RESEARCH ARTICLE

Aspirin therapy reduces the ability of platelets to promote colon and pancreatic cancer cell proliferation: Implications for the oncoprotein c-MYC

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Mitrugno A, Sylman JL, Ngo AT, Pang J, Sears RC, Williams CD, McCarty OJ. Aspirin therapy reduces the ability of platelets to promote colon and pancreatic cancer cell proliferation: Implications for the oncoprotein c-MYC. Am J Physiol Cell Physiol 312: C176–C189, 2017. First published November 30, 2016; doi:10.1152/ajpcell.00196.2016.—Aspirin, an anti-inflammatory and antithrombotic drug, has become the focus of intense research as a potential anticancer agent owing to its ability to reduce tumor proliferation in vitro and to prevent tumorigenesis in patients. Studies have found an anticancer effect of aspirin when used in low, antiplatelet doses. However, the mechanisms through which low-dose aspirin works are poorly understood. In this study, we aimed to determine the effect of aspirin on the cross talk between platelets and cancer cells. For our study, we used two colon cancer cell lines isolated from the same donor but characterized by different metastatic potential, SW480 (nonmetastatic) and SW620 (metastatic) cancer cells, and a pancreatic cancer cell line, PANC-1 (nonmetastatic). We found that SW480 and PANC-1 cancer cell proliferation was potentiated by human platelets in a manner dependent on the upregulation and activation of the oncoprotein c-MYC. The ability of platelets to upregulate c-MYC and cancer cell proliferation was reversed by an antiplatelet concentration of aspirin. In conclusion, we show for the first time that inhibition of platelets by aspirin can affect their ability to induce cancer cell proliferation through the modulation of the c-MYC oncoprotein.

cancer; aspirin; platelets; proliferation; c-MYC

ASPIRIN (acetylsalicylic acid, ASA) therapy has been the subject of extensive investigation for more than a century because of its anti-inflammatory and antiplatelet properties. In addition to its cardioprotective effect, aspirin use, taken for several years, has been correlated with reduced long-term risk of some cancers, particularly colorectal cancer (43). A significant gap in our understanding of the anticancer effect of aspirin lies in understanding how much of this effect is derived from direct inhibition of cancer cells and how much is due to inhibition of platelet activation and function. Importantly, retrospective epidemiological studies suggest that low, antiplatelet doses of aspirin (75 mg/day; 15–20 μM of salicylic acid in plasma) are more efficacious in decreasing the incidence and mortality associated with colorectal cancer compared with high, anti-inflammatory doses (325–1,200 mg/day; 0.5–2.5 mM of salicylic acid in plasma) (13, 38). A number of in vitro studies have suggested that the chemopreventive action of anti-inflammatory doses of aspirin exerts an antiproliferative effect attributable to the inhibition of functionally different oncoproteins, such as the cyclooxygenase (COX) enzyme COX-2 and the transcription factor c-MYC (1, 13, 43). The molecular underpinnings behind the anticancer effect of antiplatelet doses of aspirin remain ill defined.

Blood platelets have been shown to play a key role in promoting tumor survival, growth, proliferation, and metastasis (28, 31–33). It has been hypothesized that the release of growth factors, such as transforming growth factor-β (TGF-β), from platelet α-granules triggers an epithelial-to-mesenchymal transition (EMT) in circulating tumor cells (CTCs), resulting in increased invasive and migratory cell properties (23). Additionally, activated platelets may contribute to metastasis by protecting CTCs from the immune response (22, 36). In mouse models, platelets have been shown to enhance the proliferation of select ovarian cancer cells (9). We have shown that platelets support the recruitment of colon cancer cells from the bloodstream under physiologically relevant levels of shear flow (4, 29). The present study was designed to determine the effect of inhibition of platelet activation and function by aspirin therapy on colon and pancreatic cancer cell proliferation.

The presence and the interaction of platelets with cancer cells create a highly diverse microenvironment rich in growth factors. Tumor cells respond to these soluble factors by transducing and integrating signals to upregulate proteins that are necessary for their proliferation and survival. One of such proteins is c-MYC, a transcription factor that orchestrates the expression of >15% of all genes, including genes involved in cell cycle, survival, protein synthesis, and cell metabolism (21, 41). The oncoprotein c-MYC is overexpressed in a large number of human cancers, including colon, pancreas, breast, lung, and prostate cancers (39, 41). Moreover, c-MYC is frequently found constitutively activated and overexpressed in tumor cells at the metastatic niche compared with primary tumors, in a small part attributable to mutations in the c-MYC gene but more commonly through pathways triggered by mi-
crosstalk between platelets and cancer cells. Our results suggest that low-dose aspirin might be efficacious in reducing the proliferation of cancer cells through the inhibition of platelet-derived signals required for the upregulation of the oncoprotein c-MYC.

**Materials and Methods**

**Reagents.** All the chemicals and reagents were purchased from Sigma-Aldrich (St Louis, MO) or previously mentioned sources unless specified otherwise (4, 44). Anti-c-MYC (Y69, rabbit monoclonal) and anti-Ki-67 (rabbit polyclonal) antibodies were purchased from Abcam (Cambridge, MA). COX-2 (C20, goat monoclonal) and p300 (C20, rabbit polyclonal) primary antibodies and goat anti-rabbit/anti-mouse and mouse anti-goat horseradish peroxidase secondary antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX). CD42b (N19, goat polyclonal) and tubulin (rabbit monoclonal) antibodies were from Cell Signaling Technology (Danvers, MA). CD41a antibody was purchased from Millipore (Billerica, MA). The c-MYC inhibitor 10058-F4 and the platelet-derived growth factor receptor (PDGF-R) inhibitor AC710 were obtained from Tocris (Bristol, UK). The αIβ3 integrin inhibitor integrilin was purchased from Novaplus Pharmaceuticals (Lake Zurich, IL).

