Molecular Pathways: The complex roles of inflammation pathways in the development and treatment of liver cancer

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Abstract

Inflammatory signals from the surrounding microenvironment play important roles in tumor promotion. Key inflammatory mediators and pathways that induce and sustain tumorigenesis have recently been identified in many different cancers. Hepatocellular carcinoma is a paradigm for inflammation-induced cancer, since it most frequently develops on grounds of chronic hepatitis, consecutive cellular damage and compensatory regeneration. Recent studies revealed that liver damage-mediated inflammation and carcinogenesis is triggered by a complex crosstalk between NFκB, JNK and Stat3 signaling pathways. Molecular dissection of the mechanisms involved in the interplay between these pathways identified promising new targets for therapeutic intervention. Targeting different components of the signaling cascades may provide efficient means for blocking the apparently irreversible sequence of events initiated by chronic liver inflammation culminating in liver cancer.

Background

Most of the malignant tumors contain somatic mutations in genes involved directly or indirectly in the regulation of cell growth or cell death. Epidemiological studies suggest that these mutations are likely generated by different external factors including radiation, exposure to environmental pollutants or infectious agents, tobacco use or diet (1). Besides inducing directly uncontrolled proliferation, these factors also trigger the activation of several immune cell types, which infiltrate the affected tissue and foster tumor development. While the link between inflammation and carcinogenesis was first raised by Virchow at the 19th century, mechanistic insights into the process were obtained only during the past decade. Hepatocellular carcinoma (HCC), the third most common cause of cancer mortality worldwide (2), is the most extensively investigated inflammation-based carcinogenic process.
More than 90% of the HCC cases are associated with chronic inflammation, which arises from hepatitis B virus (HBV), hepatitis C virus (HCV) infection, hemochromatosis and alcoholic or non-alcoholic steatosis (3). Recent studies on mouse models provided important mechanistic insights into the pathogenesis by revealing the role of multiple signaling pathways that link chronic inflammation to HCC. These include the nuclear factor-κB (NFκB), the stress-responsive mitogen-activated protein kinase (MAPK) and the signal transducer and activator of transcription (STAT) pathways. Importantly, it was shown that these signaling pathways are linked to each other in a highly regulated manner, forming a highly complex signaling network that allows quality control and extensive communication between inflammatory cells and hepatocytes.

**Interplay between NFκB and JNK signaling pathway**

The NFκB family consists of five transcription factors, p50, p52, p65, cRel and RelB, which share an N-terminal Rel-homology DNA binding and dimerization domain (4). NFκB homo and heterodimers are sequestered in the cytoplasm via non-covalent interactions with IκB proteins (Figure 1, red symbols and arrows). Upon stimulation, IκB is phosphorylated by the IκB Kinase (IKK) complex, which consists of the IKKα and IKKβ catalytic and the IKKγ/NEMO regulatory subunit (4, 5). Phosphorylation of IκBs results in their K48-linked ubiquitination and subsequent degradation by the 26S proteasome complex (6). The free NFκB dimmers can then translocate to the nucleus and activate the transcription of genes encoding cytokines, chemokines and anti-apoptotic factors that promote cell growth and survival (7). NFκB is also a potent inducer of the caspase 8 homologue FLICE-interacting protein (cFLIP) a repressor of death receptor-induced apoptosis (Figure 1, red arrows) (8).

The NFκB pathway is activated by various stimuli including LPS, and anti-inflammatory cytokines, like tumor necrosis factor (TNFα) and interleukin-1 (IL-1), which elicit their effects through binding to Toll-like receptors (TLRs) and to the TNF or IL-1 receptors
(TNFR-1 or IL1R), respectively (4, 9). These cytokines are produced by inflammatory cells, which accumulate in the liver upon virus infection-induced hepatitis or through the action of other causative agents of inflammation. Upon stimulation by the corresponding ligands, rapid assembly of complexes containing TRADD, TRAF and RIP1 proteins occur at the TLR/IL-R or TNFR, which recruit and activate TAK1 through TRAF6 or TRAF2, respectively. TAK1 subsequently phosphorylate IKKβ and MKK4/7, which in turn activate NFκB and JNK, respectively (10-12) (Figure 1). The assembly step of activated receptor complexes involves TRAF2 and cIAP1/2-mediated K63-linked ubiquitination of several of the components, including TRAF2, TRAF6, TAK1, RIP1 and NEMO, which facilitates protein-protein interactions and the assembly of the signalling complexes (10-14). The main enzyme that removes poly-ubiquitin chains from the above proteins is the cylindromatosis tumor suppressor Cyld (15-20). As a result of Cyld-mediated deubiquitination of TAK1 and other components of the complex, NFκB signalling is inhibited. Importantly, the Cyld gene is transcriptionally activated by NFκB, which provides a negative feedback regulatory loop that could function in balancing activated NFκB levels (21) (Figure 1).

