Nuclear factor κ B activation is a potential target for preventing pancreatic carcinoma by aspirin

ARTICLE *in* CANCER · JUNE 2005

Impact Factor: 4.89 · DOI: 10.1002/cncr.21075 · Source: PubMed

7 AUTHORS, INCLUDING:

Guido Sclabas
Mayo Foundation for Medical Education a…
46 PUBLICATIONS **1,903** CITATIONS
SEE PROFILE

Christian Schmidt
Fraunhofer-Gesellschaft zur Förderung de…
36 PUBLICATIONS **1,570** CITATIONS
SEE PROFILE

Ken Hess
University of Texas MD Anderson Cancer C…
325 PUBLICATIONS **22,642** CITATIONS
SEE PROFILE

James L Abbruzzese
Duke University
572 PUBLICATIONS **30,069** CITATIONS
SEE PROFILE

Nuclear Factor Kappa B Activation Is a Potential Target for Preventing Pancreatic Carcinoma by Aspirin

Guido M. Sclabas, M.D.[1]
Tadashi Uwagawa, M.D.[1]
Christian Schmidt, Ph.D.[1]
Kenneth R. Hess, Ph.D.[2]
Douglas B. Evans, M.D.[1]
James L. Abbruzzese, M.D.[3]
Paul J. Chiao, Ph.D.[1,4,5]

[1] Department of Surgical Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas.

[2] Department of Biostatistics, The University of Texas M. D. Anderson Cancer Center, Houston, Texas.

[3] Department of Gastrointestinal Medical Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas.

[4] Department of Molecular and Cellular Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas.

[5] Graduate Program in Cancer Biology, The University of Texas Graduate School of Biomedical Sciences at Houston, Houston, Texas.

Supported, in part, by U.S. Public Services Grants R01CA097159 to P.J.C., P20-CA101936 to J.L.A., by a Cancer Center Supporting grant from the National Cancer Institute, by grants from the Lockton Fund for Pancreatic Cancer Research to D.B.E. and P.J.C., and by the Topfer Fund for Pancreatic Cancer Research to J.L.A. and P.J.C. G.M.S. is a recipient of a Fellowship from the Cancer League of Bern, Switzerland.

The authors thank Sanofi for generously providing Aspégic and Kate Ó. Súilleabháin (Department of Scientific Publications, The University of Texas M. D. Anderson Cancer Center) and Jonathan F. Hannay for editorial assistance.

Address for reprints: Paul J. Chiao, Ph.D., Department of Surgical Oncology, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Unit 107, Houston, TX 77030; Fax: (713) 794-4830; E-mail: pjchiao@mdanderson.org

Received December 10, 2004; revision received February 22, 2005; accepted March 2, 2005.

BACKGROUND. Pancreatic carcinoma exhibits a unique genetic profile of mutations that may play key roles in its progression to malignant phenotypes. Constitutive activation of transcription factor nuclear factor kappa B (NF-κB) is a frequent molecular alteration in pancreatic carcinoma, suggesting a possible link between inflammation and cancer. The aims of the current study were to determine the effects of aspirin on pancreatic carcinoma prevention and to reveal a possible mechanism of aspirin-mediated cancer chemoprevention.

METHODS. An orthotopic mouse model with human pancreatic carcinoma cell lines PANC-1, PANC-1/Puro, and PANC-1/IκBαM was used to study the inhibitory effects of aspirin on pancreatic tumor formation.

RESULTS. Aspirin inhibited constitutive NF-κB activity in culture and, in turn, decreased the expression of the NF-κB downstream target gene, Cox-2, in PANC-1 or PANC-1/Puro cells, without significantly inhibiting the in vitro growth of PANC-1/Puro cells. All animals inoculated with either PANC-1 or PANC-1/Puro cells, and not given aspirin, developed pancreatic tumors, whereas none of the mice injected with PANC-1/IκBαM cells showed any evidence of pancreatic tumor formation. Animals given aspirin for 6 days before, or at the time of, orthotopic tumor cell injection showed a significantly lower incidence of tumor formation compared with those receiving aspirin 2 weeks after inoculation and controls receiving no aspirin.

