Chapter

Molecular and Cellular Aspects of Cirrhosis and How an Adenosine Derivative Could Revert Fibrosis

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Abstract

Hepatic fibrosis occurs in response to persistent liver damage and is characterized by an excessive accumulation of extracellular matrix. When the damage is prolonged, there is a chronic inflammation and persistent hepatic fibrosis eventually leads to cirrhosis, where in addition to the scar, there is an important vascular remodeling associated with portal hypertension and, if decompensated, leads to death or can develop hepatocellular carcinoma. We have been studying the pharmacologic functions of adenosine, finding that a derivative of this nucleoside, IFC-305, shows hepatoprotective effects in a CCl_4-induced rat cirrhosis model where it reverses liver fibrosis through modulation of fibrosis-related genes and by ameliorating hepatic function. Furthermore, this compound has the property to rescue cell cycle inhibition *in vivo*, prevents hepatic stellate cell activation, modulates anti-inflammatory macrophage polarization, and favors a chromatin context that could decrease the genomic instability and characteristics of cirrhosis, enabling the recovery of gene expression profile. Here we show results that contribute to the comprehension of molecular and cellular mechanism of cirrhosis, give the opportunity to suggest biomarkers to the early diagnostic of this pathology, and constitute the fundaments to suggest IFC-305 as a coadjuvant for treatment of this disease.

Keywords: liver fibrosis reversion, adenosine, Kupffer cells, hepatic stellate cells, cell cycle, epigenetics

1. Introduction

Cirrhosis is the 14th dead cause worldwide in adults [1]; this pathology represents an hepatocellular alteration, which is defined histologically by a vascular remodeling that triggers formation of fibrotic interconnected septum that wraps the entire liver tissue and divides the parenchyma in nodules [2]; as a consequence, there is a reduction in hepatocellular mass as well as liver function and blood flow alterations. These pathologic characteristics are independent of disease etiology, which mainly could be alcoholic, biliary, and directed by viral or chronic hepatitis [3].

In the past, cirrhosis was considered as an irreversible illness, but there is evidence, which suggests that when there is an arrest of the viral or nonviral mayor cirrhotic-generator insult, it is possible to resolve fibrosis [4–9]. This is evident in successfully chronic hepatitis C treatment, in fibrosis resolution in hemochromatosis patients with effective treatment, and also in alcoholic liver illness patient who has suspended alcohol consumption [10]. A group of histopathological injuries of "reverted cirrhosis" has been described in the "hepatic reparatory complex" [9] including a thin, incomplete and perforated septum, through which hepatocytes are evident; there is a hepatocyte growing in terminal hepatic venules and little cumulus of thick collagen fibers in the parenchymal sinusoids [2]. Nevertheless, this disease could be considered as a pre-neoplastic state considering that 80% of hepatocellular carcinoma originated from cirrhosis [11].

This pathology is a silence one; generally it courses without symptoms, whereby its development confers a significant morbidity and mortality risk, and progression to this terminal state of chronic liver injury is slow, around 20–40 years [12]. Thanks to advances in the understanding of chromatin organization and rapid progress in sequencing technology, it has been clear that not only genetics but also epigenetics influence both normal human biology and diseases [13]; the combinatorial of these both factors could influence on speed of disease development, generating changes in chromatin, an inflammatory process and hepatic stellate cell (HSC) activation, triggering a pro-fibrotic environment and if it is perpetuated, the cirrhosis establishment. In this section, we will get deep into molecular and cellular aspects of cirrhosis and how an adenosine derivative could reverse this pathology through generating an anti-inflammatory environment and blocking HSC activation, modulating cell cycle and mediating epigenetic changes which reduce altered expressed genes.

2. Biochemical and physiological alteration of liver during cirrhosis and hepatoprotective effects of an adenosine derivative

Cirrhosis has a complex cellular and molecular dynamic that should be approached using laboratory models that could be animal or cell cultures. One of the most studied cirrhotic models employs carbon tetrachloride (CCl_4) to generate the pathology. This compound is a hepatic and renal toxic, whose effect is produced by two different mechanisms: the first one is related with the alteration of hepatocyte capacity to bind triacylglycerides to transporter lipoproteins, triggering an intracellular lipid accumulation and fatty degeneration of the liver; the second mechanism consists in the formation of metabolites extremely toxic, which lead to cell death and centrilobular hepatic necrosis [14]. This toxic compound is a substrate of P450 cytochrome that transforms it into $CCl_3\cdot$ radicals, and these radicals generate $CCl_3OO\cdot$ when it reacts with molecular oxygen. Since $CCl_3\cdot$ radicals react with cell membranes inducing lipid peroxidation, it has been proposed that the main cause of hepatic illness by CCl_4 is the membrane damage by the free radical chain reaction, and probably, the initial event is related with mitochondrion membrane damage [15]. Nevertheless, it is important to say that this model, unlike what happens in the human, progresses to hepatocellular carcinoma with low frequency, so that it permits only the evaluation of cirrhotic state.

Rats are intraperitoneal treated with 0.04 g/kg body weight of CCl_4 three times per week, during 10 weeks. After this time it is possible to observe a liver distortion, the formation of nodules, bilirubin accumulation and hepatomegaly [16], the generation of a fibrotic area of 16% of the tissue, and a reduction in parenchyma surface to around 78%. As a consequence of these liver architecture changes, there are alterations of liver function: serum samples of cirrhotic rats display elevated AST,

ALT, and bilirubin levels and albumin are relatively reduced [17]. Furthermore, there is a reduction in ATP level with slow recovery after 5 weeks with saline. All of these altered parameters reflect chronic hepato-biliary injury.

