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Decorin deficiency promotes hepatic carcinogenesis

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ABSTRACT

Hepatocellular carcinoma represents one of the most-rapidly spreading cancers in the world. In the majority of cases, an inflammation-driven fibrosis or cirrhosis precedes the development of the tumor. During malignant transformation, the tumor microenvironment undergoes qualitative and quantitative changes that modulate the behavior of the malignant cells. A key constituent for the hepatic microenvironment is the small leucine-rich proteoglycan decorin, known to interfere with cellular events of tumorigenesis mainly by blocking various receptor tyrosine kinases (RTK) such as EGFR, Met, IGF-IR, PDGFR and VEGFR2. In this study, we characterized cell signaling events evoked by decorin deficiency in two experimental models of hepatocarcinogenesis using thioacetamide or diethyl nitrosamine as carcinogens. Genetic ablation of decorin led to enhanced tumor occurrence as compared to wild-type animals. These findings correlated with decreased levels of the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ and a concurrent elevation in retinoblastoma protein phosphorylation via cyclin dependent kinase 4. Decreased steady state $p21^{Waf1/Cip1}$ levels correlated with enhanced expression of transcription factor AP4, a known transcriptional repressor of $p21^{Waf1/Cip1}$, and enhanced c-Myc protein levels. In addition, translocation of β-catenin was a typical event in diethyl nitrosamine-evoked tumors. In parallel, decreased phosphorylation of both c-Myc and β-catenin was observed in $Dcn^{-/-}$ livers likely due to the hindered GSK3β-mediated targeting of these proteins to proteasomal degradation. We discovered that in a genetic background lacking decorin, four RTKs were constitutively activated (phosphorylated), including three known targets of decorin such as PDGFRα, EGFR, IGF-IR, and a novel RTK MSPR/RON. Our findings provide powerful genetic evidence for a crucial in vivo role of decorin during hepatocarcinogenesis as lack of decorin in the liver and hepatic stroma facilitates experimental carcinogenesis by providing an environment devoid of this potent pan-RTK inhibitor. Thus, our results support future utilization of decorin as an antitumor agent in liver cancer.

1. Introduction

Hepatocellular carcinoma (HCC) represents the most frequent type of primary liver tumors, and it is the third most common fatal malignancy disease worldwide (El-Serag and Rudolph, 2007). The highest HCC incidence and mortality are observed in Eastern Asia and central Africa, but its frequency has been rapidly increasing in Europe and in the United States in the last decades. The major risk factors are hepatitis B and C infection, aflatoxin B1 intake from contaminated food and excessive alcohol abuse (Llovet et al., 2003; Sherman, 2010). Primary HCC often evolves on cirrhotic or chronic inflammation induced fibrotic background, although this is not essential for tumor formation.

The extracellular matrix (ECM) is an acellular compartment of organs, made of macromolecules providing support for the cells, they embrace. It is a key participant in the tissue specific organization of cells and the establishment of their differentiated function. ECM together with the nonparenchymal cells creates the microenvironment for the parenchymal cells in the tissues. The phenotype and behavior of parenchymal cells are influenced by their interrelationship with the stromal elements in a great extent. However, not only differentiated, but also malignant phenotype is proved to be driven by the microenvironment of cancer cells, including hepatomas. Their growth, local invasion and metastatic ability all depend on their microenvironment.

During malignant transformation, the tumorous ECM undergoes qualitative and quantitative changes. As a result, the matrix is capable to provide the proper environment for tumor progression. Accordingly,

Abbreviations: SLRP, small leucine-rich proteoglycan; RTK, receptor tyrosine kinase; TA, thioacetamide; DEN, diethyl nitrosamine; HCC, hepatocellular carcinoma; $Dcn^{-/-}$, decorin null; WT, wild type; $p21^{WAF1/CIP1}$, cyclin-dependent kinase inhibitor p21; AP4, transcription factor AP4; Rb, retinoblastoma protein; GSK3β, Glycogen synthase kinase 3β; GS, glutamine synthetase; AFP, alpha fetoprotein; CDK4, cyclin dependent kinase 4; EGFR, epidermal growth factor receptor; IGF-IR, insulin-like growth factor receptor I; PDGFR, platelet-derived growth factor receptor; MSPR, macrophage stimulating protein receptor; ECM, extracellular matrix; MAPK, mitogen activated protein kinase; ERK1/2, extracellular signal regulated kinase 1/2.

