Diclofenac Inhibits Tumor Growth in a Murine Model of Pancreatic Cancer by Modulation of VEGF Levels and Arginase Activity

Nina Mayorek*, Nili Naftali-Shani, Myriam Grunewald

Developmental Biology and Cancer Research, Institute for Medical Research Israel-Canada (IMRIC), The Hebrew University-Hadassah Medical School, Jerusalem, Israel

Abstract

Background: Diclofenac is one of the oldest anti-inflammatory drugs in use. In addition to its inhibition of cyclooxygenases (COX), diclofenac potently inhibits phospholipase A2 (PLA2), thus yielding a broad anti-inflammatory effect. Since inflammation is an important factor in the development of pancreatic tumors we explored the potential of diclofenac to inhibit tumor growth in mice inoculated with PANC02 cells orthotopically.

Methodology/Principal Findings: We found that diclofenac treatment (30 mg/kg/bw for 11 days) of mice inoculated with PANC02 cells, reduced the tumor weight by 60%, correlating with increased apoptosis of tumor cells. Since this effect was not observed in vitro on cultured PANCO2 cells, we theorized that diclofenac beneficial treatment involved other mediators present in vivo. Indeed, diclofenac drastically decreased tumor vascularization by downregulating VEGF in the tumor and in abdominal cavity fluid. Furthermore, diclofenac directly inhibited vascular sprouting ex vivo. Surprisingly, in contrast to other COX-2 inhibitors, diclofenac increased arginase activity/arginase 1 protein content in tumor stroma cells, peritoneal macrophages and white blood cells by 2.4, 4.8 and 2 fold, respectively. We propose that the subsequent arginine depletion and decrease in NO levels, both in serum and peritoneal cavity, adds to tumor growth inhibition by malnourishment and poor vasculature development.

Conclusion/Significance: In conclusion, diclofenac shows pronounced antitumoral properties in pancreatic cancer model that can contribute to further treatment development. The ability of diclofenac to induce arginase activity in tumor stroma, peritoneal macrophages and white blood cells provides a tool to study a controversial issue of pro-and antitumoral effects of arginine depletion.

Citation: Mayorek N, Naftali-Shani N, Grunewald M (2010) Diclofenac Inhibits Tumor Growth in a Murine Model of Pancreatic Cancer by Modulation of VEGF Levels and Arginase Activity. PLoS ONE 5(9): e12715. doi:10.1371/journal.pone.0012715

Editor: Irene Oi Lin Ng, The University of Hong Kong, Hong Kong

Received January 31, 2010; Accepted August 6, 2010; Published September 15, 2010

Copyright: © 2010 Mayorek et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by the Hebrew University-Hadassah Medical School (grant # 0463435). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: ninam@ekmd.huji.ac.il

Introduction

Inflammation is highly related to both carcinogenesis and to progression of tumor growth [1]. Epidemiological studies show that chronic inflammation predisposes patients to cancer development and long-term use of non-steroidal anti-inflammatory drugs (NSAIDs) reduces the risk of several cancers [2].

Cyclooxygenases (COX-1 and -2) are rate-limiting enzymes in the production of prostaglandins (PGs) from arachidonic acid. COX-1 expression is generally constitutive, while COX-2 is usually induced by stimuli involved in inflammatory responses. Prostaglandin E2 (PGE2), a primary metabolite of COX-2, has been shown to promote cell survival, proliferation, and angiogenesis and inhibit apoptosis and antitumor immune response, all processes promoting cancer development [3].

Pancreatic cancer is a lethal disease with very limited options for treatment. Chronic pancreatitis predisposes individuals to developing pancreatic ductal adenocarcinoma [4] and pancreatic tumor stroma is characterized by the variety of inflammatory cells [5] suggesting the involvement of inflammatory processes in this cancer. COX-2 is overexpressed in approximately 75% of human carcinomas including those of the pancreas. Recent studies in transgenic mice [6,7,8] have shown that COX-2 overexpression in the exocrine pancreas induced pancreatitis-like-state. The onset of the cancer in these mice was prevented by maintaining mice on selective COX-2 inhibitors, further demonstrating the important role of inflammation on pancreatic cancer development.