While NFκB activation has pro-survival, anti-apoptotic effects, JNK signalling (Figure 1, green symbols and arrows) has been implicated in the induction of either cell proliferation or apoptosis (22-24). JNK can phosphorylate various substrates, including c-Jun, JunB, JunD ATF2, p53, Bcl2, Bcl-xL, Bid, Bad and Bax proteins, which regulate cell growth and death (25). One mechanism by which JNK contributes to cell survival involves JunD phosphorylation, which transcriptionally activates anti-apoptotic genes (Figure 1). For example, the potent apoptosis repressor gene cIAP2 contains a composite promoter with tandem AP1 and NFκB binding sites, through which JunD/Fos and NFκB dimers cooperate and activate transcription in a synergistic manner (26). This generates a positive feedback regulatory circuit: NFκB– and JNK-activated JunD induces cIAP expression, which promotes
K63-linked polyubiquitination of upstream signaling molecules leading to TAK1 activation. TAK1 in turn phosphorylate IKKβ and MKK4/7 to activate NFκB and JNK (Figure 1).

While the initial TNFR1-mediated JNK activation is transient and promotes cell survival and proliferation, the opposite effect is seen when JNK activation is sustained for a prolonged period time. Chronic JNK activation induces a Bax/Bak-dependent apoptotic pathway, via mitochondrial release of cytochrome-c, which in turn activates Apaf1 and various caspases (Figure 1) (27, 28). The pro-apoptotic effect of JNK is also exerted through transcriptional activation of apoptosis inducing genes like TNFα, Fas-L or Bak, or by phosphorylation of the tumor suppressor p53 and via phosphorylation of the E3 ubiquitin ligase Itch homologue (29-32). Itch facilitates cell death by promoting the degradation of the NFκB-induced antiapoptotic caspase 8-inhibitor cFLIP (32).

In most conditions there is an inverse correlation between the activation of the NFκB and JNK signaling axis, which is at odds with the findings that both pathways are induced by the same upstream kinase TAK1. The negative cross-regulation is mainly achieved by NFκB-dependent transcriptional activation of the Gadd45b gene, which represses MKK4/7-mediated phosphorylation of JNK1/2 (Figure 1) (33, 34). In conditions when reactive oxygen species (ROS) accumulate, the opposite regulation of the two pathways is elicited by a complex negative feedback circuit, which involves NFκB-mediated induction of the ROS-metabolizing enzyme, superoxide dismutase 2 (SOD2) (35). This regulatory axis prevents ROS accumulation, which otherwise causes chronic JNK activation via inhibiting JNK phosphatases (Figure 1) (36).

**Signal transduction pathways establish communication between different cell types during hepatocarcinogenesis**

The importance of NFκB and JNK signalling in the development of inflammation-associated hepatocellular carcinoma has been demonstrated by recent studies using relevant research.
animal models. NFκB seems to have a pro-tumorigenic effect in Mdr2-/- mice, which exhibit low-grade chronic inflammation and spontaneous development of HCC (37). Inhibition of the NFκB pathway by the expression of non-degradable IκB mutant prevented HCC formation and increased apoptosis of premalignant hepatocytes (38). Similar protective effects were observed after the administration of anti-inflammatory or anti-TNF drugs to Mdr2-deficient mice. Transgenic mice expressing lymphotoxins α and β (Ltαβ) in the liver display inflammation, fibrosis and develop HCC (39). In this model the occurrence of inflammation and HCC depends on IKKβ expression in hepatocytes, but is independent of TNFR1 function (39).

