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OSTEOPONTIN, AN OXIDANT STRESS-SENSITIVE CYTOKINE, UP-REGULATES COLLAGEN-I VIA INTEGRIN $\alpha_v\beta_3$ ENGAGEMENT AND PI3K-pAkt-NF κ B SIGNALING

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Abstract

Background & Aim—A key feature in the pathogenesis of liver fibrosis is fibrillar collagen-I deposition; yet, mediators that could be key therapeutic targets remain elusive. We hypothesized that osteopontin (OPN), an extracellular matrix (ECM) cytokine expressed in hepatic stellate cells (HSC), could drive fibrogenesis by modulating the HSC profibrogenic phenotype and collagen-I expression.

Results—rOPN up-regulated collagen-I protein in primary HSC in a TGF β -independent fashion whereas it down-regulated matrix metalloproteinase-13 (MMP13) thus favoring scarring. rOPN activated primary HSC—confirmed by increased α -smooth muscle actin (α -SMA) expression—and enhanced their invasive and wound-healing potential. HSC isolated from wild type (WT) mice were more profibrogenic than those from *Opn*^{−/−} mice and infection of primary HSC with an Ad-OPN increased collagen-I, indicating correlation between both proteins. The OPN induction of collagen-I occurred via integrin $\alpha_v\beta_3$ engagement and activation of the PI3K-pAkt-NF κ B signaling pathway, while CD44-binding and mTOR-p70S6K were not involved. Neutralization of integrin $\alpha_v\beta_3$ prevented the OPN-mediated activation of the PI3K-pAkt-NF κ B signaling cascade and collagen-I up-regulation. Likewise, inhibition of PI3K and NF κ B blocked the OPN-mediated collagen-I increase. HCV-cirrhotic patients showed co-induction of collagen-I and cleaved OPN compared to healthy individuals. Acute and chronic liver injury by carbon tetrachloride (CCl₄)-injection or thioacetamide (TAA)-treatment elevated OPN expression. Reactive oxygen species up-regulated OPN *in vitro* and *in vivo* and antioxidants prevented this effect. *Opn*^{HEP} Tg mice developed spontaneous liver fibrosis compared to WT mice. Lastly, chronic CCl₄-injection and TAA-treatment caused more liver fibrosis to WT than to *Opn*^{−/−} mice and the reverse occurred in *Opn*^{HEP} Tg mice.

Conclusion—OPN emerges as a cytokine within the ECM protein network driving the increase in collagen-I protein contributing to scarring and liver fibrosis.

Keywords

Extracellular matrix; fibrosis; hepatic stellate cells; oxidant stress

Fibrogenesis, or activation of the wound-healing response to persistent liver injury, is characterized by changes in the composition and quantity of the ECM deposits distorting the

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normal hepatic architecture by forming fibrotic scars. Failure to degrade accumulated ECM is a major reason why fibrosis progresses to cirrhosis. Emerging antifibrogenic therapies aim at inhibiting the activation of profibrogenic cells to prevent fibrillar collagen-I deposition, at degrading the excessive ECM to recover the normal liver architecture and at restoring functional liver mass.

Although different cell types contribute to the increase in fibrillar collagen-I during hepatic fibrogenesis, they all undergo a common process of differentiation and acquisition of a classical myofibroblast-like phenotype. HSC are considered a central ECM-producing cell within the injured liver (1), playing a significant role in collagen-I deposition when hepatocellular injury is concentrated within the liver lobule and sinusoids. In normal liver, they reside in the sinusoidal *space of Disse*; however, during chronic injury, they activate while acquiring motile, proinflammatory and profibrogenic properties (2). Activated HSC migrate and accumulate at the sites of tissue repair, secreting large amounts of ECM, mostly collagen-I, and regulating ECM remodeling. Up-regulation of fibrillar collagen-I is thus a key event leading to scarring, the pathophysiological hallmark of liver fibrosis.

While some current therapies have proven beneficial, dissecting key profibrogenic mechanisms, pathways and mediators of disease progression is vital. Several studies have identified OPN as significantly up-regulated during liver injury and in HSC (3-6). OPN is a soluble cytokine and a matrix-bound protein that can remain intracellular or is secreted; hence, allowing autocrine and paracrine signaling (7-8). OPN, as a matricellular phosphoglycoprotein, functions as adaptor and modulator of cell-matrix interactions (8). Among its many roles, it regulates cell migration, ECM-invasion and cell adhesion due to its ability to bind integrins -via its RGD motif- or CD44 -via a cryptic site (SVVYGLR)- exposed after cleavage by thrombin, plasminogen, plasmin, cathepsin-B and some MMPs (5, 9). OPN expression increases in tumorigenesis, angiogenesis and in response to inflammation, cellular stress and injury (10-14). OPN plays an important role in regulating tissue remodeling and cell survival as well as in chemoattracting inflammatory cells (15). Moreover, *Opr^{-/-}* mice show matrix disarrangement and alteration of collagen fibrillogenesis in cartilage compared to their WT littermates (16).

