Mini-review

Contradictory functions of NF-κB in liver physiology and cancer

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Received 7 March 2008; received in revised form 7 March 2008; accepted 13 March 2008

Abstract

Rudolf Virchow (1821–1902), one of the founding fathers of modern pathology, hypothesized that cancer and inflammatory processes are linked, due to the presence of leukocytes in the tumor tissue. Today, chronic inflammation is believed to be one of the major causes for cancer development, accounting for nearly 20% of cancer cases worldwide. Hepatocellular carcinoma (HCC) is the third leading cause of cancer mortality throughout the world, and its incidence is increasing in the United States. HCC is widely accepted to be the outcome of continuous injury and chronic inflammation, and thus provides a good model to gain insight into inflammatory related cancer processes.

Nuclear Factor- kappa B (NF-κB) was first identified as an enhancer protein of the kappa light-chain gene in B lymphocytes. Later it was realized that there are five NF-κB transcription factors with important roles in inflammation, innate immunity, cancer and apoptosis aborting. Consequently, NF-κB was shown to link inflammation and cancer, but recent reports have revealed it to play a much more complex role, where in some disease processes it promotes cancer and in others it impedes carcinogenesis. In this review, we will focus on the seemingly contradictory role of NF-κB in liver homeostasis, as well as in liver cancer.

Keywords: Cancer; Inflammation; NF-κB; Liver; Hepatocellular carcinoma; HCC

1. The NF-κB signaling pathway in liver embryogenesis and adult physiology

It is now common knowledge that at least one of the NF-κB factors is found in essentially every vertebrate cell type. As a result of their distinctive regulation, the NF-κBs are unique in their ability to rapidly re-program an exceptionally large number of genes in stressful situations [1–3]. The ability of the NF-κB pathway to react to several stimuli, the presence of canonical and non-canonical activation and the NF-κB members’ ability to form homo- and heterodimers can explain its complex and sometimes seemingly conflicting effect in different biological systems and pleiotropic phenotypic traits in which it is involved [4–7].

To date, there are five known members of the NF-κB/Rel protein family: p65 (RelA), c-Rel, RelB and p50/p105 (NF-κBα), p52/100 (NF-κBβ) [8]. The last two proteins, p105 and p100, are synthesized as precursor-proteins which are processed by the proteasome to p50 and p52, respectively. All NF-κBs...
share the Rel-homology domain (RHD). The RHD contains a nuclear localization signal, and mediates the dimerization as well as the sequence-specific DNA binding and the interaction with the NF-κB inhibitors, the IκBs [1]. Normally, dimers of RelA/p65 and c-Rel/p55 are held in the cytoplasm by the inhibitors of NF-κB (IκBs). Conditions such as infection and inflammation induce the IκB kinases (IKKs), a dedicated kinase complex, which phosphorylates the IκBs’ N-terminal serine residues. This phosphorylation subsequently subjects the IκBs to ubiquitination and degradation by the 26S proteasome, consequently resulting in the nuclear translocation of NF-κB and activation of its target genes [9]. The classic IKK is composed of three protein units named IKKα, IKKβ and IKKγ/NEMO. The first two are the catalytic subunits, while the last is an essential regulator. The IKKβ and IKKγ/NEMO are considered necessary for the canonical (classic) NF-κB pathway, while IKKα is also involved in the non-canonical pathway. Although, the canonical and non-canonical NF-κB pathways lead to the activation of NF-κB, the differences in the involved IKK subsequently results in different NF-κB dimers involvement, which eventually induce a different subset of genes [10,11].

Upon activation, the NF-κB dimer accumulates in the nucleus and is modified post-transcriptionally by phosphorylation and acetylation, and, as a consequence, controls the extent of the response [12,13]. Numerous promoter enhancers harbor the 10 bp κB binding motif, 5′-GGGPuNNPypyc̅CC-3′, where Pu is purine, Py is pyrimidine and N is any nucleotide [14,15], giving further support to the extensive re-programming of which NF-κB is capable.

Several lines of evidence prove that NF-κB is a potent anti-apoptotic agent. One of the fundamental indications is the massive apoptosis of liver cells in-vivo when genetically deprived of their NF-κB signaling pathway, as will be subsequently explained in detail. In addition, the NF-κB signaling pathway is necessary for the normal embryogenesis of the skin and for the long-term survival of some normal hematopoietic cells. Much work has been done to reveal the precise mechanism behind NF-κB’s anti-apoptotic activity: NF-κB blocks the apoptotic process, extrinsic or intrinsic, at many points along its way, through the many of the genes that it regulates, as reviewed by Lin and Karin [16].