Since 1967, our laboratory has been studying the pharmacologic effects of adenosine on hepatic metabolism [18], finding that this nucleoside increases hepatocyte energetic charge [19], an effect which is able to increase metabolic fluxes [20]; it increases glycogen synthesis, blocks fatty acid oxidation [21], and maintains cell redox state. Some of the pharmacologic effects of adenosine on hepatotoxicity are: prevention of fatty liver disease [21], recovery of basal energetic state which was reduced by toxic agents [22], maintenance of redox balance between cytosol and mitochondrion [23], prevention of CCl_4-induced necrosis [24], avoiding free radical propagation during CCl_4 metabolism [25], and modulation of the blood flux of hepatic artery [23]. Furthermore, adenosine is able to reduce 50% collagen accumulation in a cirrhosis prevention model, thanks to the increase of liver collagenolytic activity hand by hand with an improvement of liver function [26–31]. These findings allowed us to propose adenosine as possible treatment to reverse cirrhosis.

In order to understand the mechanism of action of adenosine, we generated several derivatives and compared their hepatoprotective properties against adenosine, because this nucleoside is subject of an active metabolism within the cell resulting in a short half-life of the nucleoside but with capability of metabolic modulation; it could be phosphorylated by adenosine kinase, deaminated to inosine by adenosine deaminase, or transformed to S-adenosylhomocysteine by S-adenosylhomocysteine hydrolase [32].We found that aspartic salt of this nucleoside, now denominated IFC-305, presents a better protection against lethal dose of CCl_4 using a fourth of the dose than the parental compound; this effect could be understandable because IFC-305 presented a delay in the maximal absorption than adenosine (20 vs. 30 min), but adenosine level rapidly declined to practically undetectable levels between 60 and 120 min, while IFC-305 presented a significant liver concentration even 120 min after its administration; this behavior could be explained by a 20% diminution of the activity of the adenosine deaminase, in the presence of IFC-305, an enzyme responsible to transform adenosine to inosine. These results suggest that IFC-305 clearance is much slower than that of adenosine [33]. With these results, we decided to explore the hepatoprotective properties of this adenosine compound, treating rats (50 mg/kg body weight, three times per week) during 5 weeks after cirrhosis induction with CCl_4.

Cirrhotic rats treated with IFC-305 present a healthy-like liver phenotype compared with cirrhotic rats and also with cirrhotic rats treated with saline during 5 weeks after cirrhosis induction. Besides, decreased fibrosis was evident in response to IFC-305 treatment, accelerating fibrosis resolution, leaving only 4% of fibrotic area, while increasing parenchymal liver area from 87 to 90%, and collagen was decreased to half-level as compared to saline-treated rats. This improvement in liver architecture was in accordance with liver physiological amelioration; IFC-305 reduced significantly bilirubin and serum transaminase activities [17], and ATP levels were equivalent to those of healthy liver [34], corroborating that IFC-305 presents the same hepatoprotective properties reverting cirrhosis than adenosine but with a lower dose.

3. Cell cycle inhibition during cirrhosis and its recovery by IFC-305

Liver has a well-known capability to regenerate after resection [35]; the severity of liver fibrosis is considered to be related with impaired regenerative capacity, suggesting the arrest of cell cycle [36]. The fibrogenesis process is accompanied by

energetic imbalance as well as oxidative damage generated by oxygen species that could result in chromosomal instability, which induces injury in the check points of the cellular cycle triggering an impaired regenerative capacity [34].

Cell cycle molecules play essential roles in hepatocyte proliferation. Specifically, G1-phase related molecules are important because they are a requisite to enter into cell cycle from quiescent state [34]. Considering adenosine is able to increase DNA synthesis as well as the mitotic index and the expression of proliferating cell nuclear antigen (PCNA) in a pre-established cirrhosis [31], over and above accelerating progression of cell cycle during liver regeneration in rats subjected to one-third hepatectomy [37], we have explored cell cycle state during cirrhosis and changes mediated by IFC-305.

During cirrhosis, there is no evident change on PCNA, which is an auxiliary protein of DNA polymerase delta and is an excellent marker of cell proliferation and it is present at the beginning of the S phase; but IFC-305 treatment generates a 10-fold increase of this protein, supporting the effect on proliferation activation mediated by this compound; this result was validated by immunohistochemistry [34]. Regarding cell cycle cyclins, cyclin D1 levels in cirrhotic state was not altered, but treatment with IFC-305 showed a 77% protein increase; this behavior correlates with expression levels of that cyclin. On the other hand, Cyclin B1 did not change in cirrhotic rats, but IFC-305 treatment reduces by 30% the protein level [34]. Cyclin D1 belongs to G1 phase and is fundamental to initiate cell cycle and requires the association with cyclin-dependent kinase 4 or 6 (CDK4/CDK6) to form an active complex and allows the progression of cell cycle to S phase, whereas degradation of cyclin B1 is important for metaphase-anaphase transition and progression of cell cycle [38]. Evaluating the levels of CDK4, we did not find changes in cirrhosis but IFC-305 generates a high increment of this protein. In the case of CDK6, the protein is present in cirrhosis, the cessation of CCl_4 and saline solution administration reduces its levels, meanwhile IFC-305 treatment maintains elevated the presence of CDK6 [33]. These results suggest that both CDK4 and CDK6 could form a complex with Cyclin D1 and favor cell cycle progress in response to IFC-305 treatment. The complex Cyclin D/CDK4/6 is responsible for Rb protein phosphorylation, promoting the release of E2F1, which can induce transcription of several genes involved in cell cycle entry into S phase and induction or inhibition of apoptosis [39]. In livers from cirrhotic rats, there is a reduction of phospho-Rb (Ser 795), and IFC-305 restores the healthy levels; *Rb* gene expression correlates with protein levels. Analysis of E2F1 protein levels reveals a decrease of this protein in cirrhotic livers and administration with saline solution during 5 weeks after cirrhosis inductions partially reestablishes the levels of that protein but IFC-305 generates a higher increment than the one reached with saline; this increment together with Rb gain supports the reactivation of cell cycle, suggesting the entry to S phase of cell cycle [34].