There is limited information on the contribution of OPN to the HSC profibrogenic behavior and the molecular mechanisms and signaling pathways involved in governing collagen-I protein expression during the fibrogenic response to liver injury (3-6, 17). Since OPN is expressed in HSC (3-6), we hypothesized that OPN could trigger signals capable of up-regulating collagen-I *per se*; hence, acting as a feed-forward mechanism promoting scarring. Therefore, the major goal of this work was to determine how OPN could become a profibrogenic 'switch' and to characterize the underlying cellular mechanism for this effect. In the present study, we identified a role for intracellular OPN in increasing collagen-I, the HSC membrane proteins engaged by extracellular OPN, the proximal signaling molecules/ stress-sensitive kinases activated upon binding that trigger the profibrogenic cascade, the ability of OPN to respond to oxidant stress and the effect of *Opr* ablation or overexpression on collagen-I deposition *in vivo*.

MATERIAL AND METHODS

Please see Supplementary Experimental Procedures

RESULTS

rOPN slightly increases HSC proliferation rates and promotes HSC migration

rOPN did not alter HSC viability but slightly induced proliferation rates both in rat and in human HSC (Supplementary Figure 1); however, rOPN caused a 2-fold increase in the invasive potential or chemotaxis (Supplementary Figure 2A-2B) and enhanced the wound closure ability of rat HSC (Supplementary Figure 2C), important functions gained by HSC during their activation that contribute to their profibrogenic ability. Neutralizing Abs to $\alpha_v\beta_3$ integrin and OPN blocked the effects on HSC invasion (not shown) and on closure ability (Supplementary Figure 2C).

rOPN induces profibrogenic effects in primary HSC

Upon stimulation with rOPN, rat HSC up-regulated intra- and extracellular collagen-I in a time-dependent fashion (Figure 1A, left). Denatured rOPN did not elevate collagen-I; thus, confirming the specificity of the rOPN effect on collagen-I in HSC (not shown). rOPN lowered extracellular MMP13 protein by 50%, contributing to extracellular collagen-I accumulation. Reciprocal modulation of MMP13 and collagen-I has been previously described in rat HSC (18). Extracellular pro-, intermediate and active MMP2 and MMP9 remained unchanged (Figure 1A, left). Likewise, tissue inhibitor of MMP1 was comparable (not shown). rOPN induced rat HSC activation as shown by the increase in collagen-I and α -SMA proteins (Figure 1A, right). Analogous results were observed in human HSC (Figure 1B).

Because of the ability of HSC to secrete TGF β (19) along with its well-known profibrogenic effect (20), rat HSC were treated with anti-TGF β Ab. Neutralization of TGF β did not alter the rOPN-mediated induction of collagen-I; thus, implying a mechanism independent of TGF β production by HSC (Supplementary Figure 3).

To dissect whether intracellular OPN could play an autocrine role in modulating collagen-I expression, HSC were isolated from WT and *Opn*^{-/-} mice. WT HSC appeared more profibrogenic than *Opn*^{-/-} HSC since intracellular collagen-I expression at 7 days of culture was higher in WT HSC than in *Opn*^{-/-} HSC (Figure 1C). Infection of rat HSC with an Ad-OPN increased intracellular collagen-I and intra- and extracellular OPN compared to HSC infected with Ad-LacZ (Figure 1D). Therefore, a novel autocrine role for intracellular OPN in modulating collagen-I deposition was identified.

Anti- $\alpha_v\beta_3$ integrin Ab blocks the rOPN-driven collagen-I increase

Since OPN is also a soluble cytokine and a matrix-bound protein, next we evaluated the role of extracellular OPN-mediated signaling (paracrine role) on collagen-I induction in HSC. OPN signals via integrins - mostly integrin $\alpha_v\beta_3$ highly expressed in HSC- (21-23) and via CD44 -also expressed in HSC- (24). Incubation with anti- $\alpha_v\beta_3$ integrin blocked the rOPN-driven total collagen-I (intra-plus extracellular) increase in rat HSC, while no major effect was observed by anti-CD44 (Figure 2A). Neutralization of other integrins (i.e. β_1 , β_5 and β_6) failed to prevent the increase in collagen-I by rOPN (not shown). Similar results were observed in human HSC (not shown).

rOPN increases collagen-I protein via activation of the PI3K-pAkt-NF κ B signaling pathway

Given that collagen-I protein is highly responsive to oxidant stress-sensitive kinases, we analyzed the expression of protein kinases involved in regulating collagen-I expression such as pp38 (25-26), pERK1/2 (27), pJNK (28-29), PI3K and pAkt (26, 30). Only PI3K and the ratio pAkt⁴⁷³Ser/Akt were elevated time-dependently by rOPN up to 3 hours in rat HSC (Figure 2B) and up to 1 hour in human HSC (Supplementary Figure 4A).