NF-κB activation is strongly associated with cancer. It was found abnormally activated in Hodgkin’s and non-Hodgkin’s lymphomas [17], breast carcinoma [18], HCC [19] and others. NF-κB anti-apoptotic activity and aberrant activation in many tumors marked it as a therapeutic target for cancer research [16,20,21]. Additionally, it has been shown that NF-κB is an important player in preventing apoptotic death after DNA-damaging treatments, such as chemotherapy and irradiation, clearly affecting the efficacy of these treatments [2,22]. More evidence linking NF-κB to cancer came through its activation by many oncogenes, the dependence of transformed cells on it and the oncogenicity of the viral Rel protein (v-Rel) [23]. Recently, studies in mouse models provided direct genetic evidence that the NF-κB pathway is required for inflammation-induced carcinogenesis, which emphasizes even further that NF-κB is an important factor in carcinogenesis [24,25]. NF-κB has a pivotal role in the normal liver biology and pathophysiology as has been clearly illustrated with several constitutive knockout (KO) mice. Knocking out p65 (p65−/− mice) results in mid-gestation in-utero death due to massive liver apoptosis [26]. This massive liver apoptosis is induced by tumor necrosis factor (TNF) as backcrossing the p65 KO mice with TNF or TNF receptor deficient mice reverses the lethal phenotype [26–28]. As IKKβ−/− mice [29,30] and IKKγ−/−/C0 mice [31,32] also died in-utero because of massive liver apoptosis, the NF-κB pathway is considered a major anti-apoptotic agent in the liver in-vivo. To circumvent the lethality issue and decipher the role of the IKKs in the adult liver, several indirect methods were employed. In one study, adenoviruses harboring dominant negative forms of IKKα or IKKβ were used. Although, this study also confirmed the necessity of IKKβ as a hepatocyte protector from TNF signals, it was criticized because of the adenovirus effects on the liver and the short term non-physiological levels of expression it produced [33]. Several other researchers inhibited the NF-κB pathway by indirect means, using inhibitors or degradation-resistant Iκβ, “super-repressor” Iκβ, and were able to shed some light on the role of NF-κB in the adult liver. In these studies, NF-κB also seemed to play a role in preventing TNF-induced apoptosis [34,35]. Lavon et al. showed that by expressing degradation-resistant IκBα specifically in hepatocytes, the mice were more sensitive to bacterial infection and sepsis [35]. Although the mice showed no signs of liver dysfunction even at the age of 15 months, they were unable to clear certain bacteria from the liver and suc-
cumbed to sepsis, in spite having intact immuno-
cytes and inflammatory cells. Therefore, activation
of hepatocyte NF-κB, an innate immunity modula-
tor, is essential to fight some systemic infections.

The generation of several conditional KO mice
based on the Cre Lox technology was crucial for
facilitating studies of NF-κB function in the liver.
However, no technology is without caveats [36,37].
As emphasized recently, Cre toxicity is a major issue
of this powerful genetic method. In a recent article
from the Rajewsky laboratory, several pitfalls of
this method are emphasized [37]. They pointed out
that cryptic loxP sites are present in the mammalian
genome. It has been suggested that cryptic loxP sites
can be found every 1.2 million bases in the mouse
genome, although Cre recognizes them in lower
affinity [38,39]. This effect should be especially
emphasized in mouse cancer models as it is likely
that nicking these sites will induce a DNA damage
response in the cells. Another toxic effect of the
Cre occurs when a large amount of the protein is
expressed, and the Cre protein overload may induce
growth arrest [40]. Even a well-designed study of a
lymphoma using a CreERT2, a chimeric protein of
Cre, and tamoxifen high affinity estrogen recep-
tor, showed significant toxicity when tamoxifen
was administered [37]. With all the evidence of Cre
recombinase toxicity to mammalian cells, the
authors revealed their surprise at the fact that most
Cre-transgenic mice in the literature are described as
indistinguishable from the wild-type mice. They
postulate that it is likely that this is not the case,
and that the Cre-expressing strains are not com-
pletely normal, but overcome the toxic effects of
the Cre protein by adaptation and selection pro-
cesses. They advocate including Cre-expressing mice
as an essential control, and a highly significant pub-
lisher issued a special toxicity alert [41]. Further
proof for Cre toxicity comes from the fact that
RIP-Cre mice which express Cre in the pancreatic
β-cells are glucose intolerant [42]. Most of the arti-
cles mentioned hereafter are based upon the Cre-
lox system. Cre-based KO of specific genes in the
NF-κB pathway has aided investigators in elaborat-
ing the role of the pathway in the liver. However,
since KF-κB pathway is very fundamental to the
liver homeostasis, obliterating it may render the
hepatocytes more sensitive to different assaults,
including to Cre toxic effects. Nevertheless, it is
notable that several of the articles, described in
detail later, did not carry out a Cre expressing mice
control.