Another level to regulate cell cycle progression is related with its inhibitors; with regard to this, p21 is reduced 40% in cirrhotic animals and IFC-305 is able to reassemble the healthy liver levels; p27, another cell cycle inhibitor, did not show effects that could be related with cirrhosis establishment neither to IFC-305 treatment during 5 weeks [34], so it is possible to suggest that one of the key cell cycle inhibitors in cirrhosis development is p21.

Trying to understand which could be the signal that generates this reactivation of cell cycle, we evaluated hepatocyte growth factor (HGF) levels in serum and liver; HGF originally identified and cloned as a potent mitogen for hepatocytes, is a strong protective and trophic factor for many tissues and organs [40]. Since HGF is produced mainly by mesenchymal cells and c-Met, its specific receptor tyrosine kinase is expressed in most epithelial, endothelial, and somatic stem cells [41]. In cirrhosis, there is a little increment of HGF in serum but in liver, there are no

significant changes; meanwhile, rats treated with IFC-305 trigger a threefold increase of serum HGF versus healthy animals and a 35% increase versus cirrhotic animals; in liver, there is a trend to increase the levels of HGF compared with both healthy and cirrhotic rats. On the other hand, c-Met receptors present a 25% increase in cirrhotic animals with further increase after 5 weeks of progress, but treatment with IFC-305 induced a diminution in relation with cirrhotic rats administered with saline [34]. With these results, we could suggest that IFC-305 is able to increase HGF levels in serum of cirrhotic rats, which could interact with c-Met in liver, being the mitogenic signal which could trigger the reactivation of cell cycle recovery.

4. Inflammation, the beginning of liver disease and a key of IFC-305-mediated cirrhosis resolution

In recent years, it has been demonstrated that the immune response is one of the main mechanisms involved in the progression and repair of liver pathologies [42].

Liver injuries provide a proper model of inflammation and repair, showing a complex interaction of parenchymal, no-parenchymal cells and the extracellular matrix, all of them, components of the mammalian wound-healing response. In almost all etiologies, cirrhosis is preceded by fibrosis and inflammation, with elements of innate and adaptive immune response that are crucial in regulating these processes [43]. Recent efforts to confront these fibrotic diseases are focused on finding specific marks that transform an acute inflammation to a chronic one, and to use them as therapeutic aims for treatment and reversion of this phenomenon [44]. The immune response plays an essential role in this transformation, mainly by diverse cellular phenotypes [45]. The participation of immune cells, such as Kupffer cells (KCs), the liver macrophages, as initial effectors, is one of the main responsible of cirrhosis development [46, 47]. They are antigen presenting cells and represent an immune cell population related to liver fibrosis treatment.

The KCs present diverse activation phenotypes: M1 related to inflammation and M2 anti-inflammation related with resolution of inflammation processes [48–50], both are regulated by extracellular signals such as adenosine [51] and are directly connected with other immune cells types as B and T cells.

In liver diseases, the phenomenon, in a canonical way, occurs when the activated KCs regulate the hepatic stellate cells and other molecular and cell interactions associated with the establishment of cirrhosis [52, 53]. KCs also interact with other cells, like neutrophils, hepatocytes, etc., mainly through molecules directly associated with inflammation, tissue damage, and fibrosis, like cytokines and chemokines, such as IL-1β, IL-6, TNF-α, and MCP-1 [54] or ROS (reactive oxygen species) which promote the inflammatory response; KCs could be contributing to anti-inflammatory effects with IL-10 and other cytokines involved in tissue repair [55]. The liver is the main organ that produces and removes cytokines; all cell types in the liver are capable of cytokine production, parenchymal and non-parenchymal cells [56, 57].

By their destructiveness, macrophages guide the course of the inflammatory response and are involved in the synthesis and repair of damaged tissue during the inflammatory process, participating actively in the resolution of inflammation [58]. There are two proposed macrophage subtypes; activated by two ways: the classical pathway (M1) or the alternative pathway (M2) [59]. These different polarization states will depend on the microenvironment and the source of damage that has occurred. The classical activation is critical for the initiation and maintenance of the inflammatory process and to the response against pathogens and immune response.

Classical activation or M1 is produced by the interaction of TLR4 receptor with PAMPs such as LPS, from Gram-negative bacteria wall or by specific cytokines such as TNF-α or γ-IFN. This group of classically activated macrophages produces large amounts of proinflammatory cytokines such as TNF-α, interleukins (IL-1β, IL-6, and IL-12), proinflammatory chemokines such as MCP-1, and nitric oxide (NO), promoting activation, migration of other cells, and tissue damage [58].

In the case of alternative activation, the Th2 cells secrete cytokines such as IL-4 or IL-13 and induce the macrophage alternative M2 phenotype [60]. These M2 macrophages have very little capacity to present antigens while secrete high levels of anti-inflammatory cytokines such as IL-10. Unlike classical activation, these macrophages are not able to produce nitric oxide from L-arginine and also fail to control the growth of intracellular pathogens [61]. However, they are capable of producing a high quantity of arginase 1 enzyme that metabolizes L-arginine to produce proline, glutamate, and polyamines promoting tissue repair [62].

We have demonstrated that in the experimental model of CCl_4-induced cirrhosis, the IFC-305 treatment generates several changes in the inflammatory process, mediated by cytokines and immune cells [63]. During the development of cirrhosis, we observed an increment in the liver inflammatory cytokines, IL-6, IL-1β, MCP-1, and TNF-α, in plasma and liver tissue, as well as an increment of M1 macrophages (CD163+/CD11b+). The IFC-305 treatment decreased these inflammatory cytokines, reduced the M1 inflammatory macrophages, and increased the M2 anti-inflammatory macrophages (HIS36+/CD11b+). The anti-inflammatory role of IFC-305 was also supported by elevation of IL-10, an enhanced metabolic activity of arginase, reduction of NO levels in serum rats, a diminution of the protein levels of inducible nitric oxide synthetase, and an increment of the protein levels of arginase 1 in the liver. These results suggest that the IFC-305 modulates the immune response in cirrhosis and supports the hepatic protective action through an anti-inflammatory role, mainly mediated by Kupffer cells [64].