Since PI3K/pAkt are upstream of IKK and the IKK complex is central for the activation of NF κ B to regulate collagen-I (26, 31), we focused on analyzing this signaling pathway. There was up-regulation of the ratios pIKK α , β 176/180Ser/IKK α , β and pI κ B α 32Ser/I κ B α as well as of nuclear/cytosolic p65 in OPN-treated rat HSC (Figure 2C). However, involvement of the mTOR-p70S6K cascade, a translational regulatory mechanism downstream of PI3K and pAkt⁴⁷³Ser for regulating collagen-I (32), was precluded since rOPN neither altered mTOR and p70S6K expression (Figure 2D) nor induced mTOR phosphorylation at 2448Ser or²⁴⁸¹Ser in rat HSC (undetectable).

To define further the molecular mechanism for the collagen-I induction under rOPN challenge, we evaluated the potential role of the activation of these two stress-sensitive kinases (i.e. PI3K and pAkt) and of the NF κ B signaling pathway. Wortmannin, a PI3K inhibitor, neither altered rat HSC viability (100% by the MTT assay), morphology nor proliferation rates (Supplementary Figure 4B and not shown); however, three different doses of wortmannin down-regulated total collagen-I expression in rat HSC co-treated with rOPN (Figure 3A, top). Similar effects were observed by co-incubation with LY294002, a second PI3K inhibitor (Figure 3A, bottom); thus, linking OPN, PI3K-pAkt activation and collagen-I up-regulation in rat HSC. Comparable results were observed in human HSC (Supplementary Figure 4C). Lastly, inhibitors of pp38, pERK1/2 and pJNK signaling did not prevent the increase in collagen-I by rOPN (not shown).

Addition of pyrrolidine dithiocarbamate (PDTC) to block NF κ B signaling prevented the rOPN-driven increase in collagen-I in rat HSC (Figure 3B, top). Analogous effects were observed by co-incubation with CAY10512 -a second inhibitor of NF κ B signaling- (Figure 3B, middle). Moreover, HSC infected with Ad-NF κ B-Luc and treated with rOPN for 24 hours showed a 2-fold increase in luciferase activity compared to non-rOPN-treated Ad-NF κ B-Luc-infected cells (Figure 3B, bottom). Both, wortmannin and an $\alpha_v\beta_3$ integrin neutralizing Ab, blunted the rOPN-mediated effect on the ratios pIKK α , β 176/180Ser/IKK α , β and pI κ B α 32Ser/I κ B α as well as on nuclear/cytosolic p65 (Figure 3C), suggesting engagement of OPN with integrin $\alpha_v\beta_3$, PI3K-pAkt activation and NF κ B signaling to up-regulate collagen-I expression in rat HSC. Lastly, blocking $\alpha_v\beta_3$ integrin prevented the increase in PI3K, the ratio pAkt⁴⁷³Ser/Akt and collagen-I by rOPN in rat HSC (Figure 3D). In summary, these results established a connection among rOPN, $\alpha_v\beta_3$ integrin, PI3K-pAkt activation and the NF κ B signaling pathway to drive collagen-I up-regulation in rat HSC in a paracrine manner.

OPN expression is up-regulated in liver fibrosis

Samples from stage-3 HCV-cirrhotic patients displayed correlation between elevated collagen-I and cleaved OPN protein (~55, ~42 and ~25 kDa isoforms) compared to healthy individuals. Fully modified (glycosylated and phosphorylated) monomeric OPN, typically running at ~75 kDa, was not detectable (Figure 4A).

To determine whether OPN also increased during liver injury in mice, we used well-established *in vivo* models to induce liver fibrosis such as CCl₄-injection and TAA-treatment (33). These drugs undergo cytochrome P450 metabolism leading to significant oxidant stress, inflammation and hepatocyte necrosis within hours. The ~25 kDa OPN form was markedly induced in acute and chronic models of liver injury, while the ~55 kDa OPN form was elevated only under chronic CCl₄-injection and TAA-treatment (Figure 4B). Hence, there was association between OPN induction, OPN proteolytic processing and the extent of liver fibrosis both in humans and in mice.

