Liver carcinogenesis: from naughty chemicals to soothing fat and the surprising role of NRF2

Michael Karin¹,²,³,* and Debanjan Dhar¹

¹Laboratory of Gene Regulation and Signal Transduction, Department of Pharmacology, ²Department of Pathology and ³Moores Cancer Center, UCSD School of Medicine, 9500 Gilman Drive, La Jolla, CA 92039, USA

*To whom correspondence should be addressed. Tel: +1 858 534 1361; Fax: +1 858 534 8158; Email: karinoffice@ucsd.edu

Abstract

The liver is a key metabolic organ that is essential for production of blood proteins, lipid and sugar metabolism and detoxification of naturally occurring and foreign harmful chemicals. To maintain its mass and many essential functions, the liver possesses remarkable regenerative capacity, but the latter also renders it highly susceptible to carcinogenesis. In fact, liver cancer often develops in the context of chronic liver injury. Currently, primary liver cancer is the second leading cause of cancer-related deaths, and as the rates of other cancers have been declining, the incidence of liver cancer continues to rise with an alarming rate. Although much remains to be accomplished in regards to liver cancer therapy, we have learned a great deal about the molecular etiology of the most common form of primary liver cancer, hepatocellular carcinoma (HCC). Much of this knowledge has been obtained from studies of mouse models, using either toxic chemicals, a combination of fatty foods and endoplasmic reticulum stress or chronic activation of specific metabolic pathways. Surprisingly, NRF2, a transcription factor that was initially thought to protect the liver from oxidative stress, was found to play a key role in promoting HCC pathogenesis.

Introduction

One of the main tenets of chemically induced or naturally occurring carcinogenesis is that carcinogens act by inducing oncogenic mutations and tumor promoters induce the proliferation of oncogenically initiated cells or lead to expansion of a cell population that has been targeted by carcinogens (1). However, as our understanding of cancer and its etiology is growing, it has become obvious that these early assumptions and theories are too simplistic. Sequencing of numerous cancer genomes had revealed that a typical cancer initiating cell accumulates at least two to eight driver mutations (2). Obviously, all of these mutations need to accumulate within a single cell, otherwise cancer will not happen (Figure 1). However, given the extremely low rates of somatic mutations (estimated at ~10⁻⁸ per nucleotide site per generation) (3) and the existence of multiple tumor suppressive and genome surveillance mechanisms (4–6), as well as immunosurveillance that nips early tumors in the bud (7), it is quite surprising that cancer can ever evolve. Tumor suppressive mechanisms either kill initiated and genetically damaged cells or induce their senescence (oncogene-induced senescence), and thereby prevent the accumulation of multiple initiating mutations. Given these safety valves and obstacles, it is obvious that carcinogenesis is much more complicated than the mere activation of oncogenes or loss of tumor suppressors. Given that an initiated cell can either undergo cell death or oncogene-induced senescence, and thus never give rise to cancer, specific tumor promoting mechanisms must exist to make cancer a reality, a harsh one indeed.

Our studies of hepatocellular carcinogenesis, focusing on the molecular etiology of hepatocellular carcinoma (HCC), the major form of primary liver cancer, have taught us a great deal about the carcinogenic process in whole animal systems. Currently, HCC is the sixth most common cancer worldwide and the second leading cause of cancer-related deaths (8). HCC usually develops as the end stage of chronic liver inflammation (hepatitis) and damage, caused either by viral infections [hepatitis B virus (HBV) or
hepatitis C virus (HCV)), excessive alcohol consumption, which leads to alcoholic steatohepatitis or hypernutrition, which causes non-alcoholic steatohepatitis (NASH). A variety of genetic diseases, such as Wilson’s disease and hemochromatosis, also cause chronic liver damage and inflammation and therefore increases HCC risk (9,10). HCC is not a rapidly growing cancer and takes 20–30 years to evolve, but because it is often detected too late, it is one of the most lethal cancers. Another striking feature of HCC is its pronounced male bias (11,12). Although the rates of most cancers in the USA have been declining by 1–2% per year, HCC rates have been increasing up to 2.8% per year (13). Although much of the growth has been attributed to the spread of HCV, non-viral HCC due to obesity has been rising rapidly, showing a 300% increase during the past 20 years (14,15). Sequencing studies have revealed a great deal of heterogeneity in HCC, with somewhere between 8 and 11 major signaling pathways and biological processes undergoing recurrent oncogenic activation (16–18).