CONCLUSIONS. Aspirin repressed tumor formation by PANC-1 cells in vivo in a prophylactic setting, suggesting a possible mechanism for aspirin's preventive effect in pancreatic carcinoma through inhibition of NF-κB activation and a mechanistic link between inflammation and tumorigenesis. Aspirin-mediated antiinflammatory approaches might be an effective strategy to prevent pancreatic carcinoma. ***Cancer* 2005;103:2485–90.** *© 2005 American Cancer Society.*

KEYWORDS: pancreatic carcinoma, chemoprevention, inflammation, tumorigenesis, aspirin, genetic alterations, nuclear factor kappa B.

Pancreatic carcinoma, the fourth leading cause of cancer death in the United States, is a highly malignant neoplasm characterized by locally advanced unresectable disease or metastasis at the time of diagnosis.[1] Current therapies are largely ineffective in this disease and the 5-year survival rate remains dismal (1–4%).[2] Although early detection and effective therapeutic strategies for pancreatic carcinoma remain to be developed, genetic analysis suggests that this malignancy exhibits a unique profile of mutations that might be involved in tumor progression.[2] Constitutive activation of nuclear factor kappa B (NF-κB) is a frequent molecular alteration in pancreatic carcinoma and is also found in human pancreatic carcinoma cell lines but not in immortalized, nontumorigenic pancreatic epithelial cells.[3] The tran-

DOI 10.1002/cncr.21075
Published online 28 April 2005 in Wiley InterScience (www.interscience.wiley.com).

scriptional activity of NF-κB proteins is regulated by a nuclear-cytoplasmic shuttling mechanism that is itself mediated by association with the family of NF-κB inhibitors known as IκB, and this interaction results in the formation of inactive NF-κB:IκB complexes in the cytoplasm.[4] NF-κB orchestrates the expression of genes that encode key determinants in inflammation, tumorigenesis, and apoptosis, and thus promotes the cardinal clinical features of pancreatic carcinoma of locally aggressive growth and metastasis.[4–6] Several studies have shown that inhibition of constitutive NF-κB activity by a phosphorylation-defective IκBα (S32, 36A) (IκBαM) suppresses tumorigenesis of pancreatic carcinoma cells in an orthotopic nude mouse model, suggesting that constitutive NF-κB activity plays a key role in pancreatic carcinoma.[6]

Aspirin inhibits NF-κB activation through specific inhibition of IKK-2 activity by binding to IKK-2 and reducing adenosine triphosphate (ATP) binding.[7,8] Recent epidemiologic studies investigating the preventive value of aspirin in pancreatic carcinoma reached conflicting findings.[9–11] To determine the effects of aspirin on pancreatic carcinoma cells and to reveal a possible mechanism of aspirin in cancer prevention, we designed the following experiments utilizing an orthotopic mouse model with the human pancreatic carcinoma cell lines PANC-1/Puro and PANC-1/IκBαM as we previously described.[6]

MATERIALS AND METHODS

Cells and Cell Culture

Cells of the human pancreatic carcinoma cell line PANC-1 were obtained from the American Type Culture Collection (Manassas, VA), and PANC-1/Puro and PANC-1/IκBαM cell lines were generated as previously described.[6] Cell lines were grown in Dulbecco's modified Eagle's medium (Life Technologies, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin (Life Technologies), and 10 μg/mL streptomycin (Life Technologies) in a 37 °C incubator with 5% CO_2.

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assay (EMSA) was performed using nuclear extracts as described previously.[6] We used the κB probe (5′-CTCAACAGAGGGGACTTTCCGAGAGGCCAT-3′) that contained the κB site (underlined) found in the human immunodeficiency virus long terminal repeat. Oct-1 probe (5′-TGTCGAATGCAAATCACTAGAA-3′) was used as a loading control. A competitive binding experiment was performed with a 50-fold excess of wild-type or mutant κB oligonucleotides (5′-CTCAACAG AGTTGACTTTTCGAGAGGCCAT-3′).