Human trials to prevent colon cancer were recently conducted using COX-2 inhibitors. Despite encouraging results in colon cancer prevention and reduction in the incidence of colon adenomas, NSAIDs and selective COX-2 inhibitors can cause serious cardiovascular and gastrointestinal side effects and therefore are not routinely recommended for these diseases [9,10].

Diclofenac, one of the oldest NSAIDs has been in use since 1976. Diclofenac inhibits cyclooxygenases non-selectively. Studies in humans demonstrated that it reduces 94% of COX-2 and 49% of COX-1 activity [11]. In vitro it potently inhibits phospholipase A2 (PLA2) [12], the enzyme which liberates arachidonic acid and lysophospholipid to generate a family of pro-inflammatory eicosanoids (including PGE2) and platelet activating factor.
Additional evidence suggests that diclofenac also inhibits PLA2 in vivo. PLA2 is believed to play a key role in the first steps of the inflammatory cascade leading to acute pancreatitis [12]. The initial study [13], followed by several other groups, has shown that diclofenac can significantly decrease the rate of acute pancreatitis caused by endoscopic retrograde cholangiopancreatography.

In light of diclofenac ability to inhibit potentially both COX-2 and PLA2 we have explored its anticancer potential in a mouse model of pancreatic cancer. Here we report that diclofenac treatment causes a 60% decrease in tumor size. This is due to increased apoptosis of tumor cells. We provide evidence that this effect is indirect and operates through at least two mechanisms. First, diclofenac yields a significant antiangiogenic effect, as demonstrated by a strong reduction in vascular endothelial growth factor (VEGF) tumor content, decreased microvascular density and morphological changes in tumor blood vessels. Second, diclofenac treatment remarkably increases arginase activity in tumor stroma cells, peritoneal macrophages and white blood cells. This is an unexpected finding since COX-2 inhibitors were shown to reduce tumor growth through arginase inhibition [14,15]. Our results are in line with earlier studies, which point to antitumoral [16] and antiangiogenic effect [17] of arginine depletion and support the rather controversial approach of anticancer treatment by enhancing arginase activity [18,19].

Methods

Ethics statement
Experimental animal procedures were approved by the Ethical Committee of the Hebrew University, which acts under the surveillance of the AAALAC International.

Animals and PANCO2 cell line
Female CB6F1 mice (9–10 weeks old) and male Sprague-Dawley rats (200 g) were from Harlan, Israel. Female CX3CR1GFP/+ mice [20] were generously provided by S. Jung (Rehovot, Israel). In these mice a GFP reporter is driven by the CX3CR1 promoter. Activity of this chemokine receptor promoter is restricted to mononuclear myeloid cells, including all circulating monocytes, and is essentially absent from cells of the lymphoid lineage. All experiments except the experiment presented in Fig S3 were performed using CB6F1 mice.

PANCO2 cells were a generous gift from M Dauer (Munich, Germany). Cells were maintained in RPMI with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.

Orthotopic-syngeneic model of pancreatic cancer
Mice were anesthetized with a mixture of ketamine-xylazine. The pancreas was injected with 4×10^5 CB6F1 mice or 6×10^5 CX3CR1GFP/+ mice) PANCO2 cells in 30 µl phosphate buffered saline (PBS). Mice were removed from an experiment if a peritoneal leakage was suspected. Sham operated mice underwent the same procedure and were injected with 30 µl PBS. Mice were divided into groups of 6 to 8 animals per group. The treated group received 30 mg/kg b.w. diclofenac in drinking water starting from day 3 after inoculation. The dosage was calculated based on the estimate that a mouse drinks about 3 ml water per day. Mice were killed 14 days after inoculation unless specified otherwise.

Blood for white blood cells (WBC) preparation, VEGF and nitrogen oxide (NO) concentration was taken from heart. Peritoneal cavity was flushed with 2 ml of PBS with 0.1% bovine serum albumin (BSA) for macrophages preparation, for measurement of VEGF and for NO concentrations.