**Cell line culture and aspiration treatment.** The human colon adenocarcinoma cell lines, SW480 and SW620, and the human epithelial cancer cell line, PANC-1, were purchased from American Type Culture Collection. SW480 was derived from a colon carcinoma in a 50-yr-old male patient, whereas SW620 was derived from the lymph node metastasis in the same patient 1 yr later (26). PANC-1 cancer cells were isolated from the excrine pancreas of a 56-yr-old male. Cells were grown as monolayers (37°C in 5% CO2) in DMEM (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were plated in 96-well or 6-well plates (Corning Costar, Corning, NY) at select concentrations and allowed to grow for 24 h before treatment with aspirin or vehicle control (DMSO). Aspirin (Sigma-Aldrich) was prepared daily as a 2.5 M stock solution in DMSO. On the day of an experiment, cells were washed twice with PBS and treated with increasing doses of aspirin (0.25, 0.50, 1.00, 2.00, 2.50, or 5.00 μM) or with DMSO in serum-complete medium for 24 or 48 h.

**MTT-like cell proliferation assay.** Cell proliferation was measured by Cell Titer 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI) following the instructions supplied by the manufacturer. SW620, SW480, or PANC-1 cancer cells (5,000 per well) were plated in 96-well plates in FBS-complete medium and treated with increasing concentrations of aspirin or vehicle control at 37°C in a CO2 incubator for 24 or 48 h. The assay was performed in duplicate in three independent experiments, and the absorbance at 570-nm wavelength was measured with an Infinite M200 spectrophotometer (TECAN, Männedorf, Switzerland). A reference wavelength of 560 nm was used to reduce nonspecific absorbance background. The culture medium with vehicle was used as a negative control, and the average baseline absorbance values were subtracted from all experimental wells to yield the corrected absorbance at 570 nm.

**Isolation of washed platelets and platelet releasate.** Platelets were isolated from human venous blood drawn from healthy volunteers by venipuncture into sodium citrate (1:9 vol/vol), in accordance with an Institutional Review Board-approved protocol at Oregon Health and Science University as previously described (3). Briefly, anticoagulated blood was centrifuged (200 g, 20 min) to obtain platelet-rich plasma (PRP). PRP was centrifuged (1,000 g, 10 min in the presence of prostacyclin (0.1 μg/ml) to obtain a platelet pellet. The platelet pellet was resuspended in modified HEPES/Tyrode buffer (129.00 mm NaCl, 0.34 mm NaH2PO4, 2.90 mm KCl, 12.00 mm NaHCO3, 20.00 mm HEPES, 5.00 mm glucose, and 1.00 mm MgCl2, pH 7.3) and washed once via centrifugation at 1,000 g for 10 min in modified HEPES/Tyrode buffer in the presence of prostacyclin (0.1 μg/ml). Purified platelets were resuspended in modified HEPES/Tyrode buffer at the indicated concentrations. When required, washed platelets were pretreated with aspirin (20 μM) or vehicle control (DMSO) for 30 min at 37°C under gentle shaking before activation with thrombin and/or coculture with cancer cells. The antiplatelet concentration of aspirin used in this research is based on previous studies (2, 11). Platelet-free releasate was isolated and purified as previously described (10). Briefly, thrombin-activated platelets were pelleted by centrifugation at 2,000 g for 10 min. The supernatant was collected and centrifuged at 13,000 g for 10 min to remove any microparticles. Hirudin (40 μg/ml) was added to the supernatant to neutralize thrombin activity.

**Coculture of human cancer cells with human washed platelets or platelet releasate.** Cancer cells were seeded on a six-well plate or on ibiTreat 15μ-Slide eight-well microscopy chambers at a starting concentration of 105 cells/well. The cancer cell monolayer was cultured in complete medium for 24 h. Serum-supplemented medium was removed, and the cells were washed twice with PBS and serum-starved for 24 h in fresh serum-free medium. Cells were then washed once with PBS before 24-h incubation at 37°C in a 5% CO2 with resting or thrombin-activated human washed platelets (3 × 105 platelets/ml) or platelet releasate in the presence of serum-free medium. Control samples were incubated with serum-free medium in the presence of equivalent volume of platelet buffer or medium supplemented with 10% FBS. Following coculture with platelets, cancer cells were washed twice with PBS. In select experiments, cancer cells were treated with wortmannin [phosphatidylinositol 3-kinase (PI3K) inhibitor, 100 nM], AC710 (PDGF-BB receptor inhibitor, 10 μM), or 10058-F4 (c-MYC inhibitor, 60 μM) for 20 min under cell culture conditions before the addition of washed platelets and further incubation for 24 h. The concentration of the inhibitors was chosen according to previous studies (17, 27, 37).

**Transwell assay.** A transwell device (0.4-μm polyester membrane, Corning Costar) was used to prevent the physical interaction of cancer cells with platelets, as previously described (5). Cancer cells (105 cells/well) were loaded into the lower chamber of the well and cultured as described above. Resting or thrombin-activated washed platelets (3 × 105 platelet/ml) were added to the upper chamber, and the coculture proceeded as detailed above. Control samples were incubated with serum-free medium in the presence of an equivalent volume of platelet buffer.

**Ki-67 immunofluorescence.** For immunofluorescence experiments, cancer cells were fixed in 4% paraformaldehyde for 15 min, washed twice with PBS, and permeabilized with 0.2% Triton X-100/PBS for 5 min followed by two washes in PBS. Following 20-min blocking with 1% BSA/PBS, cells were stained with an anti-Ki-67 (proliferation marker) primary antibody (10 μg/ml) overnight at 4°C. Cells were then washed three times with 1% BSA/PBS and incubated with Alexa Fluor 488 anti-rabbit secondary antibodies (20 μg/ml) (green fluorescence), and actin was stained with anti-stain phalloidin–TRITC (1 μg/ml; red fluorescence) for 1 h in the dark. After three washes with 1% BSA/PBS, the nuclei was stained with Hoechst 33342 (1 μg/ml; blue fluorescence) in the dark for 30 min. Images of cells were acquired at ×10 and ×63 with Zeiss Axio Imager 2 microscope 6 (Carl Zeiss MicroImaging, Jena, Germany). For analysis of the percentage of Ki-67-positive cells, three random fields at a ×10 magnification were chosen and processed using ImageJ software. The Otsu method was used to threshold for DAPI positivity (blue, total cell

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number) and FITC positivity (green, Ki-67-positive cells). Thresholded FITC-labeled images were also diluted to merge together multiple Ki-67-positive isolated events within one cell. The Particle Analyzer plugin was employed to obtain a count of particles $> 10 \mu m^2$. Cell counts in each field were averaged, and the fraction of Ki-67 positivity (%) was calculated by dividing the number of Ki-67-positive cells by the total number of DAPI-positive cells.