2. NF-κB’s paradoxical roles in the adult liver

The Cre-lox technique has already been imple-
mented in several mouse models especially designed
to point out the role of NF-κB in the adult liver.
The first of these conditional KOs was the IKKβ
KO in hepatocytes. Maeda and colleagues [43] pro-
duced IKKβfl/flAlb-Cre (IKKβAhep) mice, and stud-
iied TNF’s effects on them. These mice showed a
very low NF-κB activity after insults, suggesting a
quite efficient block of NF-κB signaling. On the
other hand, they found that the IKKβAhep hepato-
cytes were not so sensitive to a challenge with
LPS, a potent inducer of soluble TNF, in-vivo,
but showed massive liver injury upon challenge with
ConcavalinA (ConA), a potent inducer of cell-
bound TNF. Furthermore, cultured primary hep-
atoctyes derived from the IKKβAhep mice showed sig-
ificant apoptotic resistance to soluble TNF, but
were sensitive to the non-soluble membrane bound
TNF fraction. They concluded that the IKKβ defi-
ciency is not sufficient to induce massive liver apop-
tosis in the presence of a high soluble TNF level,
but is sufficient in the presence of non-soluble TNF.
Within a short time a contradictory report about
the role of IKKβ was published [44]. Luedde and
colleagues generated a different exon spanning
IKKβfl/fl mouse and bred it with Alfp-Cre, generat-
ing another type of IKKβAhep mouse. Surprisingly,
this mouse’s NF-κB response to TNF was almost
normal as judged by the NF-κB activity. This
response can probably explain the fact that these
mice were not sensitive to mTNF, LPS and ConA
challenges. In addition, by using AS602868, a novel
IKKβ inhibitor, they observed matching phenotypic
presentation upon identical insults, confirming their
finding. One possible explanation is based on the
fact that these mice have different flox sites. As a
result, a truncated protein or its degradation prod-
ucts can exert some influence on the cell, as is
known in the case of IKKβ [45]. Comparing these
two strains in one lab, and by so doing, eliminating
the effects of different animal facilities and protocol
variations might help resolve this paradox.