Peritoneal cells and peritoneal macrophages isolation
Peritoneal cells were obtained by centrifugation of peritoneal cavity flush. Peritoneal macrophages were isolated from peritoneal cells by differential adhesion to plastic. The macrophages were cultured in RPMI with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin for 20 hrs. To measure arginase activity, cells were washed with PBS and dissolved in 0.1% TritonX100 in water for 30 min at room temperature.

Tumor homogenates
Tumor homogenates were prepared from tumor samples which were stored at −80°C, in water containing 0.1% Triton X 100 and protease inhibitor cocktail (Sigma P8340). Homogenates were centrifuged for 30 min at 13400 g and the supernatants were used for measuring arginase activity, arginase 1, smooth muscle actin and VEGF content and caspase-3 activation.

Bone marrow cells isolation
Tibias and femurs of tumor bearing mice were removed and flushed with PBS. After centrifugation of harvested cells on a Ficoll gradient at 4000 g for 20 min at room temperature mononuclear cells were isolated with PE-conjugated primary antibody anti–CD 115 and anti-PE microbeads (Biolegend). CD-115 positive and negative cells were analyzed for arginase activity.

White blood cells isolation (WBC)
WBC were isolated using Ficoll density gradient (Amersham). WBC fraction was washed, cells were counted, lysed in water containing 0.1% Triton X 100 and analyzed for arginase 1 protein content.

Aortic ring sprouting assay
Aortic rings were prepared from thoracic aortas of Sprague-Dawley rats, as described [21]. Rings were embedded in collagen gel and maintained in Bio MgpM medium (Biological Industries, Israel). The treatment with 10 µM diclofenac started one day after seeding and lasted for 5 days. Rings were then fixed in 4% buffered formalin, stained with 0.02% crystal violet in absolute ethanol. Sprouting area was evaluated using the Image Pro program.

Caspase-3 activation
Caspase-3 activation was measured in tumor homogenates by separating the non-cleaved and cleaved caspase-3 on a 20% acrylamide SDS-PAGE electrophoresis gel and blotting with monoclonal anti–caspase-3 (Cell Signaling).

VEGF and NO content
VEGF content was measured in serum, in supernatant of peritoneal cavity flush, in tumor homogenates, PANCO2 and cultured macrophages using the mouse VEGF Quantikine immunoassay kit (R &D systems).

NO content was measured in the serum and in the supernatant of peritoneal cavity flush using a nitrate/nitrite colorimetric assay kit (Cayman Chemical Company).

Arginase activity
Arginase activity was measured as described [22]. 100 µg of protein was assayed for 10 min at 37°C for peritoneal cells and macrophages, 30 min for tumor homogenates and 2 hours for
bone marrow cells. Protein content was measured in each sample using the micro BCA protein assay kit (Thermo Scientific).

**Arginase 1 and smooth muscle actin protein content**

Proteins from tumor homogenates, WBC lysates and GFP positive cells isolated from tumors were separated on 10% acrylamide SDS-PAGE electrophoresis gels and blotted either with monoclonal arginase-1 (BD Transduction Laboratories) or monoclonal smooth muscle actin (Sigma).

**Isolation of mononuclear myeloid cells from tumors**

Tumors were extracted from CX3CR1^{GFP+} mice and digested with collagenase (400 units/ml; Sigma and Dnase 0.33 mg/ml; Sigma) for 45 min at 37°C. GFP positive population was purified by high speed cell sorting using FACS Aria (Becton, Dickinson). Cells were counted and lysed in water containing 0.1% Triton X 100 and analyzed for arginase 1 protein content.

**PANCO2 cells rate of growth**

PANCO2 cells were seeded in RPMI supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Diclofenac (10 or 50 μM) was added the following day and after 4 days of treatment, cells content was measured using a cell proliferation kit (Biological Industries, Israel).

**Immunohistochemistry, TUNEL staining and morphometry**

Paraffin sections of tumors were stained using the following primary antibodies: rat anti-mouse Fk/80 (Serotec), monoclonal anti- arginase-1 (BD Transduction Laboratories), monoclonal anti-α-smooth muscle actin (Sigma), monoclonal anti- Ki 67 (Neomarkers), rabbit anti- phosphor-histone H3 (Cell Signaling), rabbit anti-VEGF (Calbiochem) and monoclonal anti- von Willebrand factor (DACO). Horseradish peroxidase (HRP) conjugated secondary antibodies were used for chromogenic detection.