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in the last decade scientific activities have been directed toward the better understanding of the relationship between the tumor and its matrix.

Decorin is a member of the small leucine-rich proteoglycan (SLRP) gene family (Iozzo and Murdoch, 1996; Iozzo, 1999; Iozzo et al., 2011) that is expressed in the stroma of various forms of cancer (Iozzo and Cohen, 1993) and thus has been recently proposed to act as a guardian from the matrix (Neill et al., 2012b), in analogy to p53, the guardian of the genome. Although this proteoglycan is ubiquitously expressed, practically no cells of epithelial origin synthesize it. This implies that decorin is a mesenchyme-specific gene product and that it exerts its effects in a paracrine fashion on endothelial and epithelial cells including cancer cells. Functionally, soluble and matrix-bound decorin modulate various biological processes including collagen fibrillogenesis, wound healing, myogenesis, bone physiology, stem cell biology, immunity, angiogenesis and fibrosis (Reed and Iozzo, 2002; Robinson et al., 2005; Zhang et al., 2009; Seidler et al., 2011; Ichii et al., 2012; Neill et al., 2012a, 2013; Brandan and Gutierrez, 2013; Chen and Birk, 2013; Dunkman et al., 2013; Sofeu Feugaing et al., 2013). Initially identified as a natural inhibitor of transforming growth factor-β (Yamaguchi et al., 1990; Ruoslahti and Yamaguchi, 1991), soluble decorin is emerging as a pan-RTK inhibitor targeting a multitude of RTKs with various affinities, including EGFR, Met, IGF-IR, VEGFR2 and PDGFR (Iozzo, 1999; Schonherr et al., 2005; Schaefer et al., 2007; Goldoni et al., 2009; Iozzo et al., 2011; Khan et al., 2011; Nikitovic et al., 2012; Schaefer and Iozzo, 2012; Seidler, 2012; Baghy et al., 2013; Buraschi et al., 2013; Morrione et al., 2013). Besides initiating signaling, this decorin/RTK interaction can induce caveosomal internalization and receptor degradation (Zhu et al., 2005).

Notably, a combined genetic ablation of decorin and of the tumor suppressor p53 induces the formation of early and aggressive T-cell lymphomas that lead to a premature demise of the compound mice (Iozzo et al., 1999a). These genetic studies are further supported by genetic evidence where loss of the decorin gene is permissive for tumorigenic growth of intestinal tumors with a concurrent increase in the levels of β-catenin (Bi et al., 2008, 2012). Conversely, decorin delivered via adenoviral-mediated gene transduction or systemic administration of recombinant protein to several tumor xenografts, such as breast and prostate carcinomas, inhibits tumorigenic growth (Reed et al., 2002, 2005; Tralhao et al., 2003; Araki et al., 2009; Hu et al., 2009; Buraschi et al., 2012).

A healthy liver contains only a small amount of decorin deposited around the central veins and portal tracts. However, an increased deposition of decorin was observed in the connective tissue septa during fibrogenesis (Dudas et al., 2001; Baghy et al., 2011). In hepatocellular carcinoma, deregulation of several signaling pathways has been described involving RAS/MAPK, IGF, HGF/MET, WNT/β-catenin, EGFR, VEGFR and PDGFR (Villanueva et al., 2007). The observation that decorin affects multiple signaling pathways emanating from various RTKs together with its ability to downregulate β-catenin and Myc levels (Buraschi et al., 2010) and to concurrently induce $p21^{WAF1/CIP1}$ (Santra et al., 1997), prompted us to investigate the role of decorin in mouse models of hepatocarcinogenesis evoked by TA or DEN. We found that a genetic background lacking one single SLRP caused a constitutive activation of various RTKs thus providing a mechanistic explanation for the increased incidence of hepatocellular carcinomas in decorin-null livers following experimental carcinogenesis.