5. Hepatic stellate cells, generators of extracellular matrix components which trigger fibrosis and its activation prevention by IFC-305

Liver fibrosis is characterized by an accumulation of collagen types I and III that are secreted by liver myofibroblast. These cells are originated mainly from hepatic stellate cells (HSCs) and in a less number from the periportal fibroblast or bone marrow cells. In normal liver, HSCs represent almost 10% of all resident cells of the liver. They are quiescent cells specialized in lipid storage, mostly retinyl esters. When there is a liver damage, the HSC become activated or transdifferentiate to myofibroblast phenotype, characterized for being proliferative, pro-inflammatory, and contractile and for increased synthesis of ECM proteins [65].

The activation of HSCs is promoted by stimuli from resident and infiltrating inflammatory cells that produce fibrogenic, proliferative, and inflammatory cytokines such as TGF-β, PDGF, and TNF-α, among others, in addition to reactive oxygen species [65].

In order to clarify the hepatoprotector role of IFC-305 in the CCl_4-induced liver fibrosis at a molecular and cellular level, we explored the effect of IFC-305 on the activation of HSCs. These cells isolated from normal rat livers become activated *in vitro* after 7 days in culture, in a similar manner that occurs *in vivo* after a liver injury. We isolated HSCs from normal rat livers and cultured them for 7 days. We found that IFC-305 treatment suppresses their activation, determined by the inhibition of *Col1a1* mRNA expression, prevention of Rho activation, inhibition of

PDGF-stimulated proliferation, and increased expression of anti-fibrogenic genes such as *Pparg*, *Smad7*, and *Mmp-13* [66].

Hepatic fibrosis is characterized by ECM deposition, specially the type I collagen protein. The excess of ECM is due to an imbalance between its production and its degradation. ECM degradation is carried out by matrix metalloproteinases (MMPs), whose activity is negatively regulated by tissue inhibitors of matrix metalloproteinases (TIMPs). During progression of liver fibrosis, activated HSCs produce an excess of ECM and increase the expression of TIMP-1 and TIMP-2, resulting in an excess of ECM deposition. In rodents, the MMP-13 is the principal matrix metalloproteinase that degrades type I collagen [67]. Treatment of HSCs with IFC-305 inhibited the production of *Col1a1* mRNA but also increased the expression of *Mmp13* mRNA, which may result in an important decrease of collagen deposition.

The main fibrogenic cytokine is TGF-β, which signals into the cell through membrane kinase TGF-type I and type II receptors, which activate the intracellular Smad proteins and transduce the TGF-α signal to the nucleus. The Smad 7 acts as a negative regulator of this pathway [65]. The *Smad7* mRNA expression induced by IFC-305 could result in the inhibition of TGF-β signaling and inhibition of HSC activation.

Peroxisome Proliferator Activated Receptor gamma (PPARγ) regulates cellular fatty acid storage and adipogenesis of fibroblast. Another very important effect of IFC-305 on HSC is an increase of *Pparg* mRNA expression. PPARγ is expressed in quiescent HSC, and its expression is rapidly decreased during HSC activation *in vitro* and *in vivo* [68]. It is well documented that expression of PPARγ or treatment with its natural or synthetic ligands inhibits HSC activation or can reverse the activated HSC phenotype to the quiescent one [69]. The increased expression of *Pparg* mRNA with IFC305 in HSCs could be contributing to maintain their quiescent phenotype.

The IFC-305 also inhibited the PDGF-BB-stimulated proliferation of HSC; exploring the mechanism, we found that this effect was independent of adenosine receptors, but required their uptake into cells by adenosine transporters followed by their intracellular conversion to AMP by adenosine kinase, leading to increased levels of AMP, pyrimidine starvation, and inhibition of DNA synthesis [66].

In summary, we demonstrated that HSCs are an important target of the anti-fibrotic role of IFC-305 contributing to its hepatoprotective effect on liver fibrosis.

6. Gene expression deregulation in cirrhosis and IFC-305 modulation beyond genetics

With the interest to have a general view of molecular changes occurring in cirrhosis, we assessed the transcriptome evaluation of both cirrhotic and cirrhotic livers treated with IFC-305 and found 413 deregulated genes in cirrhosis, and IFC-305 treatment reduces the genes with deregulated expression to 263; making a gene ontology, we noticed that the highest proportion of deregulated genes is related with signal transduction, and interestingly, some of these deregulated genes are involved in TGF-β signaling pathway, lipid metabolism, urea cycle, and fibrogenesis.

Validating some of these differential expressed genes, we found an over-expression of *Fn1* (fibronectin 1) and *Col1a1* in cirrhosis; both of them are regulated by TGF-β signaling pathway, and importantly, *Col1a1* gene encodes a component of type I collagen called the pro-α1(I) chain that constitutes one of the main ECM proteins in the fibrotic liver. In fact, expression of *Tgfb1* gene was also increased in cirrhosis, and other two genes with the same behavior were the complement *C9* and

Apoa1 [17]. PPARγ has anti-fibrogenic activity inhibiting collagen I transcription [70]; there was a reduction in transcript levels of *Pparg* gene in cirrhosis state, in concordance with other reports [71, 72]. Transcriptome analysis also revealed three genes involved in ornithine and urea metabolism; the transcript level of *Ass1* gene was reduced as well as *Cps1* gene. Making a deeper analysis of the transcriptome data, we could identify a chromatin-related gene deregulated in cirrhosis; *HDAC3* histone deacetylase is over-expressed during cirrhosis. IFC-305 is able to reduce the levels to healthy-like levels for three fibrogenic genes (*Fn1*, *Col1a1*, and *Tgfb1*), *C9, Apob1* and *Hdac3;* meanwhile, this compound generates the increasing expression of *Pparg* and *Cps1* recovering the levels of the healthy liver and partially restoring the levels of *Ass1* [17]. Analyzing proteins, we could identify an increment in collagen I and HDAC3 and a reduction of PPARγ during cirrhosis, and the treatment with IFC-305 resembles the healthy-like levels of these three proteins [16]. Thus, through this quantitative analysis of expression and protein levels, IFC-305 shows capabilities to modulate the gene expression of some important genes involved in liver fibrogenesis.