Next, we evaluated the specific localization of the OPN induction in the liver. Non-treated livers showed OPN⁺ biliary epithelial cells (not shown). Primary HSC isolated from WT

mice and cultured for 6 days were OPN⁺ (Figure 4C, left). Immunohistochemical (IHC) analysis revealed OPN expression in HSC (30), biliary epithelial cells (4, 6, 34), oval cells and mostly in damaged hepatocytes in WT mice injected CCl₄ for 1 month (Figure 4C, middle). Similar results were observed under TAA-treatment, although hepatocytes showed punctated staining (Figure 4C, right). The insets show OPN⁺ HSC in both models. In the early stages of CCl₄- and TAA-mediated liver injury, Kupffer cells were also OPN⁺ (not shown); however, the staining faded with disease progression. Of note, granular OPN⁺ staining -typical of secreted proteins-appeared in focal-septal hepatocytes (Figure 4C, middle). There was co-localization of OPN⁺ staining with α -SMA⁺ (a HSC activation marker) under TAA-treatment (Figure 4D) and by CCl₄-injection (not shown).

Since liver fibrosis is associated with significant oxidant stress, to dissect whether OPN was responsive to reactive oxygen species, HSC were challenged with H₂O₂ -a prooxidant typically generated during CCl₄ metabolism- or with L-buthionine sulfoximine (BSO) - which depletes glutathione. Both treatments increased OPN expression in HSC, whereas co-treatment with glutathione-ethyl ester to restore glutathione levels, blunted this effect (Figure 4E). To validate the induction of OPN by oxidant stress *in vivo*, WT mice were CCl₄-injected for 1 month in the presence or absence of S-adenosylmethionine (SAM), an antioxidant known to restore glutathione levels. Co-injection with SAM lowered OPN protein (Figure 5A-5B) and the extent of liver fibrosis (Figure 5C-5D) by 50% when compared to mice injected CCl₄ alone. In summary, these data proved the ability of OPN to respond to drug-induced liver injury and to oxidant stress.

WT show more CCl₄-induced chronic liver injury and fibrosis than *Opn*^{-/-} mice

Fibrosis typically develops due to chronic liver injury. To decipher the role of OPN in the progression of liver disease, we tested whether chronic CCl₄-injection could lead to differences in the extent of liver fibrosis. CCl₄-injected C57BL/6J WT showed greater alanine aminotransferase (ALT) activity and more inflammation, hepatocyte ballooning degeneration and necrosis than *Opn*^{-/-} mice (Figure 6A-6E). Cytochrome P450 2E1 (CYP2E1) expression was similar in WT and *Opn*^{-/-} mice, indicating that the extent of liver injury in these mice was not due to different CCl₄metabolism (Figure 6F).

In addition, CCl₄-injected WT mice presented elevated collagenous proteins, portal fibrosis, bridging fibrosis, scar thickness, Brunt fibrosis score and Sirius red and collagen-I morphometry compared to *Opn*^{-/-} mice (Figure 7A-7E). The above results were validated in WT and *Opn*^{-/-} 129sv mice (Supplementary Figures 5-6). Transgenic mice overexpressing OPN in hepatocytes (*Opn*^{HEP} Tg) and injected CCl₄ for 1 month showed similar ALT activity, necrosis and inflammation but significant periportal, bridging and sinusoidal fibrosis along with increased collagen-I scar thickness compared to WT mice (Figure 8). Moreover, *Opn*^{HEP} Tg mice developed spontaneous perivenular, perisinusoidal and portal fibrosis over time (1 yr) in the absence of any profibrogenic treatment (Supplementary Figure 7). In aggregate, the data suggest that OPN plays a major role in chronic CCl₄-induced hepatic fibrosis by regulating scar formation.

WT mice show significant liver fibrosis under chronic TAA-treatment compared to *Opn*^{-/-} mice

To confirm the results obtained under chronic CCl₄-injection, we used the TAA-treatment as a second model of chronic drug-induced liver fibrosis. Sirius red/fast green staining and collagen-I IHC showed stage >3 fibrosis in TAA-treated WT and ~1-2 in *Opn*^{-/-} mice with clear induction of collagen-I deposition in TAA-treated WT compared to *Opn*^{-/-} mice, extensive portal fibrosis, bridging fibrosis and a ~3-fold increase in scar thickness (Supplementary Figure 8A-8B). Thus, fibrosis was more distinct in TAA-treated WT than in

Opn^{-/-} mice as quantified by the Brunt fibrosis score and by the Sirius red and collagen-I morphometry (Supplementary Figure 8C-8E).

Collectively, these results suggest that increased OPN expression *per se* or following chronic liver injury and oxidant stress can stimulate collagen-I deposition *in vivo*. In addition, the *in vitro* studies demonstrate that intracellular OPN plays an autocrine role in regulating collagen-I expression in HSC. Moreover, treatment with rOPN to resemble the paracrine actions of secreted OPN, increases HSC invasion, chemotaxis and wound healing potential and up-regulates collagen-I via integrin $\alpha_v\beta_3$ engagement, activation of PI3K-pAkt and NF κ B signaling (Supplementary Figure 9).