Western blotting. For Western blotting experiments, cancer cells were washed twice with PBS and lysed in M-PER Mammalian Protein Extraction Reagent (Pierce, Thermo Scientific, Rockford, IL) supplemented with a protease and phosphatase inhibitor cocktail (1× Pierce) followed by sonication for 2 × 10 pulses at 1 output and 15% duty. Lysates were denatured in an equal volume of Laemmli sample buffer (Bio-Rad, Hercules, CA) with 0.5 M dithiothreitol (100°C, 5 min). Equal amounts of protein (30 μg) were separated by SDS-PAGE, and Western blotting was carried out as previously described (3). Blot films were digitally scanned and analyzed for band intensity with ImageJ Volume 1.44 software. Band densitometry statistical analysis was performed using a paired t-test between vehicle, and each test condition for the antigen was specified. Protein blots were analyzed in three independent experiments.

Statistical analysis. Data were analyzed using GraphPad PRISM 4.0 software (San Diego, CA). To determine statistical significance, Student’s paired t-test was used for comparison between treatments. For grouped analysis, used for the MTT-proliferation study, two-way ANOVA with post hoc Bonferroni’s comparison test was performed. Results are expressed as the means ± SE or as means with range. Differences were considered significant at P values <0.05.

RESULTS

Platelets induce colon cancer cell proliferation: Implications for the oncoprotein c-MYC. Platelets are known to support cancer cell survival, metastasis, and angiogenesis, but their effect on tumor cell proliferation is ill defined (31). We first investigated whether human platelets, which are a rich source of mitogenic factors, could influence the proliferation rate of the following two colon cancer cell lines grown in serum-free DMEM (Sf-DMEM): the primary human nonmetastatic cell line SW480 and the lymph node metastatic derivative cell line SW620. For this purpose, the expression of the proliferation marker Ki-67 was detected by immunofluorescence microscopy. Cancer cells cultured in Sf-DMEM served as negative control, whereas cells grown in serum-conditioned DMEM (serum-DMEM) served as positive control of cell proliferation. As shown in Fig. 1, A and B, the proliferation rate of SW480 and SW620 cancer cells significantly increased following 24-h coculture with serum-DMEM compared with cells grown in Sf-DMEM, as observed by the upregulation in Ki-67 expression (green). We next utilized the same approach to quantify changes in Ki-67 expression in SW480 and SW620 cancer cells cocultured for 24 h with resting or thrombin-activated human washed platelets in the presence of Sf-DMEM. Our results show that resting and thrombin-activated washed platelets dramatically increased the percentage of Ki-67-positive SW480 and SW620 cancer cells compared with control cells cultured in Sf-DMEM (Fig. 1, A and B). It is noteworthy that we observed a robust degree of individual platelet binding to SW480 colon cancer cells under resting platelet conditions (Fig. 1A, white arrows). When platelets were activated with thrombin, we observed a large degree of platelet aggregate binding to SW620 cancer cells (Fig. 1B, white arrows).

The uncontrolled proliferation of many types of cancer cells is driven by the aberrant upregulation of the transcription factor c-MYC (39, 41). To investigate whether c-MYC regulates SW480 and SW620 cancer cell proliferation, we treated cancer cells with 10058-F4 (60 μM), a cell-permeable selective c-MYC inhibitor, before exposure to resting or activated platelets. Subsequently, proliferation rate was determined by Ki-67 immunostaining. As seen in Fig. 1, A and B, inhibition of c-MYC resulted in a reduction in platelet-induced proliferation of both SW480 and SW620 cancer cells, with a more dramatic effect observed with SW480 cancer cells (Fig. 1C).

Platelets induce the upregulation of the oncoprotein c-MYC in SW480 colon cancer cells. Paracrine and juxtacrine interactions between platelets and cancer cells have been shown to foster tumor growth and aid the survival of circulating tumor cells during their metastatic journey (20, 30, 31, 34). Considering that the expression of the oncoprotein c-MYC in cancer cells is thought to be regulated by the host microenvironment (39, 41), we next asked whether platelets are able to upregulate c-MYC in SW480 and SW620 human colon cancer cells. Cancer cells were serum starved for 24 h before being cocultured with resting or thrombin-activated human washed platelets and examined after 24 h for c-MYC expression. As shown in the representative blots in Fig. 2A, the oncoprotein c-MYC is only expressed at exceedingly low levels in SW480 cancer cells at baseline when cultured in Sf-DMEM. Interestingly, c-MYC expression was upregulated when serum-starved SW480 cancer cells were cocultured with human resting or activated washed platelets. In contrast, SW620 cancer cells expressed high levels of c-MYC oncoprotein at baseline when cultured in serum-free medium; c-MYC expression levels in SW620 cells were insensitive to coculture with either resting or activated platelets (Fig. 2A). We assessed the degree of platelet adhesion to cancer cell lines by quantifying the levels of the platelet-specific integrin αIIBβ3 and glycoprotein Ib (GPIb) by immunoblot of whole cell lysates of cancer cells with a CD41 and CD42 antibody, respectively. As shown in the representative blots in Fig. 2A, incubation of either SW480 or SW620 cancer cells with platelets resulted in an increase in CD41a and CD42b expression in the cell lysates, confirming that platelets remained bound to the cancer cells.