During CCl_4-induced cirrhosis, besides hepatocellular damage previously mentioned, there is chromosome instability [73] that could be induced by hypomethylation on DNA, and contributes to carcinogenesis [74]. Considering cirrhosis as a pre-neoplastic state (because 80% of hepatocellular carcinoma cases are preceded by cirrhosis) [11], it is possible that the big changes in gene expression could be directed by chromosomal instability generated by CCl_4, but beyond the genetic alterations that probably are occurring, many of these changes could be related to regulation of gene expression at epigenetic level even more because chromosomal instability could be occurring by DNA hypomethylation. Also, it is important to remember that HDAC3 was incremented in cirrhosis, so some changes in gene expression could also be modified by changes in chromatin.

Every process which is able to influence in heritable gene expression without affecting DNA sequence is considered as an epigenetic regulation process [75]. DNA methylation is probably the most studied epigenetic modification [76, 77]; it consists in the incorporation of methyl group in 5 position of cytosine from CpG dinucleotide. This incorporation does not modify DNA sequence and can influence directly in transcriptional activity [78]. Methylated DNA distribution along genome shows an enrichment on noncoding regions, repetitive elements [75], and further, it inactivates mobile elements of the genome as transposons and sequences of viral origin, having a function in genome stability maintenance, blocking undesired recombination events [76]. On the other hand, on CpG islands of active genes, there is no enrichment of this DNA modification [75] and participates in permanent gene silencing in different steps of development [78, 79]. DNA methylation is directed by DNA methyltransferase enzymes which use S-adenosylmethionine (SAM) as methyl donor from methionine cycle and the product of this reaction is S-adenosylhomocysteine (SAH). It is important to mention that one important factor to modulate biological methylation reactions (to DNA, RNA, proteins, and phospholipids) is the hepatocellular ratio SAM/SAH [80].

The discovery of an active DNA demethylation pathway that involves the conversion of 5mC to oxidized forms, like 5hmC, by DNA dioxygenases TETs and DNA repair through the base excision system, incorporates a dynamic reversibility of DNA methylation [81–84]. Ever since the discovery of this dynamics, strenuous efforts have been made to characterize the precise role of 5hmC; such roles are becoming more evident as we learn about 5hmC-specific genomic localization, its relative stability, and recognition by other proteins [85], and current studies have shown that this DNA modification has an antagonic role to 5mC [86].

We have previously shown that adenosine can modulate trans-methylation reactions, like methylation of phospholipids, via regulation of

S-adenosylmethionine (SAM) levels [87], so that we make an approach to SAM levels and found that during cirrhosis, the amount of this molecule is diminished, whereas IFC-305 treatment restored the physiological levels; this suggests that during cirrhosis, it could be an imbalance of methylation reactions, and IFC-305, like adenosine, could also regulate this process [16].

Epigenetic changes in cirrhosis are little understood; some studies demonstrated a reduction on DNA methylation through the genome in CCl_4-induced cirrhosis [15, 88], and analyzing in a global way this modification, we obtained the same behavior. With these results and in order to assess DNA methylation dynamics, 5hmC levels were measured and a similar behavior to the one observed with 5mC, a reduction of 5hmC in cirrhosis in concordance with group of Mann findings, was found [89]. Treatment with IFC-305 triggers a regaining of both 5mC and 5hmC [16]. These results together suggest that there is a perturbation of DNA methylation dynamic during cirrhosis, while IFC-305 is able to modulate this dynamic, possibly reducing chromosome instability.

Another level of epigenetic regulation is related with genome packaging in chromatin, which has a direct repercussion in transcriptional activity, being mandatory its remodeling, space specifically, and in a time defined way to carry out gene expression [90]. Histones are a fundamental component of chromatin structure, being a target of a big variety of post-translational modifications (PTMs), which allows the formation of particular and regulated chromatin states; furthermore these modifications could be inherited post-mitotically [91]. We want to highlight among histone PTMs, lysine acetylation. DNA association with histone *core* is facilitated by electrical charge difference between both molecules, but histone acetylation neutralizes lysine positive charge, weakening nucleosome-DNA interaction and triggering a less compact conformation which favors transcription [92].

Considering the finding that histone deacetylase HDAC3 level was high during cirrhosis, global histone H4 acetylation was assessed, finding a reduction in this histone PTM in cirrhotic livers. Physiological levels of global histone H4 acetylation in cirrhotic rats treated with IFC-305 were recovered [16]. Together, these results suggest that deregulated gene expression during cirrhosis could be related with epigenetic deregulation involving DNA methylation dynamics and changes in histone acetylation; besides, IFC-305 has epigenetic properties being able to modulate 5mC, 5hmC, and histone H4 acetylation in a global way, favoring the recovery of physiological levels of each epigenetic modification and triggering the rescue of healthy-like gene expression in liver.