Western Blot Analysis

Cytoplasmic extracts were prepared as previously described.[3] Soluble protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% polyacrylamide) and electrophoretically transferred to polyvinylidene difluoride membrane (Osmonics, Westborough, MA). The membrane was blocked with 5% nonfat milk in phosphate-buffered saline (PBS) containing 0.2% Tween 20 (Fisher Scientific; Fairlawn, NJ) and incubated with monoclonal anti–Cox-2 mouse antibody (Cayman Chemical, Ann Arbor, MI). The membrane was washed in PBS containing 0.2% Tween 20 and probed with horseradish peroxidase-coupled secondary goat anti-mouse immunoglobulin G antibody (Amersham, Arlington Heights, IL). The protein was detected with Lumi-Light Western blotting substrate (Roche, Indianapolis, IN) according to the manufacturer's instructions.

In Vitro Cell Proliferation Assay

The standard thiazolyl blue (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide; MTT) cell proliferation assay was performed as described previously.[6]

Animals

The assay to determine the tumorigenic potential of a cell line using orthotopic nude mice was as described by Fujioka et al.[6] Briefly, female athymic BALB/c nude mice were purchased from Charles River Laboratories (Wilmington, MA). One million viable PANC-1, PANC-1/Puro, and PANC-1/IκBαM cells were determined by dye-exclusion assay, suspended in 50 μL PBS, and injected into the pancreatic parenchyma through a left subcostal incision while the mouse was under general anesthesia, being careful to avoid possible leakage of tumor cells from the injection site. Aspégic (Laboratoires Synthelabo, Le Plessis-Robinson, France), a soluble intravenous preparation of aspirin, was administered daily by intraperitoneal (i.p.) injection at a dosage of 200 mg/kg according to a previous report by Kort et al.[12] starting either 6 days before, on the day of, or 14 days after the tumor cell injection. Necropsy found no intraabdominal reaction to the i.p. injections and no evidence of increased disposition to bleeding caused by aspirin was observed. Animals were maintained according to institutional and National Institute of Health guidelines.

Statistical Analysis

All statistical analyses were performed with SPSS software (SPSS, Chicago, IL) using the two-tailed Fisher

exact test with a 95% binomial confidence interval. A value of $P < 0.05$ was considered significant.

RESULTS

Inhibition of Constitutive Nuclear Factor Kappa B Activity and Cox-2 Expression by Aspirin

To determine whether aspirin can inhibit constitutive activation of NF-κB and expression of its downstream target gene *Cox-2* in human PANC-1 pancreatic carcinoma cells, NF-κB DNA binding activity and Cox-2 protein expression were studied by EMSA and Western blot analysis, respectively (Fig. 1A,B). PANC-1/Puro pancreatic carcinoma cells were treated with aspirin at the indicated concentrations for 24 hours.

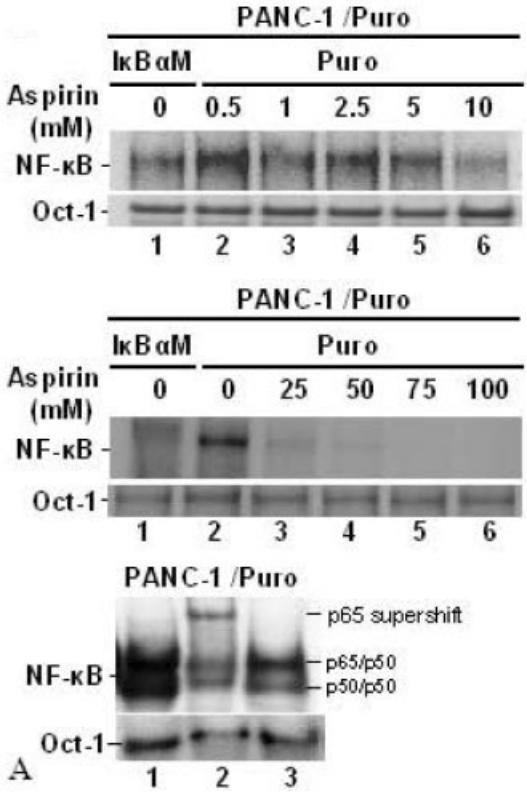
Constitutive Nuclear Factor Kappa B Activity in PANC-1/Puro cells was Inhibited by Aspirin