Previous studies have shown that the platelet integrin αIIBβ3 plays a role in mediating platelet-cancer cell binding (29, 31, 32). We therefore designed a set of experiments to determine whether the platelet integrin αIIBβ3 plays a role in the ability of platelets to upregulate c-MYC expression in SW480 cancer cells. Our results show that pretreatment of platelets with integrilin, a selective inhibitor of αIIBβ3, blocked both resting and activated platelet-induced c-MYC upregulation in SW480 cancer cells (Fig. 2B), suggesting that the platelet integrin αIIBβ3 plays a key role in upregulating c-MYC in SW480 cancer cells.

We next aimed to study the intracellular molecular mechanism through which platelets upregulate c-MYC oncoprotein in SW480 colon cancer cells. Multiple lines of evidence indicate that the PI3K pathway is involved in regulating c-MYC expression levels (30). To evaluate whether platelets upregulate c-MYC expression in a PI3K-dependent manner, we compared the levels of c-MYC expression in SW480 cancer cells treated with wortmannin (PI3K inhibitor, 100 nM) in the presence of
either resting or activated washed platelets. We demonstrated by Western blot and densitometry analysis of SW480 lysates that wortmannin significantly inhibited the ability of activated, but not resting, platelets to induce c-MYC expression levels in SW480 cancer cells (Fig. 2C), indeed suggesting that activated platelets upregulate c-MYC expression in a PI3K-dependent manner.

Platelets induce the upregulation of the oncoprotein c-MYC in PANC-1 pancreatic cancer cells. We next aimed to study whether platelets were able to induce c-MYC upregulation in the human nonmetastatic pancreatic cancer cell line PANC-1. PANC-1 cancer cells were serum starved for 24 h before being cocultured with resting or thrombin-activated human washed platelets, and c-MYC expression levels were examined after 24 h via Western blotting. As shown in the representative blots in Fig. 3A, expression levels of c-MYC were upregulated in PANC-1 cancer cells cultured in the presence of serum (Fig. 3A) compared with baseline expression levels in Sf-DMEM, indicating that serum growth factors upregulate c-MYC expression in PANC-1 cells. We found that c-MYC expression was upregulated when serum-starved PANC-1 cancer cells were cocultured with either resting or thrombin-activated platelets (Fig. 3A). However, in contrast to what we observed for SW480 colon cancer cells, blockade of integrin αIIβ3 with integrilin had no effect on platelet-induced c-MYC expression in PANC-1 pancreatic cancer cells (Fig. 3B).
To evaluate whether the PI3K pathway was required for platelets to induce c-MYC upregulation in PANC-1 cancer cells, we compared the levels of c-MYC expression in PANC-1 cancer cells treated with wortmannin in the presence of either resting or activated washed platelets. In contrast to what we observed for SW480 cancer cells (Fig. 2C), Western blot and densitometry analysis of PANC-1 lysates show that wortmannin prevented the increase in c-MYC expression induced by resting washed platelets yet had no effect on the ability of activated washed platelets to induce c-MYC expression in PANC-1 cells (Fig. 3C). These results suggest that the signaling cascade by which platelets induce the upregulation of c-MYC shifts from a PI3K-dependent to a PI3K-independent manner following platelet activation.

Platelets induce pancreatic cancer cell proliferation: Implications for the oncoprotein c-MYC. We next examined whether platelets were able to promote PANC-1 cancer cell proliferation. As shown in Fig. 4, the proliferation rate of PANC-1 cancer cells significantly increased following the culture of PANC-1 cells for 24 h with serum-DMEM compared with culture in SF-DMEM, as measured by an increase in Ki-67 expression (green). Quantification of the immunostaining of the fraction of Ki-67-positive cells (%) Ki-67 positivity) demonstrated that the addition of serum resulted in an increase in Ki-67 expression from 62.5 ± 3.6% for serum-starved PANC-1 cancer cells to 85.3 ± 3.1% and 97.2 ± 1.1% for PANC-1 cancer cells cocultured with resting or activated washed platelets, respectively (Fig. 4B). Similar to what we observed for SW480 cells, numerous platelet aggregates were observed bound to PANC-1 cancer cells (Fig. 4A, white arrows). To determine whether c-MYC activity was required for platelet-induced PANC-1 cancer cell proliferation, we treated cancer cells with 10058-F4 (60 μM) before exposure to resting or activated platelets. As shown in Fig. 4A, inhibition of c-MYC resulted in a significant reduction of PANC-1 proliferation induced by both resting and activated washed platelets. Quantification of the fraction of Ki-67-positive cells (%) demonstrated that the extent of platelet-induced PANC-1 cancer cell proliferation decreased from to 85.3 ± 3.1% and 97.2 ± 1.1% at baseline to 70.1 ± 3.5% and 73.7 ± 4.0% for cancer cells treated with 10058-F4 in the presence of resting or activated washed platelets, respectively (Fig. 4B).
An antiplatelet dose of aspirin reduces the ability of activated platelets to induce SW480 and PANC-1 cancer cell proliferation: Implications for the oncoprotein c-MYC. Clinical studies have shown an anticancer effect for aspirin although it is unclear whether this effect is a result of the antiplatelet or anti-inflammatory properties of this drug, or likely a combination of the two (43). We found that the proliferation rate of SW480, SW620, and PANC-1 cancer cells was significantly lower when cultured with platelet-conditioned medium compared to serum-conditioned medium. These effects were confirmed by immunofluorescence staining for the nuclear proliferation marker Ki-67 and actin, showing a decrease in cell proliferation and a decrease in actin localization in the cell membranes.

**Fig. 3.** Effect of human platelets on c-MYC expression in PANC-1 pancreatic cancer cells. A–C: PANC-1 pancreatic cancer cells were cultured in SF-DMEM or in DMEM conditioned with resting or activated washed platelets for 24 h and subsequently lysed and proteins (30 μg) were blotted for c-MYC and CD41a expression. The c-MYC protein expression level is graphed over the loading control, tubulin. Data are means ± SE of three or four independent experiments. *P ≤ 0.05 of cancer cells cultured in DMEM conditioned with serum or platelets vs. cancer cells cultured in SF-DMEM (A) and *P ≤ 0.05 for comparison of inhibitor-treated cells/platelets vs. vehicle-treated cells/platelets (B and C).