DISCUSSION

It is becoming clearer that OPN is significantly induced during liver injury both in humans and in rodents (4-6, 17). In the past few years, work from several groups (4-6, 17, 34) studied the potential role of OPN in liver fibrosis albeit with inconclusive results. Studies by Lee *et al* (5) demonstrated an OPN increase in the culture medium from culture-activated HSC and under oral CCl₄-administration; however, no mechanistic studies were performed to dissect how OPN regulates collagen-I protein deposition. Lorena *et al* (6) suggested increased susceptibility to CCl₄-injection in *Opn*^{-/-} mice. Although the authors claimed that the protection observed in WT mice was due to enhancement of hepatocyte survival and reduction in NOS2 expression; yet, they neither provided IHC for cell survival markers nor measured the concentration of NO $\cdot$ or ONOO $\cdot$ to support their conclusions, and no studies on collagen-I regulation were performed. Lastly, a recent publication from Syn *et al* (4) proposes a role for the Hedgehog signaling pathway in activating OPN and promoting fibrosis progression in non-alcoholic steatohepatitis; however, it is not clear which OPN isoform the authors are referring to and it is *Gli1*, and not *Gli2*, expression that it is widely considered the most reliable readout for cells undergoing active Hedgehog signaling.

Thus, there is a well timed need for dissecting the molecular mechanism on how this matricellular protein could regulate the fibrogenic response to liver injury, and specifically collagen-I protein expression by HSC. Currently, there are many unresolved questions on how OPN could act as a feed-forward mechanism to promote scarring, among these are: Does extracellular OPN have the ability to increase the HSC profibrogenic potential and HSC-derived collagen-I protein? Which HSC receptors are involved in the profibrogenic cascade triggered by OPN? What intracellular signals are activated upon OPN-receptor binding that drive the collagen-I increase in HSC? Is autocrine OPN signaling involved in regulating collagen-I in HSC? Is OPN sensitive to oxidant stress? Which OPN isoforms appear during the course of liver fibrosis? Does OPN induce sinusoidal fibrosis?

Thus, the overall goal of this work was to address these questions, identify the mechanism for the OPN-driven collagen-I up-regulation in HSC and determine the functional role of OPN in the pathogenesis of liver fibrosis. The idea that OPN mediates liver fibrosis is relevant for several reasons. First, because the observation that OPN is up-regulated in HSC during hepatic injury provides an excellent conceptual advance in our understanding of liver fibrogenesis, as it appears that OPN up-regulates HSC collagen-I protein in an autocrine and paracrine fashion. Second, it supports the clinicopathological finding that injury occurring in the central region is accompanied by fibrosis. Third, it opens the possibility of linking a soluble cytokine/matricellular protein with fibrogenesis. Lastly, the identification of the mechanism and mediators involved in the profibrogenic actions of OPN could help in devising strategies for therapeutic targeting.

Our *in vitro* experiments validated the hypothesis of the profibrogenic and proinvasive actions of OPN in HSC. Mechanistic studies identified the HSC membrane proteins engaged by OPN and the proximal signaling molecules/oxidant stress-sensitive kinases activated upon OPN binding that trigger the fibrogenic cascade. The experimental data identified integrin $\alpha_v\beta_3$ as an efficient conveyor of the OPN-mediated profibrogenic actions in HSC and pointed at the PI3K-pAkt activation and the NF κ B signaling pathway as highly involved in this process.

Since OPN signals via integrins and CD44, it is feasible that following liver injury, a ligand for $\alpha_v\beta_3$ integrin such as OPN accumulates in the *space of Disse* and acts in a $\alpha_v\beta_3$ integrin-dependent manner to maintain collagen-I induction, HSC activation, invasion and migration. Because OPN binds ECM proteins (35-36), this binding ability may enhance HSC activation, migration and invasion, key HSC features for the development of fibrosis. The finding that blocking CD44 did not prevent the effect of rOPN on collagen-I may be related to the ability of hyaluronic acid -a glycosaminoglycan synthesized during HSC activation (24, 37)- to bind CD44; thus, competitive inhibition between hyaluronic acid and rOPN for CD44 binding could occur in HSC, although this possibility needs further investigation.

Several observations support the role for the PI3K-pAkt activation and the NF κ B signaling pathway in the effects mediated by rOPN on collagen-I. First, rOPN rapidly increased PI3K, the ratios pAkt 473Ser/Akt, pIKK α,β 176/180Ser/IKK α,β and pI κ B α 32Ser/I κ B α as well as nuclear translocation of p65. Second, inhibitors of PI3K activation and NF κ B signaling blunted the rOPN-mediated increase in intra- and extracellular collagen-I protein. Third, blockade of $\alpha_v\beta_3$ integrin signaling with a neutralizing Ab and incubation with wortmannin or LY294002 prevented the induction of PI3K, the increase in the ratios pAkt 473Ser/Akt, pIKK α,β 176/180Ser/IKK α,β and pI κ B α 32Ser/I κ B α , nuclear translocation of p65 and the up-regulation of collagen-I protein by rOPN. Involvement of the mTOR cascade was ruled out since rOPN altered neither mTOR-p70S6K expression nor mTOR phosphorylation. Therefore, this study linked extracellular and/or secreted OPN (i.e. paracrine effect) with $\alpha_v\beta_3$ integrin binding, PI3K-pAkt activation, NF κ B signaling and scarring.