TUNEL staining [23] was performed using in situ cell death detection kit (Roche).

Quantification of immunostaining data was performed either by high power field analysis counting of at least 10 areas/slide of 4 different tumors in each group or with the aid of image analysis system (Ariol SL-50).

**Statistical analysis**

All data were analyzed with SigmaStat software (Aspire software international). A Mann-Whitney test was used to calculate significant differences between untreated and diclofenac treated samples. A value of p≤0.05 was accepted as significant.

Sample size and number of repetitions are indicated in legends.

**Results**

**Diclofenac inhibits tumor growth in an orthotopic model of pancreatic cancer in mice**

PANCO2 cells were injected into the tail of the pancreas. After 4 days cancer cells formed small tumors of approximately 4 mm length. Tumors developed rapidly; at day 8 they had doubled in length and consistently metastasized to the peritoneal area of the abdominal incision executed for tumor inoculation. At day 14, tumors which grew at the site of the inoculation (primary tumors), weighed 300–500 mg and were highly vascularized. The metastatic tumors at the abdominal cut and in spleen were also prominent (Fig 1A).

Three to four weeks after inoculation, the peritoneal cavity was full of bloody ascites. Numerous metastatic tumors were found in the peritoneal cavity, including lymph nodes of the alimentary track, and mice began to die. Metastatic tumors on liver or lungs were not detected.

Subsequent experiments were therefore terminated at day 14 after inoculation. The mice appeared normal at this stage and there was no loss of body weight.

Diclofenac (30 mg/kg bw) was given daily in drinking water starting at day 3 after inoculation. Its effects were examined after 11 days of treatment. As shown in Fig 1B, the weight of the primary tumor (growing in the pancreas) was significantly lower (60%) in diclofenac treated mice as compared to untreated animals. Metastatic tumors which grew in the abdominal cut also weighed less in diclofenac treated mice, in all experiments, yet this effect did not achieve statistical significance.

**Diclofenac increases apoptosis of tumor cells in vivo, but not in vitro**

The decreased tumor weight observed in diclofenac treated mice led us to examine proliferation and apoptosis of tumor cells.

Tumors stained for Ki 67 (present during all active phases of the cell cycle: G1, S, G2, and mitosis) and of phospho-histone H3 (the specific marker of mitotic cells) showed no difference between untreated and diclofenac treated mice, Fig 2B.

However, as shown in Fig 2, diclofenac treatment increased apoptosis by 6 fold, as indicated by TUNEL staining of tumors and quantification (Fig 2A). This finding was further supported by an enhanced presence of cleaved caspase 3 in homogenates from tumors excised from diclofenac treated mice (Fig 2B).

The enhanced apoptosis caused by diclofenac in vivo could not be recapitulated in vitro. PANCO2 cells cultured for 4 days in the presence of 10 and 50 μM of diclofenac grew at the same rate as untreated cells (Fig 2C) indicating that the in vivo effects of diclofenac require some mediators that are absent in the in vitro system.

**Antiangiogenic effect of diclofenac in vivo and ex vivo**

Tumors from diclofenac treated animals were very pale (Fig 1A), suggesting that the treatment caused an antiangiogenic effect. Indeed, as shown in Fig 3A and B, staining for vascular endothelial marker von Willebrand Factor and counting of blood vessels revealed a 4 fold drop in the number of large peripheral vessels and a 2 fold drop in capillary density in tumors of diclofenac treated mice. Furthermore, smooth muscle actin staining (Fig 3C) revealed thin, straight vessels in treated tumors when compared to numerous, wide, tortuous blood vessels from tumors of untreated mice. Analysis of smooth muscle actin expression in tumor homogenates by western blot showed a significant decrease (2.5 fold) in diclofenac treated mice (Fig 3D and E).