**Fig. 4.** Proliferative effect of human platelets on PANC-1 pancreatic cancer cells. A: immunofluorescence staining of PANC-1 pancreatic cancer cells for the nuclear proliferation marker Ki-67 (green), actin (red), and nuclei (blue). After 24 h culture of starved PANC-1 cancer cells with platelet buffer in SF-DMEM (I), serum-complete DMEM (II), and resting and thrombin (1 U/ml)-activated WP-DMEM (III and V), the cancer proliferation rate was measured by immunofluorescence for Ki-67 expression. In IV and VI, cancer cells were pretreated with the c-MYC inhibitor 10058-F4 (60 μM) for 20 min before 24 h coculture with platelets. DMSO (0.01%) was used as vehicle control. The white arrows point to platelet aggregates. Representative images at ×10 are shown. For each sample, 3 different fields were imaged. Scale bar, 20 μm.

B: bar graph showing the percentage of Ki-67-positive cells relative to the total number of nuclei (DAPI-positive cells). From each sample, 3 fields were counted at magnification of ×10 using ImageJ software. Data are means ± SE of three independent experiments. #P ≤ 0.05 for comparison of cancer cells cultured in serum or WP-DMEM vs. cells cultured in SF-DMEM. *P ≤ 0.05 for comparison of cancer cells cultured in WP-DMEM in the presence of 10058-F4 vs. vehicle control.
enhanced in the presence of resting or activated platelets (Figs. 1 and 4). We next sought to determine whether aspirin at an antiplatelet dose (20 μM) could reduce the ability of platelets to potentiate colon and pancreatic cancer cell line proliferation. To test this hypothesis, platelets were pretreated with 20 μM aspirin for 30 min and left resting or activated with thrombin prior to coculture with SW480, SW620, or PANC-1 cancer cells for 24 h. The ability of cancer cells to proliferate was quantified via immunostaining for the proliferation marker Ki-67. Our results shown in Fig. 5 first confirm that the presence of washed platelets increased the degree of SW480, SW620, and PANC-1 cancer cell proliferation. We found that
pretreatment of platelets with 20 μM aspirin reversed the ability of activated, but not resting, platelets to promote SW480 and PANC-1 cancer cell proliferation, while having no effect on the ability of platelets to promote SW620 proliferation (Fig. 5). In addition, the proliferation of cancer cells cultured in SF-DMEM alone was not affected by aspirin.

We next sought to investigate whether the reduction in platelet-induced SW480 and PANC-1 cancer cell proliferation by aspirin was associated with changes in c-MYC oncoprotein levels. Washed human platelets were incubated with aspirin (20 μM) and activated with thrombin or left inactive before coculture with cancer cells for 24 h. As shown in Fig. 5, aspirin inhibited the ability of thrombin-activated platelets to induce increased c-MYC expression in SW480 and PANC-1 cancer cells. In contrast, c-MYC expression levels remained relatively unchanged in SW620 colon cancer cells in response to aspirin therapy. Taken together, these results suggest that an antplatelet concentration of aspirin inhibits c-MYC upregulation promoted by activated platelets and c-MYC-dependent proliferation in SW480 colon cancer cells and PANC-1 pancreatic cancer cells.

Platelet releasate is sufficient to upregulate c-MYC in PANC-1 pancreatic cancer cells but not SW480 colon cancer cells: Effect of an antplatelet dose of aspirin on cancer cell proliferation. We next investigated whether platelet releasate was sufficient to induce c-MYC upregulation in SW480 colon cancer cells and PANC-1 pancreatic cancer cells. We used a transwell membrane to prevent direct platelet-cancer cell contact yet allow the exchange of soluble factors between the upper and lower chamber. As shown in Fig. 6A, the increase in c-MYC expression was eliminated if resting washed platelets were separated from PANC-1 cancer cells by the transwell insert, indicating that a physical platelet-cancer cell interaction was required for resting platelets to promote c-MYC upregulation. In contrast, c-MYC expression was substantially increased in PANC-1 cancer cells following exposure to thrombin-activated washed platelets in a transwell apparatus compared with cancer cells exposed to buffer alone or to resting washed platelets (Fig. 6A). This observation suggested that soluble molecules released by thrombin-activated platelets were sufficient to induce c-MYC expression in PANC-1 cancer cells. Interestingly, we found that direct coculture of PANC-1 cancer cells with the platelet pellet fraction induced an increased c-MYC expression compared with platelet releasate alone. In contrast to the trends observed for PANC-1 cells, the transwell insert prevented both resting and activated platelets from inducing increased c-MYC expression in SW480 colon cancer cells, indicating that platelet-derived soluble molecules alone were not sufficient to upregulate c-MYC in SW480 cancer cells. However, an increase in c-MYC expression was observed when SW480 cancer cells were directly cocultured with the pellet fraction from thrombin-activated platelets, indicating that platelet-induced c-MYC upregulation in SW480 cancer cells is dependent on physical platelet-cancer cell contact.

Previous studies have indicated that mitogenic stimulation of cancer cells with PDGF-BB activates a signaling route that culminates in c-MYC expression (8). Considering that platelets are a major reservoir and blood source of PDGF-BB, we sought to explore whether PDGF-BB signaling was required for platelets to induce c-MYC expression in PANC-1 cancer cells. Our data show that treatment of PANC-1 cancer cells with AC710, which blocks the binding of PDGF-BB to its receptor, only marginally affected the expression of c-MYC in response to resting washed platelet, whereas blockade of PDGF-BB completely prevented the upregulation of c-MYC induced by thrombin-activated washed platelets (Fig. 6C). Together, these results indicate that PDGF-BB released by activated platelets is required to induce c-MYC upregulation in PANC-1 cancer cells.