In sharp contrast to the above pro-tumorigenic effects, NFκB signalling exhibits a tumor suppressor function in situations when liver inflammation is mainly driven by hepatocyte damage. For example, mice lacking IKKβ in hepatocytes exhibit a marked increase in hepatocarcinogenesis after diethylnitrosamine (DEN) treatment (40-42). In this experimental condition, hepatocyte-specific IKKβ depletion enhanced ROS production, induced JNK activation and hepatocyte death, which augmented compensatory proliferation of surviving hepatocytes. Hepatocyte death-mediated accumulation of inflammatory cells, including the activation of resident macrophages (Kupffer cells), was necessary for the carcinogenesis process (40). This was revealed by the observation of decreased hepatocarcinogenesis in mice where IKKβ was deleted in both hepatocytes and Kupffer cells (40). Since Kupffer cell activation could not take place in the absence of NFκB, the above results suggest that the NFκB pathway coordinates the inflammatory crosstalk between hepatocytes and Kupffer cells (Figure 2). Hepatocyte specific inactivation of NEMO (IKKγ) or TAK1, the upstream activators of NFκB, resulted in spontaneous hepatocyte death, liver inflammation, fibrosis and the development of HCC (43-45). Interestingly, constitutive hyperactivation of TAK1 in Cyld-deficient hepatocytes displayed similar effects (46).
together, the above studies reinforced the established view that NFκB signalling plays a central role in linking chronic inflammation to tumorigenesis.

A common mechanistic feature observed in all of the above models is the chronic activation of JNK, which triggers hepatocyte death. JNK activation in the NFκB-deficient models is elicited by decreased Gadd45b-mediated inhibition of MKK4/7 and by ROS-mediated inactivation of JNK phosphatases (Figure 1) (33, 34, 36). In the case of Cyld-deficiency, JNK activation is mediated by the constitutively active TAK1 (46). In all cases, prolonged activation of JNK induces hepatocyte death, which facilitates the activation of Kupffer cells and other inflammatory cells (Figure 2). Upon activation, Kupffer cells produce various cytokines including Tgfβ, TNFα and IL-6 (47). In response to Tgfβ, hepatic stellate cells proliferate and transdifferentiate to myofibroblasts, producing a network of extracellular matrix, the hallmark of the fibrotic scar (48). Elevated local levels of TNFα cause activation of death receptor signalling in neighbouring hepatocytes, which initiate a vicious cycle of intercellular signalling between hepatocytes and Kupffer cells leading to the amplification of hepatocyte death (46) (Figure 2). Hepatocytes that escape TNF-mediated death may respond to IL6 and related cytokines via activation of Stat3 signaling (42). Binding of IL-6 to its receptor displaces Janus kinases (JAKs), which phosphorylate Stat3 (Figure 2, Blue symbols). Phospho-Stat3 translocates to the nucleus and activates numerous oncogenic genes, resulting in enhanced hepatocyte proliferation (Figure 2) (42, 49). In human HCC samples there is an inverse correlation between NFκB and Stat3 signaling (42, 50, 51). The underlining mechanism involves feedback inhibition of STAT3 activation via tyrosine phosphatases, like SHP1/2 and SOCS3. In this pathway elevated ROS levels generated by NFκB inhibition oxidize SHP1/2. Oxidized SHP1/2 loose their enzymatic activity towards JAK2 substrate, which leads to constitutive activation of the JAK-STAT3 pathway (52) (Figure 2).
Collectively the above findings establish the view of a complex interplay between different signalling pathways that regulate distinct phases during the pathogenesis of inflammation-associated hepatocellular carcinoma. This “interpathway crosstalk” is accomplished through various feed-forward, feedback and autoregulatory loops that operate not only within individual cells, but also between inflammatory cells and hepatocytes.

Clinical-Translational Advances

Due to the impaired liver function of HCC patients, classical anti-cancer chemotherapeutics are toxic and ineffective. The recently introduced Sorfenib is a tyrosine kinase inhibitor, targeting multiple molecular pathways. Although its superior efficacy over conventional chemotherapy has been established by two large-scale clinical trials, its overall value is considered low, as it improves median life expectancy by only 3 months over placebo (53, 54). While combination of sorfenib with cytotoxic (e.g. Doxorubicin) or anti-angiogenic (eg. VEGF inhibitors) agents is being evaluated, to date there is no evidence for the potential of the currently used therapeutics to shrink cancerous lesions or to prevent cancer formation. This gives emphasis to the urgent demand for alternative approaches.