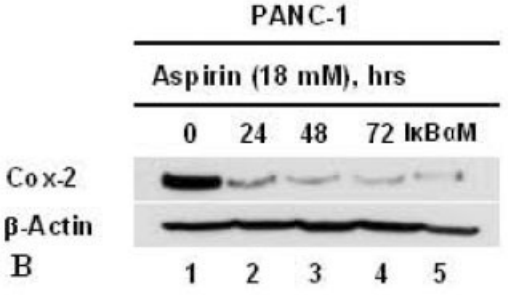
The observed inhibition of NF-κB was dose dependent (Fig. 1A). Constitutive DNA binding activity was decreased at 5 mM aspirin, a concentration measured in the serum of patients treated with aspirin for chronic inflammatory diseases,[8] strongly inhibited at 10 mM aspirin, and no longer detectable at 25 mM. As previously shown, NF-κB binding activity was completely inhibited in PANC-1/IκBαM cells which served as controls.[6]

PANC-1 cells were treated with 18 mM aspirin for ≤ 72 hours and cytoplasmic extracts were probed for Cox-2 protein expression (Fig. 1B). Cox-2 expression was strongly inhibited after 24 hours and practically abolished after 72 hours, comparable to the findings in PANC-1/IκBαM used as controls.

Effect of Aspirin on PANC-1 Cell Growth in Culture

MTT assays were performed using PANC-1 cells treated with 0 and 18 mM aspirin for the indicated times. Aspirin at a concentration of 18 mM did not





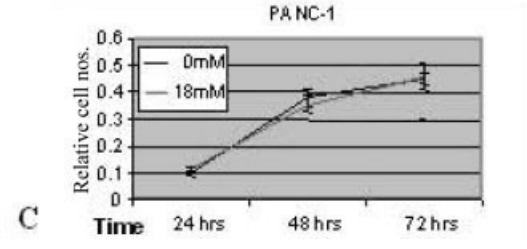


FIGURE 1. (A) Aspirin inhibits constitutive activation of nuclear factor kappa B (NF-κB) in human PANC-1 pancreatic carcinoma cells. Results of electrophoretic mobility shift assay for κB DNA binding activity are shown. PANC-1 cells were treated with aspirin at the indicated concentrations for 24 hours and nuclear extracts used in this analysis were probed with human immunodeficiency virus κB. The Oct-1 probe was used as a loading control. NF-κB supershift was performed by adding p65 antibody (lane 2), respectively p65 antibody and peptide (lane 3). (B) Inhibition of Cox-2 expression by aspirin in PANC-1 pancreatic carcinoma cells. PANC-1 cells were treated ≤ 72 hours with aspirin at a concentration of 18 mM and cytoplasmatic extracts were probed for Cox-2 expression. β-Actin was used as loading control. (C) Effect of aspirin on PANC-1 cell growth in culture. Thiazolyl blue (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide; MTT) assays were performed using PANC-1 cells treated with 0 and 18 mM aspirin for the indicated times.

TABLE 1
Pancreatic Tumor Formation in Mice without and with Aspirin Treatment

Cell line (group)	Start of aspirin treatment[a]	No. of animals	Pancreatic tumor formation (%)	95% binomial confidence interval	*p* value
PANC-1	—	5	5 (100)	0.48–1	—
PANC-1/Puro	—	10	10 (100)	0.69–1	—
PANC-1/IκBαM	—	5	0 (0)	0–0.52	—
PANC-1/Puro (A)	6 days	15	4 (27)	0.08–0.55	< 0.0006[b]
PANC-1/Puro (B)	0 days	15	6 (40)	0.16–0.68	< 0.003[b]
PANC-1/Puro (C)	+14 days	10 (8)[c]	5 (63)	0.21–0.91	NS[b]

[a] Start of aspirin treatment relative to orthotopic tumor cell injection.
[b] Compared with PANC-1/Puro cells not treated with aspirin.
[c] Two of the mice died without pathologic examination.

inhibit the growth of PANC-1 cells significantly (Fig. 1C), consistent with the growth of PANC-1/IκBαM cells and suggesting that inactivation of NF-κB by aspirin did not directly inhibit the growth of PANC-1 cells in culture.