7. Getting deeper into cirrhosis resolution or epigenetic regulation of *Pparg* and *Col1a1* by IFC-305

Once established that IFC-305 is able to reverse fibrosis, reactivating cell cycle progression in cirrhotic livers, favoring an anti-inflammatory environment, blocking hepatic stellate cell activation, and regulating gene expression through epigenetic modulation, we analyzed the regulation of two of the main genes with modified expression during cirrhosis, *Pparg* and *Col1a1.* Reminding, collagen I is the mayor ECM protein and responsible for liver fibrosis, *Col1a1,* a gene which encodes pro-α1 chain of this protein is over-regulated in cirrhosis, and there is a reduction of nuclear receptor PPARγ in this pathological condition.

PPARγ has antifibrotic properties because it is able to inhibit collagen I gene transcription. This inhibition is mediated by the ability of nuclear receptor to compete with NF-I/p300 association to the *Col1a1* gene in HSC [70]. p300 has a histone acetyltransferase activity that transfers an acetyl group to the lysine residue

[93] generating an open chromatin context in *Col1a1* gene promoter favoring its expression; whether PPARγ replaces p300 in Col1a1 gene promoter, there is a chromatin context change because of the loss of acetylation activity in this region, and the consequent blocking of the gene expression.

On the other hand, regulation of *Pparg* expression is directed by MeCP2 protein in HSC activation like in cirrhosis. MeCP2 binds to regulatory regions of the gene which are CpG enriched and recruits histone H3K9me3 writer enzymes suppressing initial transcription of the gene. Furthermore, MeCP2 is required for polycomb repressor complex 2 EZH2 component that establishes histone H3K27me mark on the downstream coding gene region, blocking transcription elongation [94]. This mechanism could explain the reduction of PPARγ levels in cirrhosis and therefore the over-expression of *Col1a1* in cirrhosis.

Considering that MeCP2 is a methyl binding protein [94], DNA methylation state was evaluated with sodium bisulfite DNA modification on *Pparg* gene promoter, and it was found that both healthy and cirrhotic livers present *Pparg* gene promoter without DNA methylation, and IFC-305 treatment does not modify this nonmethylated state [16]. Evaluating histone H4 acetylation and histone H3K27me3 on *Pparg* gene promoter through chromatin immunoprecipitation assay, we found that in cirrhosis, there is a trend to compact chromatin context mainly dictated by histone H4 acetylation reduction, which correlates with decreased expression and protein levels; after treatment with IFC-305 chromatin, there is an open chromatin context on *Pparg* gene promoter triggered by an increase on histone H4 acetylation and a reduction of histone H3K27me3, going hand by hand with over-expression and increase of protein levels [16]. These findings suggest that reduction of PPARγ on cirrhosis is coordinated by a chromatin compaction on gene promoter, and IFC-305 treatment generates a decompaction of gene promoter with the consequent increase of gene expression.

The next step was to identify if IFC-305 mediated fibrosis reversion is related with *Col1a1* gene expression blocking though PPARγ was to assess nuclear receptor deposition on *Col1a1* gene promoter, but we were not able to find an increment of this interaction in whole cirrhotic tissues treated with IFC-305; rather, we found a diminishment of PPARγ deposition [16]. Further experiments on isolated HSCs treated with IFC-305 are required to know if this molecular mechanism is occurring directly in responsible cells of fibrosis generation. So, we asses DNA methylation state on *Col1a1* gene promoter, to know if this epigenetic mechanism is involved in regulation of gene expression during cirrhosis. In healthy liver, *Col1a1* gene promoter presents a methylated state, which correlates with the absence of collagen I overproduction; in cirrhosis state and progress of the illness, there is an important reduction on methylation state on *Col1a1* gene promoter going hand by hand with accumulation of collagen I; and finally, treatment with IFC-305 generates a remethylation of gene promoter and an enrichment of methylation around transcription start site, associated with collagen reduction.

With these results, we could propose that fibrosis generation could be directed by loss of DNA methylation on *Col1a1* gene promoter, and one of the mechanisms of action that could explain cirrhosis resolution mediated by IFC-305 is the modulation of DNA methylation on *Col1a1* gene.

Blocking the prelude of hepatocellular carcinoma, a key point to avoid chronic liver injury progress.

Along this chapter, some cellular and molecular alterations that characterize cirrhosis have been described: a pathology that results in the combination of factors which alters liver environment, beginning with an immunological response originated by macrophages inflammatory polarization that triggers hepatic stellate cell activation, with alterations at chromatin level resulting in chromosome instability and altered gene expression favoring the fibrogenic process. All these changes, as a whole, could facilitate progress of the illness to hepatocellular carcinoma (HCC),

which is the most common primary carcinoma of the liver. Up to 85% of HCC cases arise in chronically inflamed and subsequently cirrhotic livers [95].

According to GLOBOCAN-IARC datasets [96], liver cancer is estimated to be at the 7th place of cancer incidence and the 4th cancer death cause in 2018. Considering cirrhosis as a prelude of HCC, it is understandable that many liver cancers follow a pattern of pathologic evolution, starting from cirrhosis to low-grade dysplastic nodules, high-grade dysplastic nodules, early HCC, progressed HCC and, finally to advance HCC. Furthermore, this progress of illness involves a more evident imbalance between genetic and epigenetic factors; regarding genetic ones, early dysplastic nodules present a genome with a very limited genetic variation, while advanced HCC has a heterogeneous genome with a range of 72–182 mutations [97, 98]. On the other hand, concerning epigenetic aspects, a genome-wide DNA hypomethylation in HCC has been described, which could be indicative of poor survival [99]. In the same sense, another study described that aberrantly methylated differential expressed genes are related with cell cycle progress, p53 signaling, and MAPK signaling in HCC [100]. Alteration on DNA methylation state could be explained by down-regulation of TET dioxygenases in HCC condition with the consequent reduction of 5hmC levels [101]. In normal tissue there is a characteristic difference between euchromatic and heterochromatic regions that is lost in cancer condition; it has been suggested that lost could be generated by a reduction of 5hmC levels which goes around 70%. Furthermore, the specific relationship between 5hmC and chromatin marks in normal tissue is largely erased in tumors and suggests that 5hmC landscape change in cancer could be associated with chromatin structure alterations and deregulation of gene expression during tumorigenesis [102].

