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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

ORIGINAL RESEARCH

Pigment Epithelium-Derived Factor (PEDF) Inhibits Wnt/β-catenin Signaling in the Liver

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SUMMARY

The absence of pigment epithelium-derived factor (PEDF) in hepatocellular carcinoma (HCC) enhances Wnt/β-catenin signaling. Genomic profiling of PEDF knockout livers correlates with gene expression signatures of human HCC associated with aberrant Wnt/β-catenin signaling. PEDF is an endogenous inhibitor of Wnt/β-catenin signaling.

BACKGROUND & AIMS: Pigment epithelium-derived factor (PEDF) is a secretory protein that inhibits multiple tumor types. PEDF inhibits the Wnt coreceptor, low-density lipoprotein receptor-related protein 6 (LRP6), in the eye, but whether the tumor-suppressive properties of PEDF occur in organs such as the liver is unknown.

METHODS: Wnt-dependent regulation of PEDF was assessed in the absence and presence of the Wnt coreceptor LRP6. Whole genome expression analysis was performed on PEDF knockout (KO) and control livers (7 months). Interrogation of Wnt/β-catenin signaling was performed in whole livers and human hepatocellular carcinoma (HCC) cell lines after RNA interference of PEDF and restoration of a PEDF-derived peptide. Western diet feeding for 6 to 8 months was used to evaluate whether the absence of PEDF was permissive for HCC formation (n = 12/group).

RESULTS: PEDF levels increased in response to canonical Wnt3a in an LRP6-dependent manner but were suppressed by noncanonical Wnt5a protein in an LRP6-independent manner. Gene set enrichment analysis (GSEA) of PEDF KO livers revealed induction of pathways associated with experimental and human HCC and a transcriptional profile characterized by Wnt/β-catenin activation. Enhanced Wnt/β-catenin signaling occurred in KO livers, and PEDF delivery in vivo reduced LRP6 activation. In human HCC cells, RNA interference of PEDF led to increased levels of activated LRP6 and β-catenin, and a PEDF 34-mer peptide decreased LRP6 activation and β-catenin signaling, and reduced Wnt target genes. PEDF KO mice fed a Western diet developed sporadic well-differentiated HCC. Human HCC specimens demonstrated decreased PEDF staining compared with hepatocytes.

CONCLUSIONS: PEDF is an endogenous inhibitor of Wnt/β-catenin signaling in the liver. *(Cell Mol Gastroenterol Hepatol 2015;1:535–549; http://dx.doi.org/10.1016/j.jcmgh.2015.06.006)*

Keywords: Extracellular Matrix; PEDF; Wnt/β-Catenin.

Hepatocellular carcinoma (HCC) is a major cause of cancer-related deaths worldwide.[1] Genomic profiling has classified HCC based on molecular "signatures" that correlate with biological characteristics and clinical outcomes.[2,3] One finding from these studies is the role of the extracellular matrix (ECM) in determining tumor behavior.[4–6] For instance, modulators of the ECM can activate developmental pathways such as Wnt/β-catenin signaling, thereby connecting liver fibrosis to a signaling pathway that drives hepatocarcinogenesis.[3]

Pigment epithelium-derived factor (PEDF) is a circulating 50-kDa protein with ECM binding domains and broad tumor suppressive properties.[7–10] In PEDF knockout (KO) mice, stromal abnormalities occur in multiple organs including the prostate, pancreas, and liver.[11–15] Endogenous liver levels of PEDF decline in experimental and human cirrhosis, and PEDF delivery ameliorates experimental liver fibrosis.[14,16] PEDF null mice crossed with the $Kras^{G12D}$ mice resulted in marked stromal changes in the pancreas and an invasive malignant phenotype not seen in the $Kras^{G12D}$ mutant mice alone.[15] These results indicate that PEDF regulates tissue matrix quiescence and its absence is permissive for malignant transformation.

The antitumor properties of PEDF are typically attributed to an antiangiogenic effect.[10,17] PEDF, however, inhibits tumor cells in culture, indicating other mechanisms.[17,18] Park et al[19] identified PEDF's ability to inhibit

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Abbreviations used in this paper: **BABB, benzyl alcohol/benzyl benzoate; CM, conditioned medium; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; FDR, false-discovery rate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GO, Gene Ontology; GSEA, gene set enrichment analysis; GSK, glycogen synthase kinase; HCC, hepatocellular carcinoma; KO, knockout; LRP6, low-density lipoprotein receptor-related protein 6; PCR, polymerase chain reaction; PEDF, pigment epithelium-derived factor; SHG, second harmonic generation; siRNA, small interfering RNA; WT, wild type.**

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Wnt/β-catenin signaling in the eye with avid binding to the Wnt coreceptor, low-density lipoprotein receptor-related protein 6 (LRP6). Whether PEDF has systemic effects beyond the eye and inhibits tumor development through an inhibitory effect on Wnt/β-catenin signaling is unclear. Because PEDF is most highly expressed by the liver, a finding recently confirmed in the Human Protein Atlas,[20,21] and modulates Wnt/β-catenin signaling,[19,22] we asked whether PEDF functions as an LRP6 antagonist in the liver.