Chemically induced HCC: inflammation and compensatory proliferation

We started our investigation of the molecular etiology of HCC by using a relatively simple model of carcinogen-induced HCC based on administration of diethylnitrosamine (DEN) to 2-week-old male mice (19). Surprisingly, we found that even chemical carcinogenesis was based on the induction of inflammation, as previously found for spontaneous HCC development in Mdr2−/− mice (20) or colorectal tumorigenesis in mice challenged with the procarcinogen azoxymethane and the colonic irritant, dextran sodium sulfate (21). To manipulate the inflammatory response, we chose to use cell type-specific ablation of IKKβ and found that its deletion in myeloid cells inhibited carcinogenesis, whereas its deletion in hepatocytes enhanced carcinogenesis (19). The procarcinogenic effect of IKKβ ablation in hepatocytes was found to be due to enhanced cell death, which leads to the release of IL-1α that in turn leads to induction of IL-6 and other tumor promoting cytokines by liver myeloid cells (19,22). Enhanced production of IL-6 in males and its suppression by estrogen in females is the main cause of the male gender bias to HCC development (12). Subsequently, we found that several other manipulations, not only the ablation of IKKβ, for instance p38α deletion, also promote hepatocyte death, trigger compensatory proliferation and augment HCC development (22). Given the propensity of HCC to develop in the context of chronic liver damage and inflammation, compensatory proliferation is one of the main drivers that governs the development of this malignancy, ensuring a sufficient proliferative impetus in a tissue that normally turns over at an extremely low rate (19,23).

Our studies have also explained why DEN is such a potent hepatic carcinogen. In addition to inducing oncogenic mutations at a high rate, DEN is hepatotoxic and leads to necrotic cell death of hepatocytes containing pre-made interleukin (IL)-1α. The release of this cytokine triggers an inflammatory chain reaction that ends up in elevated expression of IL-6, tumor necrosis factor (TNF) and hepatocyte growth factor, all of which act as mitogens for initiated hepatocytes (19,22). Compensatory proliferation and other mechanisms outlined below make sure that hepatocytes harboring initiating mutations such as BrafV600E (24) will not senesce and will continue to accumulate other oncogenic mutations until they become fully malignant (Figure 1). Consistent with this hypothesis, chronic liver damage induced by carbon tetrachloride (CCL4), a toxic chemical that is devoid of mutagenic activity, strongly potentiates DEN-induced HCC and can even lead to HCC on its own if applied frequently enough (25,26).

The HCC pioneers: HCC progenitor cells

Early work on chemically induced hepatocarcinogenesis in rodents led to identification of foci of altered hepatocytes (FAH), which are dysplastic lesions packed with small hepatocytic cells (27,28). It was proposed that these FAH, which resemble dysplastic lesions detected in cirrhotic human livers (29), represent sites or microniches within which early transformed hepatocytes proliferate (30). But it was also argued that the proliferation seen within FAH is nothing other than compensatory proliferation triggered by exposure to liver damaging chemicals (31). Since the detection of FAH relied on histological staining of paraffin-embedded fixed liver sections, there was no simple way of resolving this controversy other than isolating cells that reside within FAH and subjecting them to deep genome sequencing. This however was never done, but we found a much simpler and better solution to the FAH controversy when we discovered that extensive collagenase digestion of DEN-exposed livers, long before HCC nodules are detected, resulted in generation of collagenase-resistant aggregates in addition to the expected single-cell suspension (Figure 2A). These aggregates contained tightly packed, small, hepatocytic cells, which resembled in size the cells that reside within FAH. By introducing these cells into MUP-uPA mice, which express urokinase plasminogen activator in hepatocytes during the first few weeks of life and therefore undergo a few rounds of compensatory proliferation (32), we demonstrated that these
cells were capable of giving rise to HCC (Figure 2A). Since very few or no tumors were generated by the monodispersed collagenase sensitive fraction, we postulated that the collagenase-resistant aggregates contain HCC progenitor cells, termed HcPC (24). We have also shown that HcPC are not unique to DEN-exposed livers, but are also present in livers of young TaklΔhep mice, which exhibit spontaneous HCC development (24). Transcriptomic analysis revealed that HcPC are highly similar in their gene expression profile to ‘oval cells’ or bipotential hepatobiliary progenitors (24). But in subsequent studies we discovered that oval cells cannot give rise to HcPC or even normal hepatocytes and concluded that HcPC are most likely derived from normal, well differentiated, zone 3 (pericentral) hepatocytes that had accumulated oncogenic mutations (33). In further studies done in collaboration with Pikarsky et al., we found that HcPC may first arise within specialized microniches that contain immune and inflammatory cells that provide the HcPC with various growth promoting inflammatory cytokines, including lymphotxin, TNF and IL-6 (34). At some point, however, the HcPC are weaned from their dependence in immune cells and become capable of producing their own growth factors. One of these factors is IL-6 that is produced by HcPC that show upregulation of LIN28, an RNA binding protein that inhibits the processing and maturation of Let7 micro-RNAs that inhibit IL-6 mRNA and translation (24) (Figure 2B). Autocrine IL-6 leads to the activation of STAT3, an oncogenic transcription factor that is needed for HCC development (35).