HCC may progress silently in patients with sufficient liver function; due to vague complaints and nonspecific symptoms, HCC diagnosis is usually delayed [103]. Selected patients with localized disease may be treated with curative intents with resection, liver transplantation, or local therapy like radial frequency ablation, chemoembolization or radio-embolization. However, the majority of patients with HCC are not candidates for resection [104, 105]. For patients suffering from advanced HCC, chemotherapy failed to demonstrate a survival advantage [106, 107]; so far, sorafenib is the first and only target orientated agent approved as therapy of HCC [105], but it only extends survival of patients with advanced stage disease for 3 months, and this medication causes considerable adverse events and offers no symptom palliation [108]. This lack of effective treatment and surgical impediment highlights the importance to reinforce molecular target therapies.

Preclinical studies indicated that IFC-305 is not toxic, neither genotoxic, nor teratogenic and it is anti-carcinogenic [33]. Considering this last property, we assessed IFC-305 effects on a chronic model of liver intoxication with diethylnitrosamine and found that it could act as a HCC chemopreventive agent [109, 110]. The above suggests that some anti-fibrotic effects of the compound could prevent cancer development, being an adjuvant in chronic liver disease treatment. Currently we are studying a deep molecular level to identify IFC-305 mechanism of action in prevention of cancer establishment and potential disease reversion.

Considering recent advances in cirrhosis knowledge, we could suggest that in the not long future, drugs targeting specific molecular keys for cirrhotic and HCC will be developed and potentially they could be the first line of treatment even after surgery.

8. Conclusion

Cirrhosis is a complex pathology, which involves deregulation at different levels (**Figure 1**); some insults such as hepatitis viral infections, alcohol, high fat diet, or

even self-immune events could trigger the development of the illness, denoted by biochemical alterations like albumin reduction and increase of transaminases. In our case, we assessed the study of this disease using CCl_4, which generates hepatocellular damage. Once cell membrane is affected by free radicals from CCl_4 metabolism, through epoxide formation, KCs are activated to phagocytose damaged cells; this activation causes an inflammatory process, due to M1 macrophage activation. Whether this inflammation is perpetuated, HSCs could be activated, becoming the main producers of fibrosis (**Figure 1B**). At molecular level, cirrhotic-damaged liver loses the capability to proliferate; there is a reduction in DNA methylation, 5hmC, and histone H4 acetylation, which generates chromosome instability and therefore alteration of gene expression, affecting principally fibrogenic genes. Among important genes involved in the fibrogenic process are *Pparg* and *Col1a1*. Cirrhotic liver presents a compact chromatin context of *Pparg* gene promoter with the consequent reduction of both its transcript and protein; on the other hand, *Col1a1* gene promoter loses DNA methylation, and this correlates with gene overexpression and the increment of protein levels. Adenosine derivative, IFC-305, has hepatoprotective properties (**Figure 1C**); it is able to reduce fibrosis, ameliorate parenchymal area and recover liver function. Treatment with this compound polarizes the macrophages to an anti-inflammatory phenotype M2, increasing the levels of immunosuppressant cytokine IL-10 and arginase 1; the reduction of the inflammatory process facilitates the inhibition of HSC activation and the consequent reduction of fibrosis. At molecular level, high serum levels of HGF, principal liver mitogen, could interact with elevated levels of its receptor, c-Met, and stimulate cell cycle progression, providing once more the regenerative capacity to the liver. IFC-305 could favor a genome instability diminishment as a consequence of the