We establish that canonical Wnt3a ligand directly regulates PEDF levels. PEDF, in turn, inhibits Wnt/β-catenin signaling. Consistent with this, livers from PEDF KO mice have a transcriptional profile closely aligned with murine models of hepatocarcinogenesis and human HCC characterized by aberrant Wnt/β-catenin signaling. Knockout and knock-in experiments demonstrate that PEDF inhibits Wnt/β-catenin signaling in murine livers and human HCC cells through its ability to inhibit LRP6 and β-catenin activity. Finally, a chronic Western diet elicited sporadic HCC formation in PEDF KO mice, while the human HCC specimens demonstrated diminished PEDF staining.

Materials and Methods

Human Hepatocellular Carcinoma, Animals, and Liver Tumor Induction

Archival human HCC tissues and their corresponding adjacent livers from 14 patients were obtained from the VA Connecticut Healthcare System according to an approved institutional review board protocol. The PEDF KO mice were bred with age-matched wild-type (WT) littermates on the C57BL/6J background to generate heterozygous breeding pairs, and then PEDF KO and WT offspring were backcrossed for more than 10 generations.[11] The mice were genotyped using a commercially available polymerase chain reaction (PCR) kit (Sigma-Aldrich, St. Louis, MO). All procedures were approved by the Institutional Animal Care and Use Committee of VA CT Healthcare System. A commercial Western diet—TestDiet 4342 (TestDiet, St. Louis, MO): energy (% kcal) from fat (40%), carbohydrate (44%), protein (16%)—or standard chow was given for 26 to 32 weeks to PEDF KO and age-matched controls (n = 12/group) starting at 8 to 12 weeks of age.

RNA Extraction and Gene Arrays

Frozen whole liver tissue from five PEDF KO animals and WT controls were maintained in liquid nitrogen until total RNA extraction using the TRIzol method (Invitrogen, Carlsbad, CA). TRIzol-extracted RNA was further purified using the Qiagen RNeasy kit (Qiagen, Valencia, CA), yielding high-quality RNA suitable for microarray analyses (RNA integrity number >9). The RNA quality was verified using Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA), and the RNA was quantified by NanoDrop (NanoDrop Technologies, Wilmington, DE). For gene expression analysis, 500 ng of total RNA was used to generate biotin-labeled cRNA using the Illumina Total RNA amplification and labeling kit (Ambion, Austin, TX) according to the manufacturer's instructions. The biotinylated cRNA was labeled with fluorescent dye at the Yale Keck Genomic Core Facility (West Haven, CT), hybridized onto a MouseRef-8 v2.0 Expression BeadChip expression array bead chip (Illumina, San Diego, CA) and scanned.

Expression data were analyzed by Genespring GX12 software (Agilent Technologies) after normalization by 75th percentile shift. Only genes with a present signal (signal above background noise) in more than 50% of samples were included in the analysis. Group samples with gene expression correlation coefficients $\leq$0.95 were excluded (one KO sample). For the statistical analysis, replicate samples were averaged. Differences in gene expression were determined using a moderated t test, and multiple hypothesis testing adjustment was made using Benjamini–Hochberg method at a false-discovery rate (FDR) $\leq$ .05 and by adding a fold expression cutoff of 1.3. Genes differentially expressed in KO mice versus WT were subjected to Gene Ontology (GO) (http://www.geneontology.org) and WikiPathways (http://www.wikipathways.org) enrichment analysis using the hypergeometric test corrected by Benjamini–Yekutieli method at FDR $q \leq$0.05.

To further extend the analysis, gene set enrichment analysis (GSEA) was used (http://www.broadinstitute.org/gsea). GSEA is a computational method that determines whether an a priori defined set of genes shows statistically significant differences between two phenotypes.[23] To identify the gene sets that were statistically significantly enriched, we created a rank-order list by gene expression differences between KO and WT sets. Gene Ontology, KEGG pathways (http://www.genome.jp), Reactome (http://www.reactome.org), Biocarta (http://www.biocarta.org), Pathway interaction database (http://pid.nci.nih.gov), and curated gene sets reflecting changes induced by various chemical and genetic perturbances were used to interpret results. FDR q value was used to rank the results. Gene sets enriched at FDR q value $\leq$ .05 and nominal $P <$ .05 were considered statistically significant. Gene array data were deposited at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE63643.

PEDF and PEDF Peptide Restoration

Human full-length PEDF was generated in human embryonic kidney cells as described elsewhere, and its purity confirmed using Coomassie and silver staining (Invitrogen).[12] PEDF was administered (25 μg/kg bwt) by intraperitoneal injection on alternate days for a period of 4 weeks.[24] A 34-mer of human PEDF corresponding to amino acids 44–77 has been previously shown to inhibit neovascularization and inhibit tumor growth, but its role in Wnt signaling is unclear.[17,25] We interrogated Wnt signaling with a 34-mer that was commercially obtained (NeoBiolab, Cambridge, MA) and used at a concentration of 100μM to evaluate Wnt/β-catenin signaling in vitro.

Cell Culture

The human HCC cell lines HepG2 and Huh7 were obtained from the American Type Culture Collection (Manassas, VA), propagated, and kept at the Yale Liver