2. Results

2.1. Decorin-null mice are more susceptible to experimentally-induced liver cancer

Metabolization of thioacetamide (TA) in hepatocytes via cytochrome p450 causes fibrosis, and subsequently hepatic cirrhosis. Thus, chronic TA exposure provokes hyper-regeneration of hepatocytes initiating hepatocarcinogenesis in the cirrhotic liver (Becker, 1983; Camus-Randon et al., 1996) (Fig. 1A,B). TA-induced tumors showed abundant cytoplasm with strong eosinophilic staining, and were surrounded by a connective tissue capsule. In contrast, high dose of diethyl nitrosamine (DEN) causes DNA mutations directly without evoking overt fibrotic changes (Heindryckx et al., 2009) (Fig. 1C,D). These tumor cells had narrow basophilic cytoplasm, and often invaded blood vessels.

Thioacetamide treatment induced liver tumor in ~93% of the mice lacking the decorin gene in contrast to only ~22% tumor prevalence observed in wild-type counterparts ($n = 15$ each, $p < 0.001$, Fig. 1E). Moreover, $Dcn^{-/-}$ mice developed more tumors upon DEN treatment as compared to wild type (44% vs. 27%) (Fig. 1F), although the difference did not reach statistical significance ($n = 10$, $p = 0.12$). In parallel, significantly higher tumor volume was calculated in $Dcn^{-/-}$ animals than that of wild type ones treated with TA (100.4 mm^3 vs. 7.6 mm^3 respectively ($p < 0.01$)) as well as DEN (23.7 mm^3 tumor volume in knockout and 3.4 mm^3 in wild type mice) (Fig. 1G). Thus, ablation of decorin sensitizes liver for tumor formation, and this effect is more pronounced in hepatocarcinogenesis with a cirrhotic background such as that induced by TA.

2.2. Qualitative and quantitative changes of decorin in TA- and DEN-induced tumors

Under unchallenged conditions, decorin was primarily located in the peri-portal areas and around the central veins of the liver (Fig. 2A,B). As thioacetamide induces cirrhosis and thus a stromal activation, the amount of decorin seemed to be increased in TA-treated livers of wild type animals as detected by immunostaining (Fig. S1, Fig. 2C,D). Accumulation of decorin was seen in fibrotic septa, and focal deposits were also observed in the tumor stroma (Fig. 2C,D). The immunodistribution of decorin was overall similar in DEN- and TA-induced tumors (Fig. 2E, F). Tumor foci were surrounded by well-defined deposits of immunoreactive decorin. Interestingly, we observed not only quantitative changes but also qualitative changes in the glycanation of decorin. Unlike control samples where decorin appeared as a smear between 60 and 80 kDa, the proteoglycan in TA-treated livers was significantly retarded and centered at ~90 kDa (Fig. 2G). DEN exposure also caused a shift toward higher molecular mass, although to a lesser degree (Fig. 2G). Notably, quantification of several experiments showed a significant induction of high molecular weight decorin content in both experimental animal models ($p < 0.001$ and $p < 0.05$, respectively; Fig. 2H). These observations suggest that the expression of decorin is dynamically modulated during hepatic carcinogenesis, especially in the TA-driven tumors where the stroma plays a more prominent role than in DEN-driven tumors.

2.3. Lack of decorin accelerates cell cycle progression

Next, we investigated the status of the cyclin-dependent kinase inhibitor $p21^{Waf1/Cip1}$ since lack of decorin prevents its upregulation in TA-induced liver cancer (Baghy et al., 2013). In control animals, low levels of $p21^{Waf1/Cip1}$ were detected by immunostaining with no appreciable differences between wild-type and decorin-deficient livers (Fig. S2). Upon TA treatment, a marked induction of $p21^{Waf1/Cip1}$ was observed in the wild-type samples (Fig. 3A, B). Hepatocytes, cells of the connective tissue and tumor cells displayed intense positive staining. In contrast, no accumulation could be detected in tumor cells lacking decorin gene (Fig. 3E, F). Diethyl nitrosamine increased the amount of $p21^{Waf1/Cip1}$ as well (Fig. 3C, D), but decorin deficiency had less impact on this process, as a considerable immunopositivity was observed in $Dcn^{-/-}$ tumor cell nuclei (Fig. 3G,H).

Next, we performed qPCR for the genes encoding $p21^{Waf1/Cip1}$ (*CDKN1A*) and the transcription factor AP4 (*TFAP4*), a c-MYC-inducible basic helix–loop–helix leucine-zipper transcription factor that represses *CDKN1A* expression. TA and DEN induced a 140-fold and a 20-fold elevation of *CDKN1A*, respectively, when compared to control samples

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