The main translational benefit of studies that established an unambiguous connection between chronic inflammation and HCC is the recognition of an increased repertoire of promising new targets for the development of effective systemic therapies of HCC. An important preventive approach is the treatment with antiviral drugs against HBV and HCV, which would eliminate the main ground on which HCC develops. Unfortunately, despite extensive efforts using antiviral therapies, it is currently not possible to cure chronic viral hepatitis. The use of other anti-inflammatory drugs (e.g. non-steroidal compounds, like aspirin) has proven effective in other cancer types, but has not been evaluated in HCC.
Targeting the NFκB pathway emerges as an alternative concept for curing HCC, given its central position in the regulation of inflammatory processes. Several observations in different mouse models that can be recapitulated in human HCCs, point to the feasibility of pharmacological targeting of NFκB and NFκB-linked signalling pathways. In the majority of human subjects, where HCC was preceded by chronic HBV or HCV-mediated inflammation, the mechanism of disease progression resembles that described in Mdr2 KO mice or Ltcα/β transgenic mice, where NFκB function is absolutely required (37-39). In these cases blocking the NFκB pathway (e.g. by IKKβ inhibitors), may have beneficial effects. In the cases where HCC develops on the ground of hepatocyte damage (e.g. in alcoholic or non-alcoholic steatohepatitis or after chronic toxic assaults), the situation is more complex. Interpreting the data from the animal models where HCC is initiated by hepatocyte death raise arguments against the feasibility of using IKKβ inhibitors for treatment, because inactivation of different components in the pathway (IKKβ, NEMO or TAK1) actually promote carcinogenesis (see above). Importantly however, HCC development is halted when IKKβ is simultaneously ablated in hepatocytes and Kupffer cells, a situation that is more likely mimicked by systemic inhibitor treatment (40). On this conceptual basis, IKKβ inhibitors can be considered good candidates for HCC treatment and definitely warrant additional studies. In this regard we note that the extent of inhibition of the individual components should be taken into account, as they may greatly affect the final outcome. In genetic models the inhibition of the pathway is nearly complete, while in the case of treatment with pharmacological inhibitors the extent of blockage is partial and in most cases adjustable.

Due to the dichotomous nature of their function, other players of the pathway like TAK1 or Cyld cannot be considered promising targets. Either inactivation or hyperactivation of TAK1 leads to hepatocyte death, inflammation, fibrosis and HCC development, suggesting that balanced levels of TAK1 activity are necessary for the maintenance of physiological liver
homeostasis (46). Additional studies on its ubiquitination-mediated regulation would be important to learn more about this enzyme, since, in addition to NFκB, it can also activate the JNK pathway, which seems to represent a highly promising target for anticancer therapy.

The role of sustained JNK activation in hepatocyte death and subsequent inflammation and carcinogenesis is recapitulated in most of the mouse genetic models (IKKβ-KO, NEMO-KO, TAK1-KO and Cyld-KO) developing HCC (40-46). In addition, mice expressing hepatitis C virus core protein activate JNK through ROS production (55). Importantly, JNK1 is phosphorylated in human HCC samples (56). Direct evidence for the idea that JNK could be a promising drug target was provided by the findings that administration of the JNK inhibitor SP600125 to Cyld-deficient mice or DEN-treated rats blocked the development of HCC (46, 57). SP600125 has also been shown to sensitize tumor cells, but not normal hepatocytes to TRAIL, a major mediator of acquired immune tumour surveillance (58). Thus, JNK inhibitors that also sensitize TRAIL could be used in combination with TRAIL-targeting drugs to increase therapeutic efficiency.

The other potential target presented in this review is STAT3. In mice, prevention of STAT3 activation via inhibition of its upstream kinase JAK2 was found to be effective in blocking HCC development (42). STAT3 was detected in its activated form in more than 60% of human HCC samples and phosphorylated STAT3 levels correlated with the aggressiveness of the tumours (42). Importantly, STAT3 deletion does not affect the survival of differentiated cells, but efficiently blocks cell proliferation, suggesting that STAT3 may be a safe target for cancer therapeutics.