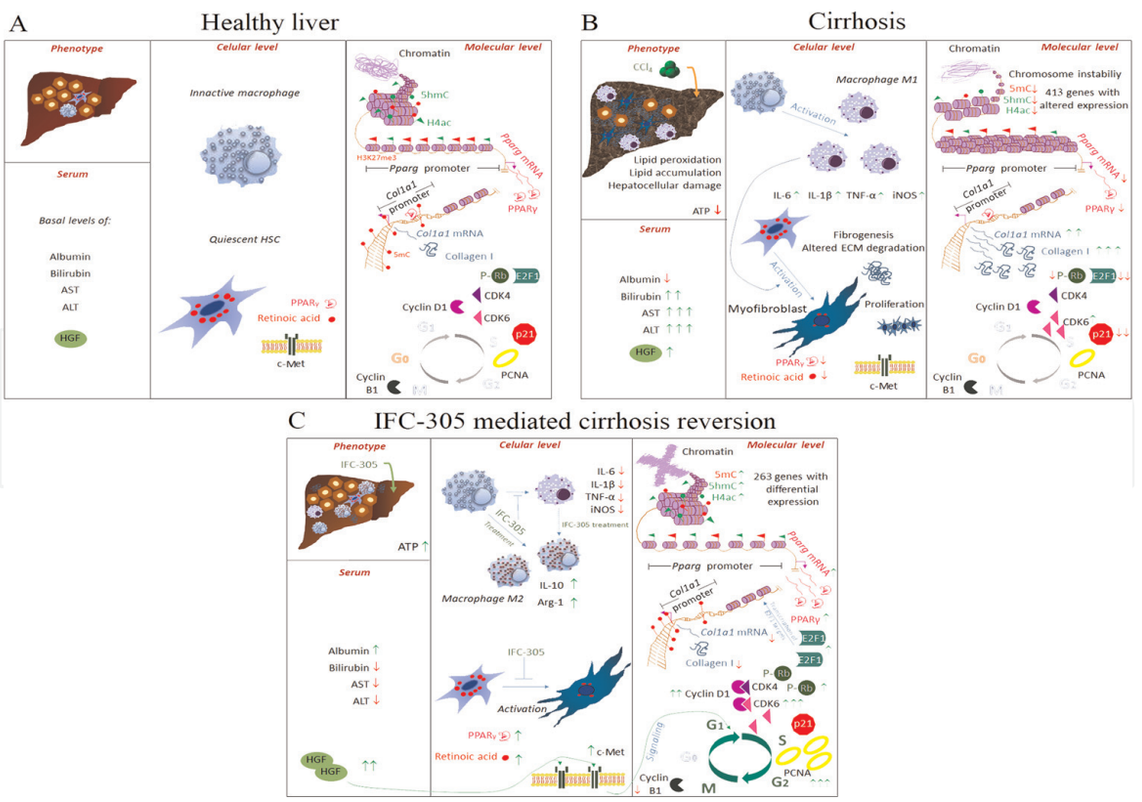

Figure 1.
Architectural, physiological, cellular, and molecular alteration during CCl_4-induced cirrhosis and hepatoprotective effects of IFC-305. We show four different analyzed levels in this section: phenotype, denoting architecture and cell composition of the liver; Serum, biochemical markers related with liver function; cellular level, changes found in non-parenchymal cells; molecular level, changes in cell cycle components, chromatin, and gene expression. (A) Healthy liver; (B) CCl_4-induced cirrhosis state; (C) pre-established cirrhosis treated with IFC-305.

reestablishment of DNA methylation levels and 5hmC and Histone H4 acetylation; these effects on chromatin trigger the recovery of healthy-like gene expression. Fibrosis resolution mediated by IFC-305 could be explained by the generation of an open chromatin context of *Pparg* gene promoter that correlates with its gene and protein up-regulation. High levels of PPARγ could act as a repressor of *Col1a1* expression in HSC; on the other hand, *Col1a1* gene promoter gains DNA methylation on promoter and TSS, this methylation state goes hand by hand with reduction of collagen I expression and protein, favoring a decrease in fibrosis, a key point in cirrhosis resolution. These studies support molecules and cell behavior modified by IFC-305 as a potential target for new drugs to treat cirrhosis, contribute to the understanding of liver fibrosis at epigenetic level, open the door to the exploration of chromatin modifications as a potential biomarker for early detection and intervention of liver diseases, and support the use of IFC-305 as therapy for liver illness. Finally, we highlight the relationship between cirrhosis and HCC, how liver fibrosis is the prelude of HCC and in what manner IFC-305 could be an adjuvant preventing HCC by its anti-cirrhotic and anti-neoplastic effects, and how recent advances could favor development of an effective treatment, preferring a less invasive to surgical one.

Acknowledgements

This work has been supported by DGAPA-UNAM, grant numbers: IN214419 to VCS and IN203917 to FRT; CONACyT, México, grant: *Fronteras de la Ciencia* 290 to FRT; *Fundación Miguel Alemán, México* "*Estímulo a Investigaciones Médicas*" to VCS; PhD Fellowships from CONACyT: CVU 508509 to JRRA, CVU 298432 to RPCV, CVU 580148 to NGC. We acknowledge the assistance of Rosario Pérez-Molina, Georgina Guerrero-Avendaño, Fernando Suaste-Olmos, Enrique Chávez, Rosario Villaseñor Ávila, and Gabriela Valdés Silva.

Conflict of interest

The authors declare no conflict of interest.

Nomenclature

5hmC	5-hydroximethylcytosine
5-LO	5-lipoxygenase
5mC	5-methylcytosine
ALT	alanine aminotransferase
AMP	adenosine monophosphate
AST	aspartate aminotransferase
ATP	adenosine triphosphate
$CCl_3\cdot$	trichloromethane radical
$CCl_3OO\cdot$	trichloromethyl peroxy radical
CCl_4	carbon tetrachloride
CDK4	cyclin-dependent kinase (for example, CDK4, CDK6, etc.)
c-Met	tyrosine-protein kinase Met or hepatocyte growth factor receptor
Col1a1	type I collagen pro-α1(I) chain gene
CpG	cytosine guanine dinucleotide
DNA	deoxyribonucleic acid

E2F1	transcription factor E2F1
ECM	extracellular matrix
H3K27me3	histone H3 trimethyl lysine 27
H4ac	hyperacetylated histone H4
HCC	hepatocellular carcinoma
HDAC	histone deacetylase
HGF	hepatocyte growth factor
HSC	hepatic stellate cells
IFC-305	aspartate salt of adenosine: 2-aminosuccinic acid–2-(6-amino-9H-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol (1:1)
IL	interleukin (for example, IL-1β, IL-10, etc.)
iNOS	inducible nitric oxide synthase
KC	Kupffer cells
LPS	lipopolysaccharide
M1	inflammatory macrophages
M2	anti-inflammatory macrophages
MAPK	mitogen-activated protein kinase
MCP-1α	macrophage protein inflammatory 1-alpha
MeCP2	methyl-CpG binding protein 2
MMP9	matrix metalloproteinase 9
mRNA	messenger ribonucleic acid
NO	nitric oxide
p21	cyclin-dependent kinase inhibitor 1
p27	cyclin-dependent kinase inhibitor 1B
P450	CYP450 cytochrome
PAMPs	pathogen associated molecular patterns
PCNA	proliferating cell nuclear antigen
PDGF	platelet-derived growth factor
PPARγ	peroxisome proliferator-activated receptor gamma
PTMs	post-translational modifications
Rb	retinoblastoma protein
RNA	ribonucleic acid
ROS	reactive oxygen species
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
STAT6	signal transducer and activator of transcription 6
TET	DNA dioxygenase ten-eleven translocation
TGF-β	transforming growth factor beta
TIMPs	tissue inhibitors of matrix metalloproteinases
TLR4	Toll-like receptor 4
TNF-α	tumoral necrosis factor alpha
VEGF	vascular endothelial growth factor
α-SMA	alpha smooth muscle actin.

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