High-fat diet: an HCC promoter and initiator

Although at the present time the main HCC risk factors are HBV and HCV infections, their relative importance, especially in the USA and other developed countries, is rapidly declining due to the development and deployment of effective HBV vaccines and anti-HCV drugs (36–39). So what accounts to the continuing surge in HCC incidence? (15). The most likely answer is the obesity epidemic (40). Indeed, an epidemiological study conducted a decade ago has shown a 4.5-fold increase in the risk of HCC-related death in men whose body mass index exceeded 35 (15). To confirm that obesity, either genetically determined or dietary, can directly potentiate HCC development and to identify the mechanisms through which it acts, we challenged leptin-deficient ob/ob mice with DEN at 2 weeks of age, prior to the onset of obesity or placed DEN-challenged wild-type mice on high-fat diet (HFD), in which 60% of the calories are fat derived. In both cases, obese mice, especially obese males, developed more HCC than lean animals (41). This was the first clear-cut demonstration that hypernutrition can directly promote cancer development. We investigated the underlying mechanisms and found that the most important tumor promoting impetus came from TNF and IL-6, whose production by myeloid cells (TNF and IL-6) as well as by hepatic stellate cells and hepatocytes (IL-6) is augmented by central obesity, resulting in liver STAT3 activation (41). Curiously, a recent study had documented that about one-third of all industrial chemicals can lead to fatty changes in the liver, causing a condition termed toxicant-associated fatty liver disease (TAFLD) (42–44), whose clinical signs are remarkably similar to those of NAFLD, a condition that affects nearly 30% of the United States population (45). Both TAFLD and NAFLD can progress to more severe conditions called toxicant-associated steatohepatitis (TASH) and NASH, respectively, characterized by pronounced liver inflammation.
(steatohepatitits), fibrosis and a substantial increase in HCC risk (43,44,46,47).

Although obesity can increase HCC risk in industrial workers suffering from TAFLD or TASH (43), most obese individuals who progress from NASH to HCC do not show evidence of toxicant exposure (48). We therefore sought to develop a mouse model that is more suitable for studying NASH-driven HCC than the simple combination of DEN challenge and HFD feeding, which does not result in severe liver inflammation and/or fibrosis (41). We took advantage of the finding that HFD feeding of MUP-uPA mice, which undergo a temporary increase in hepatocyte ER stress and damage due to transient uPA expression between weeks 3 and 7 (32) led to appearance of classic NASH signs, including ballooning degeneration of hepatocytes, inflammatory infiltrates and bridging ‘chicken-wire’ type fibrosis (49). None of these signs was exhibited by similarly fed wild-type mice and the treatment of MUP-uPA mice with chemical chaperones or overexpression of the protein chaperone Bip/Grp78 in their liver completely abrogated the NASH signs, indicating the importance of ER stress for NASH development (49). By maintaining the MUP-uPA mice on HFD for 40 weeks, we found that once NASH has appeared the mice start to develop adenomas and eventually progress to typical HCC at a very high rate (49). As found for mice kept on HFD after DEN challenge (41), the progression from NASH to HCC is highly dependent on TNF signaling via TNFR1, but instead of inducing IL-6 expression, the major pro-tumorigenic function of TNFRI signaling within hepatocytes and HcPC is to activate NF-κB signaling via IKKβ, thereby stimulating cell proliferation and survival. Inhibition of TNFR1 signaling blocked the appearance of NASH signs and NASH to HCC progression (49). Although NASH-induced oncogenic mutations are yet to be identified, our inability to detect recurrent mutations suggests that unlike DEN-induced HCC, the spectrum of oncogenic mutations induced during NASH to HCC progression is very broad. Although the carcinogens inducing these mutations remain unidentified, we suspect the involvement of reactive oxygen species (ROS), as NASH afflicted livers contain many hepatocytes that have accumulated large amounts of peroxides, as indicated by dihydroethidine staining (49).