Work from several laboratories (3-6), including our own, suggests that HSC are an important source of OPN during liver injury. To date, OPN was believed to exert its effects by binding the RGD motif in integrins and the cell-surface receptor CD44; however, an intracellular function of OPN in liver fibrosis was largely unknown. Because HSC isolated from *Opn*^{-/-} mice were less profibrogenic than those from WT mice and infection of HSC with Ad-OPN increased intracellular collagen-I, these results suggested a novel autocrine mechanism whereby intracellular OPN could modulate collagen-I deposition in HSC. Alternatively, extracellular OPN either from HSC or from neighboring cells may activate HSC through its receptor ($\alpha_v\beta_3$ integrin), as suggested above; thus, creating a positive feedback loop.

To further validate our hypothesis, we then assessed whether OPN contributed to the fibrogenic response *in vivo* using two mouse models of drug-induced liver injury. The data from human samples and from the mouse models showed that most of the OPN found in liver injury appears to be cleaved at least at the end-point of the experiments. The role of each cleaved isoform in regulating the fibrogenic response to liver injury as well as the identification of the proteases that cleave hepatic OPN is currently under active investigation in our laboratory since additional integrin binding sites, other than $\alpha_v\beta_3$ integrin, are likely to be uncovered by proteolytic processing of the protein.

Upon the onset of liver injury in mice, the increase in OPN likely results from oxidant stress because CCl₄ and TAA metabolism via cytochrome P450s generates a considerable amount

of free radicals (33), and the *in vitro* data demonstrated the OPN-responsiveness to oxidant stress, which was blocked by antioxidant treatment. Furthermore, co-treatment with SAM, known to elevate glutathione levels, prevented the increase in OPN and the fibrogenic response in WT mice injected CCl₄ for 1 month.

Although it is possible that the chronic effects of *Opn* ablation could be secondary to its effects on liver injury itself (i.e. inflammation and ductular reaction, unpublished observations), the data clearly reveal a direct action of OPN on collagen-I protein expression, a key event in liver fibrosis. Hence, OPN appears to induce scarring *per se*. This is indeed also supported by the finding that while ALT activity and the necrosis and inflammation scores were similar, there was increased portal, bridging and sinusoidal fibrosis along with enhanced width of the collagenous septa in CCl₄-injected *Opn*^{HEP} Tg mice compared to their WT littermates. Notably, *Opn*^{HEP} Tg mice developed spontaneous fibrosis over time while WT mice did not. Lastly, in line with the results using *Opn*^{HEP} Tg mice and the *in vitro* data, fibrillar collagen-I content and scar thickness was significantly lowered by OPN ablation *in vivo*. It is likely that secreted OPN allows paracrine signaling to HSC, while endogenous OPN expression in HSC signals in an autocrine fashion amplifying the fibrogenic response. The cell- and matrix-binding ability of OPN may also facilitate a proper stromal and fibrillar collagen network organization. Overall, it is reasonable to propose that OPN may drive the fibrogenic response, among others, by directly regulating collagen-I deposition. Thus, OPN emerges as a key soluble cytokine and ECM-bound molecule promoting liver fibrosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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List of Abbreviations

ALT	alanine aminotransferase
BSO	L-buthionine sulfoximine
CCl ₄	carbon tetrachloride
CYP2E1	cytochrome P450 2E1
ECM	extracellular matrix

IHC	immunohistochemistry
IOD	integrated optical density
HSC	hepatic stellate cells
MMP	matrix metalloprotease
MO	mineral oil
OPN	osteopontin
<i>Opn</i>^{-/-}	osteopontin knockout mice
<i>Opn</i>^{HEP} Tg	osteopontin transgenic mice in hepatocytes
PDTC	pyrrolidine dithiocarbamate
rOPN	recombinant OPN
SAM	<i>S</i> -adenosylmethionine
α-SMA	α -smooth muscle actin
TAA	thioacetamide
WT	wild-type

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Figure 1. Profibrogenic effects of rOPN in primary HSC