VEGF has been shown to be the major proangiogenic factor in tumors [24]. Therefore, we measured tumor VEGF levels of diclofenac treated and untreated mice (Fig 3F). Treated mice tumors contained 3 times less VEGF when compared to tumors from untreated mice, suggesting that the decline in tumor vasculature results from a down regulation of VEGF (Fig 3F, top). Since tumor cells rapidly spread through the abdominal cavity, we next measured VEGF content in the peritoneal fluid. The flushable quantity of VEGF from the peritoneal cavity of tumor-bearing mice was 13 times higher than sham-operated healthy mice. This amount decreased by 2.8 fold after diclofenac treatment (Fig 3F, center).

These pronounced effects on VEGF content, both in peritoneal cavity and in tumor tissue, were not reflected in any changes in...
VEGF serum concentration (Fig 3F, bottom). Thus, serum VEGF concentrations were similar in serum of healthy-sham operated mice, in tumor-bearing mice and in tumor-bearing mice treated with diclofenac.

To determine whether diclofenac can directly inhibit angiogenesis, we measured sprouting angiogenesis of rat aortic rings ex vivo in response to drug treatment. As shown in Fig 3G, sprouting area was inhibited by 2.5 fold, when aortic rings were incubated with 10 μM of diclofenac (C max of diclofenac-treated patients), thus showing that diclofenac can directly inhibit blood vessel development.

Diclofenac increases arginase activity in pancreatic tumors and in peritoneal macrophages, but not in bone marrow-CD 115 positive and CD 115 negative cells

One of the results of COX-2 overexpression by tumor cells is a large production of PGE2 which leads to an impaired T-cell response [14] [25]. PGE2 induces arginase 1 activity and arginine uptake in myeloid derived suppressor cells (MDSCs), thus causing arginine depletion in the tumor surrounding. The relative lack of arginine causes a defect in the CD3ζ expression of the tumor-infiltrating T cells. Since COX-2 inhibitors were shown to partially stop tumor growth through arginase inhibition in MDSC [14,25] we measured arginase activity in pancreatic tumor homogenates from non-treated and diclofenac treated mice (Fig 4A, top).

To our surprise arginase activity was not inhibited by diclofenac treatment, but rather was significantly activated by 2.4 fold. Immuno-histological staining showed arginase 1 positive cells at the periphery of tumors in both untreated and diclofenac treated mice (Fig 4B). The number of arginase 1 expressing cells and their staining intensity seemed to increase under diclofenac treatment. A significant, 1.8 fold increase in arginase 1 protein content in tumors of diclofenac treated mice was also measured by western blot analysis of tumor homogenates (Fig S2). Using an antibody against the macrophage marker F4/80 (Fig 4C) we could not detect any overlap with arginase 1 staining.

In order to further characterize the arginase 1 expressing cells in PANC02 tumors, we inoculated PANC02 cells into transgenic mice where a GFP reporter is driven by the CX3CR1 promoter. Activity of this chemokine receptor promoter is restricted to...
mononuclear myeloid cells, including all circulating CD116^+ monocytes, and is essentially absent from cells of the lymphoid lineage. We isolated GFP positive cells from tumors and found that these cells express high levels of arginase 1 (Fig S3).

Thus, the increased activity of arginase found in tumors from diclofenac treated mice is due at least in part to the activation of arginase 1 in monocyte derived CX3CR1^+ cells. Arginase activity was absent in cultured PANC02 (results not shown) and arginase 1 protein was never detected in tumor cells in vivo by immunostaining.

The striking effect of diclofenac treatment on arginase activity in pancreatic tumors led us to examine arginase activity in peritoneal macrophages. Peritoneal macrophages can be involved in tumor surveillance [26] and show an enhanced arginase expression in tumor-bearing mice [27]. Macrophages from peritoneal lavage were isolated by preferential attachment to culture dishes and cultured overnight. Immunostaining showed that they were both F4/80 positive and arginase 1 positive (results not shown). Arginase activity was upregulated (4.8 fold) in macrophages derived from tumor- bearing mice treated with diclofenac for 11 days, when compared to untreated mice, (Fig 4A center).

Since macrophage arginase expression can change in response to culture dish adhesion [28] we also measured arginase activity in non-cultured freshly isolated cells from the mouse peritoneal cavity, thereby compromising on macrophages purity, (Fig 4A bottom).

