BASIC AND TRANSLATIONAL—PANCREAS

A High-Fat Diet Activates Oncogenic Kras and COX2 to Induce Development of Pancreatic Ductal Adenocarcinoma in Mice

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BACKGROUND & AIMS: Obesity is a risk factor for pancreatic ductal adenocarcinoma (PDAC), but it is not clear how obesity contributes to pancreatic carcinogenesis. The oncogenic form of KRAS is expressed during early stages of PDAC development and is detected in almost all of these tumors. However, there is evidence that mutant KRAS requires an additional stimulus to activate its full oncogenic activity and that this stimulus involves the inflammatory response. We investigated whether the inflammation induced by a high-fat diet, and the accompanying up-regulation of cyclooxygenase-2 (COX2), increases Kras activity during pancreatic carcinogenesis in mice. **METHODS:** We studied mice with acinar cell–specific expression of KrasG12D (LSL-Kras/Ela-CreERT mice) alone or crossed with COX2 conditional knockout mice (COXKO/LSL-Kras/Ela-CreERT). We also studied LSL-Kras/PDX1-Cre mice. All mice were fed isocaloric diets with different amounts of fat, and a COX2 inhibitor was administered to some LSL-Kras/Ela-CreERT mice. Pancreata were collected from mice and analyzed for Kras activity, levels of phosphorylated extracellular-regulated kinase, inflammation, fibrosis, pancreatic intraepithelial neoplasia (PanIN), and PDACs. **RESULTS:** Pancreatic tissues from LSL-Kras/Ela-CreERT mice fed high-fat diets (HFDs) had increased Kras activity, fibrotic stroma, and numbers of PanINs and PDACs than LSL-Kras/Ela-CreERT mice fed control diets; the mice fed the HFDs also had shorter survival times than mice fed control diets. Administration of a COX2 inhibitor to LSL-Kras/Ela-CreERT mice prevented these effects of HFDs. We also observed a significant reduction in survival times of mice fed HFDs. COXKO/LSL-Kras/Ela-CreERT mice fed HFDs had no evidence for increased numbers of PanIN lesions, inflammation, or fibrosis, as opposed to the increases observed in LSL-Kras/Ela-CreERT mice fed HFDs. **CONCLUSIONS: In mice, an HFD can activate oncogenic Kras via COX2, leading to pancreatic inflammation and fibrosis and development of PanINs and PDAC. This mechanism might be involved in the association between risk for PDAC and HFDs.**

Keywords: Pancreatic Cancer; Signal Transduction; Oncogene; Prostaglandin.

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer death in the United States, with a dismal 5-year survival rate of 3%–5%.[1] In 2012, it was estimated that 43,920 Americans will be diagnosed with PDAC and 37,390 of those patients will die from the disease, most within <1 year of diagnosis.[1] Several studies have demonstrated an elevated body mass index and excessive body weight (BW) to be significant risk factors for PDAC.[2–6] This is a great concern, as obesity rates are rising worldwide.[7] The number of obese individuals in the United States alone has doubled to 59 million during the past 2 decades. One of the key causes of this surge in obesity is consumption of a high-fat diet (HFD). Therefore, determining the mechanistic effect of such a diet on PDAC development at the molecular level is critical.

Mutations of oncogenic KRAS occur almost universally in PDAC patients.[8–12] Ras is a guanine nucleotide-binding protein that acts as a binary switch to activate several important cellular signaling pathways. Normally, Ras is loaded with guanosine diphosphate and is inactive until acted upon by guanine exchange factors that are under the control of receptors for a variety of growth factors, cytokines, and other signaling molecules. Guanine exchange factors exchange guanosine diphosphate to guanosine triphosphate (GTP), allowing Ras to be active and able to stimulate downstream pathways. Downstream signaling pathways stimulated by active Ras, which are important in cell growth, differentiation, and cell survival, include phosphoinositide 3-kinase, mitogen-activated protein kinase, and Ral.[10–12] Normally, the effects of Ras activation

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Abbreviations used in this paper: α-SMA, α-smooth muscle actin; BAC, bacterial-artificial-chromosome; BW, body weight; CD, control diet; COX, cyclooxygenase; Ela, elastase; ERK, extracellular signal-regulated kinase; GTP, guanosine triphosphate; HFD, high-fat diet; IHC, immunohistochemistry; KO, knockout; PanIN, pancreatic intraepithelial neoplasia; PBS, phosphate-buffered saline; PDAC, pancreatic ductal adenocarcinoma; Pdx, pancreatic and duodenal homeobox 1; PSC, pancreatic stellate cell.

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are transient due to intrinsic GTP hydrolysis activity of the Ras molecule, which is greatly accelerated by GTPase-activating proteins. Hydrolysis of GTP to guanosine diphosphate returns Ras to its inactive state. In contrast, oncogenic KRAS results from point mutations, most specifically in codon 12, which limits the ability of GTPase-activating proteins to accelerate GTP hydrolysis.[10–12] Once loaded with GTP, oncogenic Ras displays prolonged signaling. For this reason, oncogenic Ras is often said to be constitutively active.[13] However, a recent analysis showed that this is not the case.[14] Rather, oncogenic Kras activity requires a stimulus, but once stimulated, it is slow to return to its basal state. In addition, because high Ras activity can generate inflammatory mediators via activation of several mechanisms, including nuclear factor−κB, cyclooxygenase 2 (COX2), signal transducer and activator of transcription-3, and others, activation of oncogenic Kras beyond a threshold initiates a Ras-inflammation feed-forward loop in which elevated expression of inflammatory mediators activate and prolong Kras activity.[14,15]