Primary rat HSC cultured for 7 days were treated with 0-50 nM rOPN for 6 and 24 hours. Western blot analysis of intra- and extracellular collagen-I, extracellular MMP13 and β -tubulin. Gelatine zymography showing extracellular pro-, intermediate and active MMP2 and MMP9 (A, left). Western blot analysis of intracellular collagen-I, α -SMA and actin in rat HSC treated with 0-50 nM rOPN for 24 hours (A, right). Human HSC cultured for 7 days were treated with 0-100 nM rOPN for 1 and 24 hours. Western blot analysis of intra- and extracellular collagen-I, extracellular MMP13 and MMP1 and β -tubulin. Pro-MMP2 activity was measured by gelatine zymography. Extracellular MMP9 activity was undetectable (not shown) (B). Western blot analysis of intracellular collagen-I and actin in HSC from WT and *Opn*^{-/-} mice. Extracellular collagen-I was undetectable (C). Western blot analysis of intracellular collagen-I and OPN as well as extracellular OPN in rat HSC infected with Ad-LacZ or with Ad-OPN for 48 hours. Extracellular collagen-I was undetectable (D). Results are expressed as average values. Experiments were performed in triplicates four times. ** $p < 0.01$ and *** $p < 0.001$ for rOPN, *Opn*^{-/-} and Ad-OPN vs control, WT and Ad-LacZ, respectively.

Figure 2. Role of $\alpha_v\beta_3$ integrin and the PI3K-pAkt-NF κ B signaling pathway in the rOPN-mediated effects on collagen-I

Primary rat HSC cultured for 7 days were incubated with 0-50 nM rOPN plus 5 μ g/ml of non-immune IgG, anti- $\alpha_v\beta_3$ or anti-CD44 for 6 hours. Western blot analysis of intra- and extracellular collagen-I and actin (A). Western blot analysis of PI3K, pAkt⁴⁷³Ser, Akt and β -tubulin up to 3 hours of 0-50 nM rOPN treatment in rat HSC (B). Western blot analysis of pIKK α,β ^{176/180}Ser, IKK α,β , pI κ B α ³²Ser, I κ B α , nuclear and cytosolic p65 and actin up to 30 min of 0-50 nM rOPN treatment in rat HSC (C). Western blot analysis of mTOR, p70S6K and actin up to 1 hour of 0-50 nM rOPN treatment in rat HSC (D). Results are expressed as average values. Experiments were performed in triplicates four times. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ for rOPN-treated vs control at any given time-point. • $p < 0.05$, •• $p < 0.01$ and ••• $p < 0.001$ for co-treated vs Ab-treated.

Figure 3. Blocking $\alpha_v\beta_3$ integrin, PI3K-pAkt activation and the NF κ B signaling pathway prevents the rOPN-mediated effects on collagen-I

Primary rat HSC cultured for 7 days were treated with 0-50 nM rOPN or co-treated with 0-10 μ M wortmannin, 0-10 μ M LY294002, 0-10 μ M PDTc, 0-5 μ M CAY10512 or with 5 μ g/ml of non-immune IgG or a neutralizing Ab to integrin $\alpha_v\beta_3$. Western blot analysis showing that the rOPN-mediated induction of collagen-I in HSC was blunted by 0.1, 1 and 10 μ M wortmannin (A, top), LY294002 (A, bottom), PDTc (B, top) and CAY10512 (B, middle). Infection of HSC with Ad-NF κ B-Luc for 48 hours and treatment with 50 nM rOPN for 24 hours increased luciferase activity over that of non-treated Ad-NF κ B-Luc-infected cells (B, bottom). Both, an integrin $\alpha_v\beta_3$ Ab and wortmannin blunted the rOPN-mediated induction of the ratios pIKK α , β ^{176/180}Ser/IKK α , β , pI κ Ba³²Ser/I κ Ba and nuclear/cytosolic p65 (C). A neutralizing Ab to integrin $\alpha_v\beta_3$ prevented the induction of PI3K, the ratio pAkt⁴⁷³Ser/Akt and collagen-I by rOPN in HSC (D). Results are expressed as average values. Experiments were performed in triplicates four times. * $p < 0.01$ and *** $p < 0.001$ for rOPN-treated vs control. • $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ for inhibitor or Ab-cotreated vs rOPN-treated or control.