Diclofenac effectively stimulated arginase activity in non-cultured cells derived from both tumor-free and tumor-bearing mice treated with diclofenac for 2 days only by 7 and 3 fold, respectively. Tumor presence enhanced arginase activity by 4 fold increasing the activity from 0.03 (cells from non-tumor bearing mice) to 0.12 mg urea/min/mg protein (cells from tumor -bearing mice) which is in line with previous reported results [27].

We also counted the number of peritoneal cells extracted from each mouse and found that a 2 day diclofenac treatment yielded a 3 fold increase in cells from tumor-free mice. The mean number of cells ± SE was 0.6*10^6 ± 0.06*10^6 extracted from untreated mice and 1.5*10^6±0.4*10^6 from tumor treated mice (p≤0.02, 7 mice in each group).

This observation shows that after a short diclofenac treatment, the total arginase activity in peritoneal cells (mostly macrophages) is extremely high, both because of the large increase in a specific activity of this enzyme and because of the increase in the number of activated cells.

The activation of the arginase activity by diclofenac could not be demonstrated in vitro. Incubation of peritoneal macrophages for 48 hours (Fig 4D) did not increase arginase activity, but rather yielded a certain decrease in the specific activity of this enzyme. The same results were obtained when macrophages were incubated for 96 hours with diclofenac (results not shown). Thus, similarly to the effect of diclofenac on apoptosis of tumor cells,
mediators present in vivo and absent in cultured macrophages were required in order to achieve the induction of arginase activity by this drug.

We also investigated whether arginase activation by diclofenac can be detected in bone marrow macrophage precursors. We isolated mononuclear cells from tibiae and femurs of tumor-bearing mice untreated and treated for 11 days with diclofenac. We found very low arginase activity in both CD 115+ and CD 115- cells. Thus, arginase activation by diclofenac happens either in differentiated macrophages or the mediators needed for promoting diclofenac-induced activation of arginase do not reach the bone marrow compartment.

Diclofenac decreases NO level in peritoneal cavity and in serum

We next investigated whether the pronounced activation of arginase in both tumor tissue and in peritoneal macrophages influenced NO production. Arginase and nitric oxide synthase (NOS) compete for arginine, which serves as substrate for both these enzymes. The activation of arginase can therefore lead to arginine depletion [29] yielding a decrease in NOS activity. Indeed, our measurements of the NO content in the peritoneal cavity (Fig 3top), and in the serum (Fig 3bottom), showed a significant decrease in NO in both these compartments. The NO content in the peritoneal cavity was 1.3 fold lower in tumor-bearing mice treated for 11 days with diclofenac, as compared with tumor-bearing untreated mice. The effect on serum NO concentration was even more pronounced and amounted to 3.5-fold drop in diclofenac treated animals.

In order to study the reason for this very high drop in NO serum concentration we isolated white blood cells (WBC) from untreated and diclofenac treated mice. We found that diclofenac treated mice contained 4 fold higher quantity of WBC per millimeter of blood. The mean number of cells ±SE was 0.26×10^6±0.03x10^6 isolated from untreated mice and 1.2×10^6±0.09x10^6 from diclofenac treated mice (P=0.001, 3 mice in each group).

The arginase protein level per equal number of cells was increased 2 fold Fig S4. Thus, arginase activity in diclofenac treated mice blood is about 8 fold higher as compared to untreated mice, and may explain a pronounced decrease in NO serum concentration.

Discussion

In this study we have examined the antitumor potential of diclofenac in an orthotopic model of mouse pancreatic cancer. PANC02 cells injected into the tail of the pancreas rapidly multiplied, formed large local tumors and repeatedly metastasized primarily to the cut in the abdomen performed for inoculation of (Fig 1). The tendency of tumors to implant into a wound is a concern in cancer surgery [50] and has been studied in animal models [31]. The pro-tumor factors secreted by wounds may be responsible for this phenomenon.

Diclofenac treatment caused a significant decrease in the tumor weight at the primary site of injection. This decrease was due to enhanced apoptosis of cancer cells (Fig 2). The anti-tumoral capacity of diclofenac is generally attributed to its direct effect on tumor cell viability and cell cycle [32,53]. However this effect is obtained only using very high concentrations of drug in vitro (up to 800 μM). This may not reflect the in vivo situation except for topical administration of the drug [32].