The stress activated transcription factor NRF2 as a major driver of hepatocarcinogenesis

In addition to ROS accumulation and ER stress, NASH development in MUP-uPA mice and in a second model based on administration of streptozotocin (STZ), which destroys pancreatic β cells, together with HFD, is accompanied by ER stress, reduced autophagic flux and accumulation of autophagy adaptor p62/SQSTM1 (50). In addition to being important for recruitment of polyubiquitinated proteins and organelles to the autophagic lysosomal degradation pathway, p62 is a signaling protein that can lead to activation of several signaling pathways, including NF-κB, which is activated upon binding of p62 to TRAF6, and NRF2, which is activated through titration of its negative regulator Keap1 by p62 (51,52). Of note, p62 is the major component of Mallory-Denk bodies, hyaline granules and hybrid inclusions, which are protein aggregates present in the majority of chronic liver diseases, including viral hepatitis, NASH, alcoholic steatohepatitis, hemochromatosis and HCC (53). It has been assumed that such p62-containing aggregates play a pathogenic role in the development of these diseases. Given the presence of p62-containing aggregates in livers of the mouse models of NASH used by our laboratory, as well as in livers of Tsc1+/hep mice, which undergo liver damage, inflammation and spontaneous HCC development due to the persistent mTORC1 activation (54), we decided to ablate p62 in hepatocytes of all these mouse strains. Interestingly, hepatocyte-specific p62 ablation, using p62fl/fl mice generated by Moscat et al. (55), completely abrogated HCC development in Tsc1+/hep mice and strongly reduced it in both HFD-fed MUP-uPA mice and STZ-HFD mice (50). The major oncogenic signaling pathways that are affected by the absence of p62 were found to be the mTORC1 pathway, whose downregulation also led to reduced expression of c-Myc, and the NRF2 activated antioxidant response (Figure 3) (50). Inhibition of NRF2 activity was accompanied by downregulation of numerous genes whose products are involved in glutathione biosynthesis and protection of cells against oxidative stress (56). Surprisingly, however, despite the inactivation of the antioxidant response, dihydroethidine staining did not reveal any increased accumulation of ROS.

**Figure 3.** The unexpected role of NRF2 in HCC development. Transient NRF2 activation suppresses carcinogenesis by upregulating the expression of antioxidant proteins that neutralize ROS and maintain protective amounts of reduced glutathione. However, chronic activation of NRF2 by p62 accumulation or KEAP1 and/or NRF2 mutations potentiates liver carcinogenesis by inhibiting oxidative stress induced senescence or death of initiated hepatocytes allowing these cells to accumulate numerous oncogenic mutations.
ROS in p62-deficient livers. On the contrary, p62 ablation led to a decrease in ROS accumulating cells in livers of Tsc1Δhep mice as well as in the two HFD-induced NASH models, MUP-uPA and ST2-HFD (50). We interpret these results to suggest that in the absence of NRF2-induced protection, ROS-accumulating HcPC undergo cell death and cannot contribute to HCC development (Figure 3). Of note, high p62 accumulation in non-tumor human liver is a strong predictor of HCC recurrence after curative ablation (50) and NRF2 or KEAP1 mutations that lead to constitutive NRF2 activation have been detected in 14% of human HCC (16,57,58). The presence of these mutations, which result in constitutive activation of the anti-oxidant response, does not interfere with accumulation of additional oncogenic mutations.

Conclusions

The use of mouse models to study HCC has provided us with invaluable insights into the pathogenesis of this common and highly lethal malignancy, which claims close to half a million lives every year. We conclude that regardless of etiology, with the sole exception of direct exposure to chemical carcinogenesis, HCC development is associated with chronic cellular stress that results in ROS accumulation, cell death and inflammation. In addition to causing cell death, excessive ROS accumulation results in induction of oncogenic mutations or inactivation of tumor suppressors. Importantly, a single oncogenic mutation is insufficient to induce HCC, and most well-established HCCs show mutational activation of 8–11 oncogenic signaling pathways (16). Thus, the hepatocytes that are subjected to oxidative stress and within which oncogenic mutations accumulate need to be able to survive chronic ROS accumulation and continue to proliferate under such conditions in order to accumulate the critical number of mutations needed to convert them to HcPC. Undoubtedly, the most critical player in this process is NRF2. Whereas transient NRF2 activation may be protective, its sustained activation brought about by p62 accumulation is oncogenic. Instead of attempting to use antioxidants and NRF2 activators as cancer preventative agents, we should consider the deployment of specific NRF2 inhibitors to prevent the progression of chronic liver inflammation to HCC.

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