Effect of Aspirin on Tumorigenic Potential of PANC-1/Puro Cells In Vivo

We then tested whether aspirin inhibits the tumorigenic potential of PANC-1/Puro cells using an orthotopic nude mouse model with daily i.p. injections of Aspégic, an intravenous preparation of aspirin, at a dosage of 200 mg/kg. To compare the effects of aspirin in prophylactic and therapeutic settings, 40 mice were divided into 3 groups, which were treated with different aspirin regimens for 8 weeks: Group A ($n = 15$) received a daily i.p. aspirin injection (200 mg/kg) starting 6 days before orthotopic tumor cell injection; Group B ($n = 15$) received a daily i.p. aspirin injection (200 mg/kg) starting on the day of tumor cell injection; and Group C ($n = 10$) received a daily i.p. aspirin injection (200 mg/kg) starting 14 days after tumor cell injection. At 12 weeks after injection of the cells, most of the tumor-bearing animals in Group C were significantly sick and all mice were killed. Necropsy showed that 4 (27%) of the 15 animals in Group A, 6 (40%) of the 15 animals in Group B, and 5 (63%) of 8 animals in Group C had developed macroscopic pancreatic tumors with no appreciable metastasis (Table 1, Fig. 2). Mice in the 3 control groups were not treated after being injected with PANC-1 ($n = 5$), PANC-1/Puro ($n = 10$), or PANC-1/IκBαM cells ($n = 5$). At 6 weeks, all the mice injected with PANC-1 or PANC-1/Puro cells were sick and killed. The results of pathologic examination showed that 100% of animals injected with either PANC-1 or PANC-1/Puro cells had pancreatic tumors with no visible metastasis to other organs, whereas none of the mice injected with PANC-1/IκBαM cells showed evidence of pancreatic tumor formation (Table 1). These findings are consistent with those of our previous finding that IκBαM-mediated inhibition of NF-κB activities suppressed tumorigenesis of PANC-1/Puro cells in an orthotopic nude mouse model.[6] The incidence of tumor formation was significantly lower in Groups A and B than in the untreated mice in the control group injected with PANC-1/Puro cells. There were no significant differences in the incidences of tumor formation between Group C and the control group of untreated mice injected with PANC-1/Puro cells or between Groups A and B. Our results show that aspirin has the potential to inhibit tumorigenesis in a prophylactic but not a therapeutic setting, suggesting that NF-κB activation is a possible target for aspirin in cancer prevention.

DISCUSSION

A number of studies suggest that pancreatic inflammation mediated by proinflammatory pathways is a critical component contributing to the initiation of pancreatic carcinoma.[13] Constitutive activation of NF-κB is a frequent molecular alteration in pancreatic carcinoma and is induced by autocrine stimulation of proinflammatory cytokines such as interleukin (IL)-1α.[14] Aspirin inhibits NF-κB activation through specific inhibition of IKK-2 activity by binding to IKK-2 and reducing ATP binding.[7,8] Aspirin is a potent inhibitor of Cox-2, a key regulator of inflammation, and the inhibitory effect of aspirin on Cox-2 is also partly due to the inhibition of its expression as a downstream target gene, regulated by NF-κB.[15]

As in many other types of malignant tissue, Cox-2 is also overexpressed in pancreatic carcinoma.[16] Evidence from recent clinical studies suggests that aspirin and other nonsteroidal antiinflammatory drugs are protective against gastrointestinal tract carcinomas, especially colorectal carcinoma.[17] However, recent ep-