In our model, the effect on PANC02 growth rate could not be reproduced in vitro, when cells were incubated either in the presence of 10 μM diclofenac, the higher range of C max values of the drug reported in human [34] and mice [35] studies, or in the presence of 50 μM of the drug. This prompted us to test the activity of diclofenac on other tumor growth promoting components.

Antiangiogenic activity of diclofenac was suggested in a model of subcutaneous implanted colon-26 adenocarcinoma cells, where the drug was topically applied [32]. Our study further demonstrates the antiangiogenic potential of diclofenac in intra-abdominal tumors. We found, that after 11-day treatment, diclofenac caused a significant decrease in blood content of tumors as illustrated by their pale appearance (Fig 1), decrease in blood vessels count and smooth muscle actin content (Fig 3B and Fig 3C, D and E).

VEGF has been shown to be the main angiogenic factor in tumor vascularization. Therefore we measured VEGF protein levels both in the tumor and in the peritoneum and found a pronounced decrease in diclofenac treated animals (Fig 3F). The decrease in VEGF content in tumors of diclofenac treated mice may be due to a decrease in VEGF expression in tumor cells, in stroma cells, or in both types of cells. The decrease in the peritoneal cavity can be due to the change in VEGF content of tumor or to the direct effect of diclofenac on peritoneal mesothelial cell lining. Additional studies are required to resolve this issue. The effect of diclofenac on VEGF production could not be recapitulated in vitro when cultured PANC02 or peritoneal macrophages were incubated with diclofenac (Fig S5). Previously, diclofenac has been reported to decrease mRNA-VEGF levels via COX inhibition in cultured esophageal cancer cell lines [36]. In our in vivo model it is thus likely that the direct effect of diclofenac on VEGF production leads to an antiangiogenic effect which in turn affects apoptosis of cancer cells in the growing tumor.

We have also tested the possibility that diclofenac may directly affect vascular cells. While diclofenac was unable to induce pro-apoptotic and anti-VEGF effect on cultured PANC02, its antiangiogenic effect could be demonstrated ex vivo (Fig 3G).
When 10 μM diclofenac was incubated together with rat aortic rings, it yielded a pronounced inhibition of sprouting. This suggests that diclofenac can directly inhibit the development of endothelial/smooth muscle cells.

Interestingly, we have found that peritoneal concentration of VEGF is significantly higher than that of the serum, both in tumor-free and in tumor-bearing mice. Assuming that the volume of the peritoneal fluid is about 100 ml [37], our results demonstrate that the VEGF concentration amounts to about 340 pg/ml in sham-operated-tumor-free mice and to above 4000 pg/ml in tumor-bearing mice. The high concentration of VEGF in the peritoneal cavity does not equilibrate with the blood compartment and is 4 and 55 fold higher (as compared with serum values) in sham-operated and tumor-bearing mice, respectively. In healthy pigs [38] it was also shown that the intra peritoneal VEGF does not equilibrate with the serum. Our results, as well as study [39], challenge the notion that systemic VEGF levels can predict tumor load. Furthermore it may be interesting to explore the possibility of delivering antiangiogenic antibodies for intra abdominal tumors directly into the peritoneal cavity and not systemically.

Unlike the clear antineoplastic effect of diclofenac through inhibition of angiogenesis, the striking activation of arginase activity (Fig 4), both in tumor stroma and in peritoneal macrophages is difficult to assess in context of pro- or anti-cancer
through the use of pegylated recombinant human arginase [18,19].

A significant inhibition of tumor growth. Further research is needed in peritoneal macrophages and in WBC. This activation correlates with immunosuppressive activity and tumor-promotion [25]. The authors proposed that high COX-2 activity of tumor cells releases PGE2, which activates arginase in MDSC and leads to extracellular arginine depletion and subsequent T-cell dysfunction. Along with these results, two additional studies [14,15] have shown that treatment of tumor bearing mice with COX-2 specific inhibitors completely blocked the induction of arginase activity, reduced MDSC accumulation and decreased tumor growth. However, there is evidence that tumor cells are particularly sensitive to arginine depletion [40] and that enhanced arginase activity can restrict tumor growth [18] and [16]. Furthermore it has been shown that dietary arginine restriction caused a significant reduction in both tumor incidence and tumor multiplicity in a mouse papilloma cancer model [41].