Previous studies have shown that aspirin can modulate the amount and pattern of cytokines and growth factors released by activated platelets (12). Hence, we tested whether exposure of PANC-1 cancer cells to the releasate from thrombin-activated platelets would be sufficient to support cancer cell proliferation and investigated the potential effect of an antplatelet dose of aspirin on this response. To this end, platelet releasate was isolated from thrombin-activated platelets pretreated with aspirin or vehicle control and added to PANC-1 cancer cells. After 24 h of culture with DMEM medium conditioned with releasate from activated platelets, a significant increase in PANC-1 pancreatic cancer cell proliferation was observed, as detected by Ki-67 staining, supporting the notion that platelet releasate is mitogenic in nature (Fig. 6D). Importantly, proliferation of PANC-1 cancer cells induced by platelet-derived growth factors was reduced by an antplatelet dose of aspirin (Fig. 6D).

Effect of aspirin on the proliferation rate of SW480 and SW620 colon and PANC-1 pancreatic cancer cells. We next aimed to study the direct effect of increasing concentrations of aspirin on the proliferation rate of SW480 and SW620 colon cancer cells and PANC-1 pancreatic cancer cells grown in

Fig. 5. Effect of an antplatelet dose of aspirin (acetylsalicylic acid, ASA) on human platelet-induced cancer cell proliferation and c-MYC expression in colon and pancreatic cancer cells. Immunofluorescence staining of SW480 (A), SW620 (B), and PANC-1 (F) cancer cells for the nuclear proliferation marker Ki-67 (green) is shown. Nuclear counterstaining is performed with DAPI (blue). Actin is stained in red. Cancer cells were cultured for 24 h in DMEM conditioned with washed platelets pretreated with aspirin (20 μM, D) or vehicle control (0.01% DMSO; f) for 30 min before addition of thrombin (1 U/ml; activated WP DMEM) or platelet buffer (resting WP DMEM). Platelet-free SF-DMEM containing an equivalent volume of platelet buffer and aspirin or vehicle solution were used as controls. Representative images at ×63 are shown for two (SW620) or three (SW480, PANC-1) independent experiments. For each sample, 3 different fields were imaged. Scale bar, 20 μm. C, D, and G: bar graphs showing the percentage of Ki-67-positive cells relative to the total number of nuclei (DAPI-positive cells). From each sample, 3 fields were counted at magnification of ×10 using ImageJ software. Data are means ± SE of three independent experiments or mean with range of two independent experiments. *P ≤ 0.05 for comparison of cancer cells cultured in WP-DMEM with aspirin vs. vehicle control. E and H: representative blots and quantitation of the oncoprotein c-MYC in SW480 (E, left), SW620 (E, right), and PANC-1 (H) cancer cells. Cancer cells were cultured for 24 h with SF-DMEM or resting or activated washed platelets pretreated with aspirin (20 μM) or vehicle control. Cells were then lysed and proteins (30 μg) were blotted for c-MYC and CD41a expression. The relative c-MYC protein amount was quantified for each blot by densitometry using ImageJ software. The c-MYC protein expression level is graphed over the loading control, tubulin. Data are means ± SE of three independent experiments. *P ≤ 0.05 for comparison of c-MYC expression induced in cancer cells by aspirin-treated platelets vs. vehicle-treated platelets.
serum-complete DMEM. Our results show that an increasing concentration of aspirin had no effect on the proliferation rate of SW480 cancer cells after 24 h; however, a significant reduction in proliferation rate was observed after 48 h of treatment with 5 mM aspirin compared with baseline (Fig. 7 A).

For the SW620 cancer cell line, an increasing concentration of aspirin had no effect on the proliferation rate after 48 h, whereas a significant reduction in proliferation rate was observed after 24 h in the presence of 5 mM aspirin (Fig. 7 B).

These data suggest that, in vitro, the proliferation rates of these two colon cancer cell lines are sensitive to aspirin only at a concentration of 5 mM. In contrast, the rate of PANC-1 cancer cell proliferation was significantly reduced in the presence of a minimum of 2 mM aspirin after both 24 and 48 h of incubation (Fig. 7 C).

Effect of aspirin on the expression of proliferation oncoproteins in SW480 and SW620 colon cancer cells. Finally, we wanted to assess whether aspirin affected the expression levels of the oncoprotein c-MYC as well as COX-2, considered a primary target of aspirin (25, 46), and p300, a coactivator of several transcription factors that regulate cell proliferation, including c-MYC (15). Western blot and densitometry analysis...
showed that treatment with 5 mM aspirin for 24 or 48 h dramatically decreased COX-2 expression in both SW480 and SW620 cancer cells compared with vehicle controls (Fig. 8). Moreover, we found that c-MYC expression levels were significantly reduced for both SW480 and SW620 cancer cells treated for 48 h with 5 mM aspirin compared with vehicle controls. We finally investigated the effect of aspirin on the expression of p300. As shown by the Western blot and densitometry analysis, levels of p300 protein were dramatically inhibited in both SW480 and SW620 cancer cells treated for 48 h with 5 mM aspirin compared with vehicle controls (Fig. 8). To the best of our knowledge, this is the first report suggesting p300 as a potential target of aspirin in cancer cells.

**DISCUSSION**

The observations reported in this study add new insights into the mechanisms by which blood platelets potentiate tumor cell growth and upregulation of oncoproteins, including c-MYC, and provide insights into the anticancer mechanism of action of aspirin. We show that the proliferation rates of select colon and pancreatic cancer cells stimulated with thrombin-activated platelets are sensitive to aspirin at concentrations of 20 μM. In contrast, we demonstrate that the proliferation rates of colon cancer cells and pancreatic cancer cells stimulated with serum are sensitive to aspirin only at concentrations in excess of 2 mM. We show that human platelets potentiate cancer cell proliferation in a manner dependent on the upregulation of the oncoprotein c-MYC, unveiling, for the first time, the ability of platelets to regulate the expression of oncoproteins in cancer cells. We establish that the ability of platelets to upregulate c-MYC can be reversed by inhibition of thrombin-activated platelets with aspirin.