Initiation of PDAC is accompanied by inflammation, desmoplasia, and the formation of noninvasive ductal precancerous lesions, pancreatic intraepithelial neoplasia (PanINs).[16] PanINs are an initial step in the progression of PDAC.[16,17] Investigators previously showed that consumption of an HFD induced pancreatic inflammation and accelerated PanIN development in a mouse model, with developmental expression of oncogenic Kras using a progenitor cell marker ($p48^{Cre}$) that spontaneously generated PanINs.[18] Therefore, we hypothesized that consumption of an HFD can be an inflammatory stimulus to trigger Kras signaling beyond the pathological threshold, initiating development of PDAC. We tested this hypothesis using elastase-CreERT and Pdx1-Cre murine models to activate oncogenic Kras expression. In the elastase-CreERT model in which only adult acinar cells express Kras, minimal spontaneous PanIN formation is observed at early ages.[14,19] In addition, we previously found that COX2 was a critical component of the Ras-inflammation feed-forward loop involved in PDAC initiation by inflammatory stimuli.[14] Therefore, in the current study, we tested whether COX2 was necessary for HFD-induced pathological changes in animals expressing oncogenic Kras.

We found that consumption of an HFD in mice with oncogenic Kras expression increased PanIN formation, fibrosis, inflammation, and PDAC, leading to decreased survival. Control mice without Kras expression fed an HFD and Kras-expressing mice fed a control diet (CD) showed minimal pancreas pathological alterations. This model recapitulates the risk posed by an HFD in humans expressing oncogenic Kras in the pancreas. Feeding an HFD elevated levels of Kras activity and downstream signaling activity. COX2 activity was critical to the HFD-induced pancreatic changes. These observations support Kras activation and COX2 expression as key elements of PDAC initiation and provide mechanistic insight into the connection between obesity and PDAC development, suggesting exciting possibilities for interventions to prevent PDAC.

Methods

Genetically Engineered Transgenic Mice

LSL-$Kras^{G12D}$ mice[20] expressing conditional knock-in mutant $Kras^{G12D}$ were attained from the Mouse Models of Human Cancer Consortium Repository (National Institutes of Health, Bethesda, MD). A full-length elastase (Ela) gene promoter was used to drive the expression of tamoxifen-regulated CreERT specifically in adult pancreatic acinar cells in mice (Ela-CreERT) as described previously.[19] LSL-$Kras^{G12D}$ mice were bred with Ela-CreERT (bacterial-artificial-chromosome [BAC]) mice to generate LSL-Kras/Ela-CreERT (LSL/BAC) double-transgenic mice. COX2 conditional knockout mice (COX2 KO) mice[21] were bred with LSL/BAC mice to generate triple-transgenic mice (COXKO/LSL/BAC) with additional targeted deletion of COX2 in pancreatic acinar cells after Cre activation with tamoxifen. In addition, LSL-$Kras^{G12D}$ mice were crossed with pancreatic-specific Cre (Pdx-1-Cre) mice[22] to generate LSL/Pdx-1 mice.

Treatments in Animals

All animal experiments were reviewed and approved by the University of Texas, MD Anderson Cancer Center Institutional Animal Care and Use Committee. LSL/BAC and BAC mice were given tamoxifen orally for 3 days to initiate oncogenic Kras expression in acinar cells starting at the age of 40 days. Mice were fed either a CD (Test Diet DIO 58Y2; Lab Supply, Fort Worth, TX), in which 10% of energy was derived from fat (LSL/BAC, n = 21; BAC, n = 20; LSL/Pdx-1, n = 19; Pdx-1, n = 9; COXKO/BAC, n = 5; COXKO/LSL/BAC, n = 5), or an HFD (Test Diet DIO 58Y1 van Heek Series; Lab Supply), in which 60% of energy was derived from fat (LSL/BAC, n = 21; BAC, n = 13; LSL/Pdx-1, n = 10; Pdx-1, n = 6; COXKO/BAC, n = 5; COXKO/LSL/BAC, n = 10). All mice were fed for at least 30 days and the BW of each animal was measured weekly. In addition, mice were fed an HFD for 38 days and simultaneously administered either saline (BAC, n = 4; LSL/BAC, n = 15) or celecoxib at 41.1 mg/kg (Sigma-Aldrich, St Louis, MO) (BAC, n = 4; LSL/BAC, n = 16) orally. After treatments, mice were sacrificed and their pancreases harvested for histological and protein analysis.

Ras Activity Assay

Levels of GTP-occupied Ras from mice pancreas lysates were estimated using a Raf pull-down assay kit as recommended by the manufacturer (Millipore, Billerica, MA). Briefly, snap-frozen pancreas tissue were homogenized and sonicated on ice in a lysis buffer containing 25 mM HEPES, pH 7.5, 1% IGEPAL CA-630, 150 mM NaCl, 0.25% sodium deoxycholate, 10% glycerol, 25 mM NaF, 10 mM $MgCl_2$, 1 mM EDTA acid, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mM Na_3VO_4. Cellular debris was removed by centrifuging at 15,000*g* for 20 minutes at 4°C. Lysates were then incubated for 45 minutes at 4°C with agarose beads coated with the Raf-Ras binding domain. Beads were washed 3 times in lysis buffer, loading buffer added, and then vortexed, centrifuged, heated, and analyzed via Western blot protocol. Densities of active and total Ras protein bands were quantified using an Odyssey imaging system (version 3.0; LICOR Biosciences, Lincoln, NE) then standardized, averaged, and plotted using Graphpad Prism 5 (GraphPad Software, San Diego, CA).

Western Blot Analysis

Pancreas tissues from each group were homogenized using RIPA buffer and lysates were separated using sodium