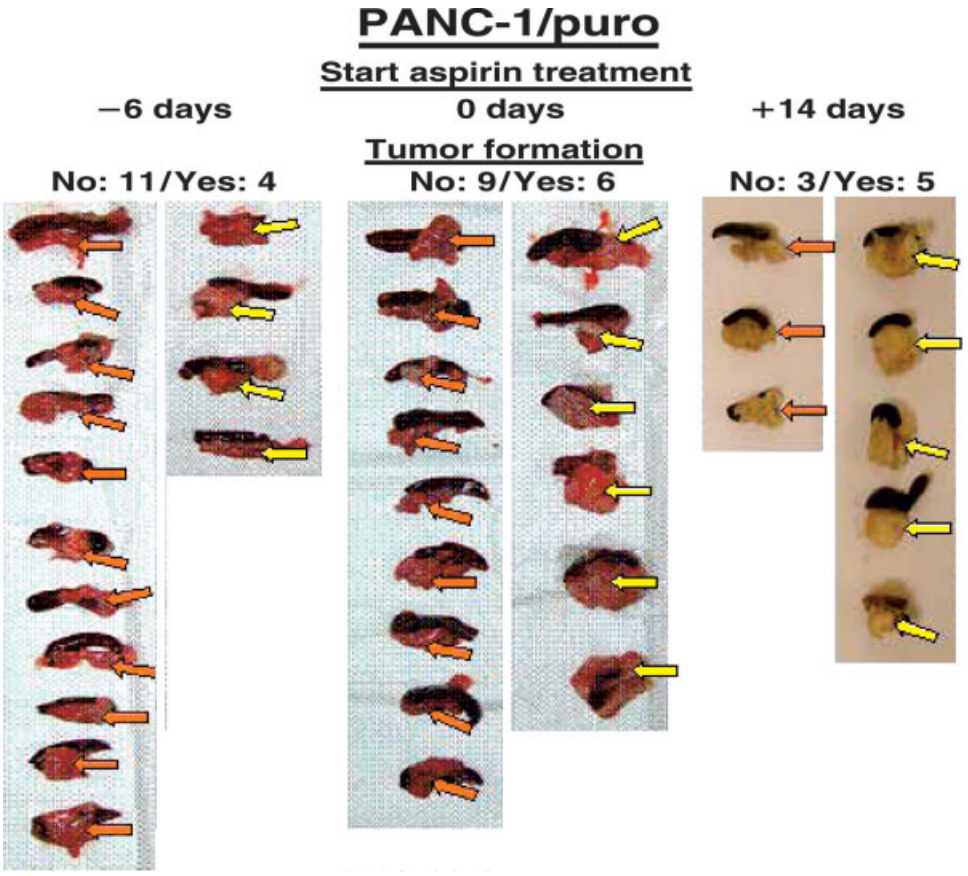


FIGURE 2. Aspirin inhibited pancreatic tumor formation in an orthotopic mouse model with a prophylactic setting. One million viable PANC-1, PANC-1/Puro, and PANC-1/IκBαM cells were determined by dye-exclusion assay, suspended in 50 μL of phosphate-buffered saline, and injected into the pancreatic parenchyma of athymic BALB/c nude mice. Aspégic, an intravenous preparation of aspirin, was administered daily by intraperitoneal injection at a dosage of 200 mg/kg starting either 6 days before, on the day of, or 14 days after the tumor cell injection as indicated. The spleen and pancreas with and without tumors are shown. Orange arrows mark normal mouse pancreas, and yellow arrows mark pancreatic tumors.

idemiologic studies investigating the preventive value of aspirin in pancreatic carcinoma reached conflicting findings,[9–11] perhaps due to the substantial differences in patient populations, exposure information (dose, duration, frequency), and outcome information. The goals of the current study were to shed further light on these conflicting findings and to reveal possible mechanisms of aspirin in cancer prevention.

Our findings show that aspirin-mediated inhibition of NF-κB activation in response to inflammation is a possible mechanism for the cancer preventive effect of aspirin. Activation of NF-κB and expression of its downstream target genes such as the proinflammatory cytokines tumor necrosis factor-α and IL-1 are mechanistic links between inflammation and tumorigenesis.[18] Our results suggest that aspirin-mediated antiinflammatory approaches could be an effective strategy to prevent pancreatic carcinoma. This is supported by recent reports showing that specific inactivation of the IKK/NF-κB pathway attenuates formation of inflammation-associated tumors.[19] In addition to suppressing apoptosis in advanced-stage tumors, IKKβ/NF-κB may link inflammation to cancer. Our laboratory is pursuing further investigations of the ability of aspirin to suppress pancreatic carcinoma formation in a chronic inflammatory state using mice, carrying a hereditary pancreatitis gene, a mutant trypsinogen (R122H), that causes chronic pancreatitis and increases the risk of pancreatic carcinoma 50-fold.[20] This model will be very useful to determine the pancreatic carcinoma preventive effect of aspirin.