Platelets have been implicated to play a role in every stage of cancer progression, providing a rationale for the therapeutic targeting of platelets in cancer. Several epidemiological studies have suggested that antiplatelet doses of aspirin (75 mg/day) are more efficacious at preventing cancer metastasis compared with anti-inflammatory doses (325–1,200 mg/day) (13, 38, 43). We therefore designed this study to define the molecular mechanisms underlying the differential effects between the anticancer actions of high- vs. low-dose aspirin. Our data show that anti-inflammatory aspirin concentrations (0.5–2.5 mM), which approximate systemic pharmacological aspirin concentrations, did not affect the rate of proliferation of SW480 or SW620 cancer cells. A significant reduction in cancer cell proliferation was only observed at supraphysiological concentrations of aspirin (5 mM) (13, 43). These results are in accordance with previous studies showing that clinically achievable doses of aspirin often fail to inhibit serum-stimulated colon cancer cell proliferation in vitro (13). In contrast, the proliferation rate of PANC-1 pancreatic cancer cells was inhibited following treatment with aspirin at a dose of 1 mM or higher. The differential response of these three cancer cell lines to the doses of aspirin tested in our study may reflect the cancer cell genotypic and phenotypic heterogeneity. In human colon cancer in particular, downregulation of COX-2 and c-MYC expression by aspirin has been correlated with decreased tumor growth (1, 25, 46). In this study, we confirm that aspirin alone is capable of decreasing the expression of COX-2 and c-MYC oncoproteins yet only when used at high doses (2.5 mM). However, we did not observe a reduction in the rate of colon cancer cell proliferation in vitro in the presence of 2.5 mM aspirin, despite observing reduced expression of COX-2 and c-MYC proteins; this may be due to the fact that our study was limited to a 2D culture system. Future experiments will be designed to determine whether anti-inflammatory doses of aspirin affects cancer cell proliferation when grown in a 3D culture system, which may better recapitulate the in vivo microenvironment (18, 35). Our study also identified a poten-

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**Fig. 7.** Direct effect of high doses of aspirin on the proliferation of colon cancer cells and pancreatic cancer cells. Colon cancer cells (10⁵ cells/ml) were plated in a 96-well plate and treated with increasing doses of aspirin (0.25–5.00 mM) or vehicle control (0.01% DMSO) in FBS (10%)–supplemented medium for 24 or 48 h. The rate of cancer cell proliferation was evaluated with the Cell Titer 96 AQueous One Solution Cell Proliferation Assay. The assay was performed in duplicate in three independent experiments, and the absorbance at 570-nm wavelength was measured. A: dose-response graph displaying the effect of the indicated doses of aspirin on the proliferation of SW480, nonmetastatic colon cancer cells. B: dose-response graph displaying the effect of the indicated doses of aspirin on the proliferation of SW620, metastatic colon cancer cells. C: dose-response graph displaying the effect of the indicated doses of aspirin on the proliferation of PANC-1, nonmetastatic pancreatic cancer cells. Data are means ± SE. **#P ≤ 0.05 and *P ≤ 0.05 for comparison of aspirin- vs. vehicle-treated cells for 24 and 48 h, respectively.**
tial effect of aspirin on p300, a transcriptional coactivator of a multitude of oncoproteins that function as regulators of cancer cell proliferation, including c-MYC (15). Aspirin at 5 mM effectively inhibited p300 expression in SW480 and SW620 cancer cells. In support of this observation, a recent study demonstrated that salicylic acid and its derivates can block the growth of leukemia cells by inhibiting p300 activation (42). Further mechanistic studies are warranted to directly define the consequence of p300 inhibition by aspirin on colon cancer survival and metastasis.

The anticancer mechanism of action of an antiplatelet dose of aspirin remains ill defined. Paracrine and juxtacrine interactions between platelets and cancer cells have been indicated to play a role in promoting tumor growth and survival (31). For instance, recent studies suggest that platelet secretion of cytokines and growth factors supports tumor vascular homeostasis, enhances tumor invasive and migratory properties, and potentiates tumor survival, proliferation, and extravasation from the circulation (4, 16, 24, 29, 40). Previous work has shown that oncoproteins, such as c-MYC, drive the proliferation of cancer (41). In our study, platelets were shown to enhance the proliferation of the colon cancer cell lines SW480 and SW620 and of the pancreatic cell line PANC-1. In SW480 and PANC-1 cancer cells, this response was associated with an increase in the expression of the oncoprotein c-MYC. This is noteworthy, as elevated expression of c-MYC is found in almost one-third of colon carcinomas and in 42% of advanced pancreatic cancer, emphasizing the oncogenic nature of this transcription factor (6, 14). Importantly, the upregulation of the oncoprotein c-MYC in colon and pancreatic cancer often occurs without apparent rearrangement or amplification of the \textit{c-myc} gene; rather, it seems to be dependent on microenvironmental signals (14). However, the cellular source(s) and molecular trigger(s) that drive c-MYC overexpression in cancer cells are, to date, unknown. Our data show for the first time that platelets, either in resting or activated conditions, trigger the upregulation of c-MYC oncoprotein in select cancer cell lines. We found that both serum-starved PANC-1 cancer cells and SW480 colon cancer cells, but not SW620 colon cancer cells, upregulated c-MYC expression following exposure to serum and resting or activated human platelets. It is noteworthy that SW620 cancer cells were found to express exceedingly high levels of c-MYC at baseline, suggesting that SW620 cancer cells might harbor a genomic amplification of the \textit{c-myc} gene that makes the ex-
pression of c-MYC oncoprotein independent of extracellular signals; future genomic studies will be necessary to confirm this hypothesis. It is also important to mention that the high expression of c-MYC in starved SW620 might have interfered with our ability to detect any direct effect that platelets might have had on this protein. In contrast, starved PANC-1 and SW480 cancer cells had a low c-MYC expression by themselves. Surprisingly, we also observed that the c-MYC inhibitor 10058-F4 was effective in reversing the ability of platelets to promote the proliferation of SW620 cancer cells, albeit with less potency compared with SW480 and PANC-1 cancer cells. In our view, the most likely scenario is that the activity of c-MYC oncoprotein is essential for the survival and proliferation of SW620 cancer cells independent of the extracellular stimuli, including platelet-derived signals. Although we propose platelet-induced c-MYC upregulation as a novel pathway associated with cancer cell proliferation, alternative, additional routes cannot be excluded. Several studies on genetic and molecular characteristics of cancer cells following exposure to platelets have identified several potential pathways controlling platelet-induced cancer cell survival and metastatic potential. Among these, the activation of the NF-κB signaling downstream of the TGF-β pathway seems to play a relevant role in select types of cancer (9, 23).