Another unexpected observation in the NF-κB
field, involves identical mice in two different labs
[46,47]. These two reports studied the liver response
to deletion of IKKγ/NEMO, another key compo-
nent of the IKK complex, using IKKγfl/flAlfp-Cre
(IKKγAhep) mice. The first of these studies, [46],
showed that by the age of 12 months IKKγAhep mice
develop multiple liver tumors. Further investigation
showed that as early as 8 weeks of age, IKKγAhep mice had elevated alanine aminotransferase (ALT) levels, apoptotic cells and oval cell activation, and the livers already showed signs of steatohepatitis, characterized by immune cell infiltration and steatosis. There were characteristic features of dysplasia with strong anisokaryosis in hepatocytes as well as fibrosis, resembling the consequences of chronic inflammation and non-alcoholic steatohepatitis (NASH) in humans [48]. Notably, even at 3 weeks of age, IKKγAhep mice displayed a considerable elevation of ALT in the serum, indicating the presence of hepatocyte damage, with already apparent increased spontaneous apoptosis of hepatocytes. The authors claim that the basis for the development of liver cancer in IKKγAhep mice is the chronic spontaneous hepatocyte death that forces a regenerative response with compensatory hepatocyte and hepatic progenitor proliferation. They conclude that IKKγ/NEMO is a liver tumor suppressor, a questionable definition as it is clear that NEMO’s role in this model is to suppress steatohepatitis and not to suppress cancer. The second article, using the same IKKγAhep mice, probed the role of IKKγ in acute liver damage [47]. IKKγ deficiency sensitized hepatocytes to TNF-induced apoptosis, but none of the mice died as a result of it, indicating that massive apoptosis did not occur as one would expect in fully blocked NF-κB hepatocytes. Curiously, while Beraza et al. stated that liver steatosis typically started at the age of 2–3 months, Luedde et al., reported that already at the age of 8 weeks mice showed very high liver enzymes levels, steatosis, liver fibrosis, regenerative proliferation, oval cells activation and ultra-structural changes. How can one reconcile these conflicting reports? As shown before, mice lacking an intact NF-κB pathway are more prone to infections [35]. Therefore, liver deletion of NF-κB components makes the mice more susceptible to liver infections. It is possible that environmental conditions at the different animal facilities used by the various researchers could explain the temporal differences, as it is likely that two animal facilities will have different flora. The fact that immunocytes infiltrating the liver can be seen as early as 3 weeks of age in only one animal facility is in agreement with the possibility that the steatohepatitis is in response to a specific pathogen to which the innate-immune-compromised mice are susceptible. It should be noted that if this is the case, then the conclusion that NEMO, and in turn NF-κB itself is a tumor suppressor is questionable. The provocative role of NF-κB as a tumor suppressor is neither supported by another recent article describing the effects of conditional RelA/p65 [49]. As in many cells, the heterodimer p65/p50 is predominant in hepatocytes. Thus, knocking out p65 is a very potent and definite way to completely block the NF-κB signaling pathway. The authors used two types of inducible systems to KO p65. The first, which is based on p65fl/flAlb-Cre mice, is hepatocyte specific. The second system based upon the interferonα1b dependent p65fl/flMxCre, is inducible by poly(I)–poly(C) administration, and showed effective recombination in Kupffer, non-parenchymal and lymphoid cells as well [50]. Both p65fl/flAlb-Cre and p65fl/flMxCre mice developed normally, showing that p65 is not essential in postnatal development and normal liver homeostasis. As anticipated, their hepatocytes were highly sensitive to TNF-induced rapid apoptosis in vitro and vivo. The fact that all the animals died shortly after TNF injection, as opposed to the situation with the IKKγ KO mice, may suggest an even greater inhibition of the NF-κB pathway in the p65 KO mice. Yet, p65 KO mice that were at least 6 months old showed no gross anatomy, histologic organization, or liver function differences, contradicting the previous reports about the IKKγ KO mice phenotype.

### 3. NF-κB’s paradoxical roles in liver carcinogenesis

Chronic inflammation is a major cause for cancer development [51–53]. Hepatocellular carcinoma (HCC), a prominent example for inflammation associated cancer, is a leading cause of cancer mortality, and its incidence is increasing in the United States [54]. In most cases HCC in humans is the outcome of continuous injury and chronic inflammation [55], thus it provides a good and realistic inflammatory related cancer model to gain insight about the role of NF-κB in the carcinogenesis.