REFERENCES

1. Hansel DE, Kern SE, Hruban RH. Molecular pathogenesis of pancreatic cancer. *Annu Rev Genomics Hum Genet.* 2003;4: 237–256.
2. Jaffee EM, Hruban RH, Canto M, Kern SE. Focus on pancreas cancer. *Cancer Cell.* 2002;2:25–28.
3. Dong QG, Sclabas GM, Fujioka S, et al. The function of multiple IkappaB: NF-kappaB complexes in the resistance of cancer cells to Taxol-induced apoptosis. *Oncogene.* 2002; 21:6510–6519.
4. Li Q, Verma IM. NF-kappaB regulation in the immune system. *Nat Rev Immunol.* 2002;2:725–734.
5. Schmidt C, Peng B, Li Z, et al. Mechanisms of proinflammatory cytokine-induced biphasic NF-kappaB activation. *Mol Cell.* 2003;12:1287–1300.
6. Fujioka S, Sclabas GM, Schmidt C, et al. Inhibition of constitutive NF-kappa B activity by I kappa B alpha M suppresses tumorigenesis. *Oncogene.* 2003;22:1365–1370.
7. Kopp E, Ghosh S. Inhibition of NF-kappa B by sodium salicylate and aspirin. *Science.* 1994;265:956–959.

8. Yin MJ, Yamamoto Y, Gaynor RB. The anti-inflammatory agents aspirin and salicylate inhibit the activity of I(kappa)B kinase-beta. *Nature*. 1998;396:77–80.
9. Anderson KE, Johnson TW, Lazovich D, Folsom AR. Association between nonsteroidal anti-inflammatory drug use and the incidence of pancreatic cancer. *J Natl Cancer Inst*. 2002; 94:1168–1171.
10. Jacobs EJ, Connell CJ, Rodriguez C, Patel AV, Calle EE, Thun MJ. Aspirin use and pancreatic cancer mortality in a large United States cohort. *J Natl Cancer Inst*. 2004;96:524–528.
11. Schernhammer ES, Kang JH, Chan AT, et al. A prospective study of aspirin use and the risk of pancreatic cancer in women. *J Natl Cancer Inst*. 2004;96:22–28.
12. Kort WJ, Hulsman LO, van Schalkwijk WP, Weijma IM, Zondervan PE, Westbroek DL. Reductive effect of aspirin treatment on primary tumor growth and metastasis of implanted fibrosarcoma in rats. *J Natl Cancer Inst*. 1986;76: 711–720.
13. Cavestro GM, Comparato G, Nouvenne A, Sianesi M, Di Mario F. The race from chronic pancreatitis to pancreatic cancer. *J Pancreas*. 2003;4:165–168.
14. Niu J, Li Z, Peng B, Chiao PJ. Identification of an autoregulatory feedback pathway involving IL-1alpha in induction of constitutive NF-kappaB activation in pancreatic cancer cells. *J Biol Chem*. 2003;279:16452–16462.
15. Yamamoto K, Arakawa T, Ueda N, Yamamoto S. Transcriptional roles of nuclear factor kappa B and nuclear factor-interleukin-6 in the tumor necrosis factor alpha-dependent induction of cyclooxygenase-2 in MC3T3–E1 cells. *J Biol Chem*. 1995;270:31315–31320.
16. Tucker ON, Dannenberg AJ, Yang EK, et al. Cyclooxygenase-2 expression is up-regulated in human pancreatic cancer. *Cancer Res*. 1999;59:987–990.
17. Higuchi T, Iwama T, Yoshinaga K, et al. A randomized, double-blind, placebo-controlled trial of the effects of rofecoxib, a selective cyclooxygenase-2 inhibitor, on rectal polyps in familial adenomatous polyposis patients. *Clin Cancer Res*. 2003;9:4756–4760.
18. Clevers H. At the crossroads of inflammation and cancer. *Cell*. 2004;118:671–674.
19. Greten FR, Eckmann L, Greten TF, et al. IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. *Cell*. 2004;118:285–296.
20. Whitcomb DC, Gorry MC, Preston RA, et al. Hereditary pancreatitis is caused by a mutation in the cationic trypsinogen gene. *Nat Genet*. 1996;14:141–145.