Figure 4. OPN expression is induced during liver injury and under oxidant stress conditions

Western blot analysis of cleaved OPN, total collagen-I and actin in livers from control and from patients with stage-3 HCV-induced cirrhosis (**A**). Western blot analysis of cleaved OPN and actin in livers of mice injected with CCl₄ for 24 hours (acute liver injury), with CCl₄ for 1 month or with TAA for 4 months (chronic liver injury) (**B**). In (**A**) and (**B**) fully modified (glycosylated and phosphorylated) monomeric OPN was not detected. Immunocytochemistry for OPN in primary HSC isolated from WT mice and cultured for 6 days (**C**, left). IHC depicting significant OPN expression in HSC, biliary epithelial cells and hepatocytes at 1 month of CCl₄-injection (**C**, middle) and in HSC, biliary epithelial cells, oval cells and hepatocytes after 4 months of TAA-treatment (**C**, right). The insets show OPN⁺ HSC in both models (→). Immunofluorescence showing co-localization of OPN⁺ with α-SMA⁺ (a HSC activation marker) after 4 months of TAA-treatment (**D**). Western blot analysis of intracellular OPN and β-tubulin in HSC in the presence of two prooxidants (H₂O₂ and BSO) and an antioxidant (GSH-EE: glutathione-ethyl ester) (**E**). Results are expressed as average values. Experiments were performed in triplicates four times. ****p*<0.001 for HCV, CCl₄, TAA or prooxidant treated *vs* control, mineral oil (MO) or water, respectively. ****p*<0.001 for BSO + GSH-EE co-treated *vs* BSO-treated.

Figure 5. SAM protects WT mice from CCl₄-induced chronic liver injury

C57BL/6J WT mice were injected MO, SAM plus MO, CCl₄ or CCl₄ plus SAM for 1 month. Co-treated mice showed decreased OPN expression (A), which was quantified by morphometry analysis (B). Likewise, fibrosis was less apparent in co-treated mice than in CCl₄-injected mice as shown by Sirius red/fast green staining (C) and morphometry analysis (D). Results are expressed as mean values $\pm$ SEM. $n=8$ /group; *** $p<0.001$ for CCl₄ or CCl₄ + SAM vs MO or SAM; ** $p<0.01$ for CCl₄ + SAM vs CCl₄.

Figure 6. WT mice show more CCl₄-induced chronic liver injury than Opn^{-/-} mice
 C57BL/6J WT and Opn^{-/-} mice were injected CCl₄ or MO for 1 month. H&E staining revealed more centrilobular necrosis (blue arrow), centrilobular inflammation (yellow arrow) and hepatocyte ballooning degeneration (orange arrow) in CCl₄-injected WT than in Opn^{-/-} mice (A). ALT activity (B), centrilobular and parenchymal inflammation scores (C), hepatocyte ballooning degeneration score (D) and centrilobular and parenchymal necrosis scores (E). A Western blot analysis showing similar CYP2E1 expression in WT and Opn^{-/-} mice (F). Results are expressed as mean values ± SEM. *n*=8/group; ****p*<0.001 for CCl₄ vs MO; **p*<0.05, ***p*<0.01 and ****p*<0.001 for Opn^{-/-} + CCl₄ vs WT + CCl₄.

Figure 7. WT mice show more CCl₄-induced liver fibrosis than *Opn*^{-/-} mice

C57BL/6J WT and *Opn*^{-/-} mice were injected CCl₄ or MO for 1 month. Sirius red/fast green staining indicated fibrosis stage ~3 in CCl₄-injected WT and ~1-2 in CCl₄-injected *Opn*^{-/-} mice (portal $\blacktriangleright$ and bridging $\blacktriangleleft$ fibrosis) as well as greater scar thickness in WT compared to *Opn*^{-/-} mice ($\leftrightarrow$) (A). Collagen-I IHC confirmed the extent of portal fibrosis ($\blacktriangleright$), bridging fibrosis ($\blacktriangleleft$) and scar thickness ($\leftrightarrow$) in CCl₄-injected mice (B). Brunt fibrosis score (C), Sirius red morphometry (D) and collagen-I morphometry analysis (E). Results are expressed as mean values $\pm$ SEM. $n=8$ /group; ** $p<0.01$ and *** $p<0.001$ for CCl₄ vs MO; ** $p<0.01$ and *** $p<0.001$ for *Opn*^{-/-} + CCl₄ vs WT + CCl₄.

Figure 8. *Opn*^{HEP} Tg mice in C57BL/6J genetic background show more CCl₄-induced fibrosis than WT mice
 WT and *Opn*^{HEP} Tg mice were injected MO or CCl₄ for 1 month. H&E staining revealed similar centrilobular necrosis (▶) and inflammation (▶) in CCl₄-injected *Opn*^{HEP} Tg and in WT mice (A). ALT activity (B). Necrosis and inflammation scores (C). Sirius red/fast green

staining and IHC for collagen-I demonstrated more portal fibrosis (✚), bridging fibrosis (✚) and sinusoidal fibrosis (✚) in CCl₄-injected *Opn*^{HEP} 4 Tg than in WT mice (**D-E**). Brunt fibrosis score, collagen-I and Sirius red/fast green morphometry (**F**). Results are expressed as mean values ± SEM. *n*=8/group; ***p*<0.01 and ****p*<0.001 for CCl₄ vs MO; ••*p*<0.01 for *Opn*^{HEP} Tg + CCl₄ vs WT + CCl₄.