Taken together, studies on mouse models have revealed a complex crosstalk between NFκB, JNK and STAT3 signaling pathways in inflammation-associated HCC. These studies have provided novel insights into the temporal order of regulated steps during the pathogenesis, which raise the possibility of developing novel means to block the sequence of
events that lead to HCC. Successful translation of the knowledge gained on NFκB, JNK and STAT3 signaling will depend on appropriate human studies that would motivate the development of safer and more effective cancer therapies.

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Figure legends

Figure 1. Regulation of cell death and proliferation by the NFκB and JNK pathways.

Complexes assembled on ligand-bound TNF or TLR and IL-1 receptors mediate TAK1 activation, whose activity is modulated by Cyld. TAK1 phosphorylates IKKβ, which in turn phosphorylate IκB, leading to its ubiquitin-proteosome mediated degradation and release of NFκB (p50-p65) factors which translocate to the nucleus to activate the transcription of pro-
survival genes. NFκB also induces the expression of cFLIP and SOD2 that inhibit apoptosis and ROS accumulation. TAK1 also phosphorylates MKK4/7, which activate JNK1/2. Short-term JNK activation induces the expression of genes involved in proliferation control, via the phosphorylation-mediated activation of JUN transcription factors. Sustained JNK activation induces the expression of pro-apoptotic genes, stabilizes p53 and the Bax/Bak-dependent apoptotic pathway. NFκB feedback regulates its own activation via modulating Cyld expression and the activation of JNK via activating Gadd45b expression, which inhibits MKK4/7. JNK activation is also modulated by ROS accumulation through DUSP1 phosphatase activity.

TNF, tumor necrosis factor; IL-1, interleukin; TRAF6, tumor necrosis factor receptor-associated factor 6; cIAP1/2, cellular inhibitor of apoptosis protein 1/2; CASP3/8, caspase3/8; RIP1, Receptor-Interacting Protein 1; TRADD, TNF receptor-associated death domain; TAK1, TGF-beta-activated kinase 1; MKK4/7, MAP kinase kinase 4/7; Gadd45, growth-arrest DNA damage-45 protein; IKK, Ikappa B kinase; TLR, Toll-like receptors; SOD2, Superoxide Dismutase 2; DUSP1, dual-specificity phosphatase 1; cFlip, Cellular FLICE-Like Inhibitory Protein; ROS, Reactive Oxygen Species; Cyld, cylindromatosis deubiquitinase; NEMO, NFκB essential modulator; IκB, inhibitor of κB; JNK1/2, c-jun N-terminal kinase; cIAP1/2, cellular inhibitor of apoptosis 1/2.

**Figure 2.** Mechanism of cell death-mediated inflammation, fibrosis and carcinogenesis.

Sustained JNK activation causes hepatocyte death. Dying hepatocytes release alarmins/damage associated molecular patterns (DAMPs), which leads to the recruitment of Kupffer cells. NFκB pathway activation in Kupffer cells leads to the expression and secretion of Tgfβ, TNFα and IL-6 cytokines. Tgfβ activates hepatic stellate cells resulting in fibrogenesis. TNFα induces apoptosis in the neighboring hepatocytes. Hepatocytes, which escape or do not complete cell death may respond to IL-6 via activation of STAT3, which
induces compensatory hepatocyte proliferation. IL-6R, IL6 receptor; JAK, Janus kinase; STAT3, signal transducer and activator of transcription 3; SHP1, src homology-containing phosphatase 1; SOCS3, suppressor of cytokine signaling 3.
Figure 2:

**Hepatocyte**
- TNFα
  - TNFR1
  - JNK1/2
  - Apoptosis
  - Alarmins, DAMPs
- Kupffer cell
  - NF-κB
  - Inflammatory cytokines
  - Proliferation
  - Inflammation

**Cell death amplification**
- JNK1/2
  - TNFR1
  - Apoptosis
  - TNFα
  - IL6

**Hepatocellular carcinoma**
- Proliferation survival
  - IKKβ
  - STAT3
  - SOCS3
  - SHP1
  - ROS
  - TNFα-A
  - IL6-R
  - IL6

**Liver fibrosis**
- Hepatic stellate cell
  - TGFβ
  - Collagen
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