploration is the possible role of P-glycoprotein (P-gp) functioning as an efflux pump for orotic acid.

Primary hepatocytes transfected with P-gp can be used to determine their ability to efflux orotic acid. mdr 1a, 1b and 1a and 1b double knockout mice are available. It should be interesting to determine the ability of the livers of these mice to efflux orotic acid. Hepatocytes exposed to an arginine deficient medium accumulate high levels of orotic acid and uridine nucleotides. It should be informative to determine the ability or the inability to efflux orotic acid of the hepatocytes with transfected P-gp and those with the mdr genes knocked out.

D. The results presented in the thesis suggest that both URPase and p-gp may play important roles in carcinogenic process.

It should therefore be interesting to determine whether inhibition of URPase and P-gp inhibit the carcinogenic process.
REFERENCES