In line with the known anti-apoptotic role of NF-κB in many cell types, the first two reports that studied the role of NF-κB in mouse carcinogenesis in vivo showed that NF-κB promotes inflammation-induced cancer, thus validating the notion that NF-κB inhibitors may be used for cancer prevention in chronic inflammatory states [24,25]. Pikarsky and colleagues hypothesized that NF-κB could constitute the missing link between inflammation and cancer [25]. To test this hypothesis, they studied the Mdr2-knockout (KO) mouse, a mouse model of chronic inflammation-induced HCC [56]. Due to
the defect in secreting phospholipids into the bile, these mice spontaneously develop cholestatic hepatitis, and by one year of age the vast majority of the Mdr2-KO mice develop HCC [57]. By monitoring the hepatitis and cancer progression in Mdr2-KO mice, they showed that the biliary-originated inflammation triggers hepatocyte NF-κB activation, which is suppressible both by anti-inflammatory and anti-TNF treatments. Moreover, they were able to investigate the direct role of NF-κB in the HCC carcinogenesis process by inhibiting its activity with an inducible IκB super-repressor (Ind-IκB-SR) using the Mdr2 Δ/ΔInd-IκB-SR hep mouse. Their results showed that NF-κB is not required for early neoplastic events, but is needed for tumor progression. Greten and colleagues applied a chemical carcinogen to mice colon, followed by an injurious substance to produce chronic colitis [24]. They found that mice that had had colonic IKKβ elimination, showed a marked decrease in tumor incidence without serious effect on the inflammation process per se. By contrast, two recently published studies showed that NF-κB inactivation in the liver caused an increase of HCC incidence [46,58]. Maeda and colleagues investigated the effect of inhibiting NF-κB in the liver, by knocking out IKKβ specifically in the hepatocytes (using the strain that showed considerable NF-κB inhibition, IKKβ fl/flAlb-Cre, IKKβΔhep), in the course of an acute liver injury due to diethylnitrosamine (DEN), a potent liver carcinogen. Notably, the DEN model mice do not develop chronic inflammation, as opposed to the MDR2 Δ/Δ model. Surprisingly, IKKβΔhep mice exhibited a marked increase in hepatocarcinogenesis caused by DEN. This correlated with enhanced reactive oxygen species production, and hepatocyte death, giving rise to augmented compensatory proliferation of surviving hepatocytes. Obviously, it is necessary to understand the basis for this contradiction and its medical implications, i.e., the consequences of NF-κB inhibition in different pathological contexts. We hypothesize that the opposing results have to do with the mechanism of tumorigenesis underlying the different models used. In chronic inflammation models, such as in the Mdr2-KO hepatitis model, NF-κB is activated over prolonged periods of time. Using an inducible super-repressor in the Mdr2-KO mouse, Pikarsky et al. showed that the effect of blocking NF-κB is most important at the promotion phase and not at the initiation phase of the experimental model. By contrast, in the DEN liver carcinogenesis model, NF-κB activation is limited to the acute injury phase following administration of the carcinogen. Blocking NF-κB at this phase results in accelerated apoptosis and compensatory hyperproliferation of mutated, thus cancer-prone hepatocytes [58]. Perhaps, this mode of carcinogenesis would even favor induction of hepatocyte mutations that obviate the need for NF-κB activation for tumor promotion. Thus, the context of NF-κB inhibition would determine the tumorigenesis outcome: if inhibition is inducing inflammation and/or TNFα induction, as in skin, or extensive tissue injury requiring intensive mending, as in carcinoma liver damage, it would facilitate tumorigenesis. If on the other hand, NF-κB inhibition compromises the survival of transformed cells, as in the chronic inflammation models and relevant human diseases predisposing to cancer, it will abolish or slow tumorigenesis. While the temporal pattern of NF-κB activation in the different model may explain the difference between the DEN and Mdr2-KO models, how can we explain the outcome of Luedde et al. where blocking NF-κB per se resulted in hepatocarcinogenesis? Luedde et al have shown that hepatocyte specific IKKγ/Nemo knockout mice develop steatohepatitis which is essential for HCC development, but as discussed earlier, steatohepatitis and HCC did not develop when NF-κB was blocked in the liver by several other methods, including IκB-SR expression, IKKβ deletion or p65 KO, suggesting that other factors are taking part in this process, be it a specific infectious agent or IKKγ/Nemo targets outside the NF-κB pathway, such as the MAPKinase/JNK signaling pathway [59].

4. Concluding remarks

Animal models are essential for gaining understanding of the role of specific pathways and molecules in pathological processes. Yet the complexity of any disease process must be taken into account when trying to draw conclusions from experiments. In the test tube scenario, one can deduce that a specific protein inhibits a process if its elimination results in higher-activity of that process. Yet in an animal model such conclusions are often incorrect. Therefore one should strive not only to inactivate a specific gene but also to explore the results of mild over-activation of the same gene, a method that should receive more attention in view of the emerging role of copy number variation in human physiology and disease. Careful inspection of the
pathogenetic processes taking part in a specific disease is instrumental in deciphering the role of different signaling pathways. Comparison with other animal models that target the same pathway/process using different tools is another critical tool. Only by combining these strategies, will we be able to harness the power of animal models towards understanding human disease and developing better treatments.

Acknowledgments

We thank Ms. Toby Reinhertz for excellent editorial assistance. Work in the laboratories of Eli Pikarsky and Yinon Ben-Neriah is supported by grants from the Israel Science Foundation the Israel Cancer Research Foundation and the Adelson Medical Research Foundation.

References