It has been reported that platelets can support cancer progression through both the release of growth factors and direct cell-cell interactions (9, 23, 40, 45). Our findings suggest that the molecular basis for platelet-induced c-MYC upregulation lies in platelet-cancer interactions that are different for each cell type and dependent on the state of activation of platelets. The inter- and intraheterogeneity of each cell line makes it difficult to identify whether a single or multiple platelet-derived molecules determine the change in c-MYC expression. A big data screening approach using a complete bank of cancer cell lines may provide further insight into the range of responses that cancer cell lines exhibit in response to platelet binding or releasate.

We demonstrate that direct contact with platelets, either in a resting or activated state, was necessary and sufficient for the induction of c-MYC expression in SW480 cancer cells; therefore, platelet-derived soluble factors alone were unable to elicit a response. Although the juxtacrine interaction between platelets and SW480 colon cancer cells could be heterotypic in nature, we found αIIbβ3 on platelets to be a pivotal adhesive molecule in this response. In our study, inhibition of αIIbβ3 with integrilin significantly reduced the increase in c-MYC expression induced by resting and activated platelets in SW480 colon cancer cells. In contrast, our observations indicate that, in addition to cell contact, platelet-released molecules are also required to increase c-MYC expression in PANC-1 pancreatic cancer cells. Platelet contact with PANC-1 cancer cells was found to be essential when platelets were in a resting state, but not when activated, suggesting that, following adhesion to PANC-1 cancer cells, platelets may secrete their contents to promote the expression of the oncoprotein c-MYC and cancer cell proliferation. In support of this, the releasate from thrombin-activated platelets was sufficient to upregulate c-MYC and induce proliferation of PANC-1 cancer cells. Several studies have provided evidence that cancer cells can induce platelet aggregation and secretion of soluble factors that create a microenvironment favorable to cell growth and survival (31, 32). In the present study, the molecular pathways triggered in platelets following exposure to PANC-1 pancreatic cancer cells and necessary for platelet activation and c-MYC expression, while unclear, seem to be αIIbβ3 independent. For both the cancer cell lines used in our study, the membrane-bound molecules engaged by platelets to transmit signal and promote c-MYC accumulation remain to be elucidated.

A wide range of growth factors and cytokines has been shown to trigger c-MYC expression in a variety of cancer cells (19). Here we identify a critical role for PDGF-BB in promoting c-MYC upregulation in PANC-1 cancer cells; however, the presence of additional platelet-derived soluble molecules synergizing with PDGF-BB cannot be excluded. Although the intracellular molecular mechanisms through which platelets induce c-MYC expression in SW480 and PANC-1 cancer cells are ill defined and the subject of present study, we observed that the activation of the PI3K pathway is partially involved, as platelet-induced c-MYC expression in both SW480 and PANC-1 cancer cells was reversed in response to the PI3K inhibitor wortmannin. These results are in good agreement with previous literature showing the importance of the PI3K pathway as key regulator of many aspects of human malignancies, including cancer cell survival and proliferation (7).

One of the key biological functions of c-MYC is its ability to promote cell proliferation (39, 41). We demonstrate that, in the absence of serum or platelets, c-MYC expression declines to undetectable levels in SW480 and PANC-1 cancer cells. As a consequence, cancer proliferation is arrested. After addition of serum or stimulation with platelets, c-MYC expression is induced, and cells are able to proliferate. Importantly, treatment of platelets with 20 μM aspirin, before stimulation with thrombin, reversed the stimulatory effect of platelets on SW480 and PANC-1 nonmetastatic cancer cells yet had no effect on SW620 metastatic cancer cells. Moreover, taking into account that aspirin did not inhibit c-MYC upregulation induced by resting washed platelets, it is plausible to suggest that aspirin modulated the response of platelets to thrombin by altering the pattern of proteins released from platelet granules, which in turn caused differential responses in the cancer cells. Previous reports have demonstrated that aspirin can reduce the amount and alter the pattern of cytokines and growth factor released by activated platelets (12). In support of this, we demonstrate that the releasate isolated from aspirin-treated platelets was less efficient in promoting PANC-1 proliferation compared with aspirin-free platelets.

Our study reveals important differences and specificities in the mechanism of action of high- and low-dose aspirin in metastatic and nonmetastatic cancer cells with different tumor origins and suggests that the ability of aspirin to prevent platelet-induced c-MYC expression might be selective for a nonmetastatic phenotype. Future work will need to expand this study to a larger number of cancer cell types characterized by diverse genotypic and phenotypic properties. Moreover, the influence of platelets on the expression of other proliferation- and metastasis-relevant oncoproteins, beyond c-MYC, should be explored.

In summary, we showed for the first time that platelets induce the upregulation of the oncoprotein c-MYC in SW480 colon cancer cells and PANC-1 pancreatic cancer cells in a molecular fashion that is cell type specific. We demonstrated that a low antiplatelet concentration of aspirin is effective in
inhibiting cancer cell proliferation of PANC-1 nonmetastatic pancreatic cancer cells and SW480 nonmetastatic colon cancer cells, but not of the SW62 metastatic cell line, when platelets are used as growth stimuli. Most importantly, we propose a novel anticancer mechanism of action of low-dose aspirin, namely through the inhibition of platelet-induced molecular signals that cause aberrant expression of c-MYC oncoprotein in nonmetastatic cancers. On the basis of the evidence that we present here, we propose that low-dose aspirin might inhibit the proliferative ability of cancer cells by manipulating the cross talk between platelets and cancer cells. This concept might have implications for aspirin therapy in conditions in which platelet activation is important, such as during the journey of the cancer cells in the circulation. Because the interaction between platelets and cancer cells is believed to occur early following intravasation (24), the use of antiplatelet doses of aspirin might serve as a safe and efficacious preventive measure for patients at risk for cancer.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

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