To our knowledge the first we are to show that diclofenac causes pronounced arginase activation in tumor stroma CX3CR1 + cells, in peritoneal macrophages and in WBC. This activation correlates with a significant inhibition of tumor growth. Further research is needed in order to determine if this correlation is causative or coincidental.

Our study supports the development of a new therapeutic avenue through the use of pegylated recombinant human arginase [18,19].

**Supporting Information**

**Figure S1** Diclofenac does not affect proliferation. Mice were inoculated with PANC02 cells and treated with diclofenac as described in Figure 1. Ki 67 and phospho-histone H3 staining of fixed tumors was performed as described in Materials and Methods. % of Ki positive nuclei was counted, lysed and analyzed for arginase 1 protein content. Mean±SE of arbitrary units/lane of 3 untreated and 3 diclofenac treated mice. *significantly different from the untreated group P<0.05.

Found at: doi:10.1371/journal.pone.0012715.s001 (4.54 MB TIF)

**Figure S2** Diclofenac increases arginase 1 protein content in tumor homogenates. Mice were inoculated with PANC02 cells and treated with diclofenac as described in Figure 1. Tumor homogenates from 5 untreated and 6 diclofenac treated mice were prepared and arginase 1 protein content was analyzed using Western blott as described in Materials and Methods. Mean±SE of arbitrary units/lane. 50 μg protein of tumor homogenate was loaded per lane. * significantly different from the untreated group P<0.001.

Found at: doi:10.1371/journal.pone.0012715.s002 (0.17 MB TIF)

**Figure S3** Arginase 1 positive cells are of mononuclear myeloid cells origin (CX3CR1 positive). PANC02 cells were inoculated into CX3CR1GFP/+ mice. After 14 days tumors were extracted, digested and GFP positive and negative cells were isolated by high speed cell sorting using FACS, as described in Materials and Methods. 400 000 cells GFP positive and negative cells were counted, lysed and analyzed for arginase 1 protein content as described in Materials and Methods.

Found at: doi:10.1371/journal.pone.0012715.s003 (0.09 MB TIF)

**Figure S4** Diclofenac increases WBC arginase 1 content. CB6F1 mice (tumor-free) were treated for 6 days with 30 mg/kg b.w. diclofenac. WBC were isolated from 1 ml of blood using Ficoll density gradient as described in Materials and Methods and counted. 200 000 cells were lysed and analyzed for arginase 1 protein content. Mean±SE of arbitrary units/lane of 3 untreated and 3 diclofenac treated mice. *significantly different from the untreated group P<0.05.

Found at: doi:10.1371/journal.pone.0012715.s004 (0.19 MB TIF)

**Figure S5** Diclofenac does not affect VEGF production in PANC02 or macrophages in vitro. A, PANC02 (3000 cells/well) were seeded in 96–NUNC wells. The day after seeding 10 or 50 μM diclofenac was added and cells were incubated for additional 4 days. B, Peritoneal macrophages were isolated from tumor–free mice as described in Materials and Methods and incubated for 48 hours with 10 or 50 μM diclofenac. At the end of the incubation cells were washed, lysed and measured for VEGF content as described in Materials and Methods. Mean±SE of pg VEGF/mg protein in 6 wells of untreated and diclofenac incubated cells.

Found at: doi:10.1371/journal.pone.0012715.s005 (0.17 MB TIF)

**Acknowledgments**

We thank M. Dauer (Munich, Germany) for kindly providing PANC02 cells and S. Jung (Rehovot, Israel) for kindly providing CX3CR1GFP/+ mice.

We gratefully acknowledge the intellectual support provided by S. Ben-Sasson (Developmental Biology and Cancer Research-IMRIC, Hadassah Medical School, Jerusalem, Israel).

**Author Contributions**

Conceived and designed the experiments: NM MG. Performed the experiments: NM NNS MG. Analyzed the data: NM MG. Contributed reagents/materials/analysis tools: NM MG. Wrote the paper: NM MG.
References

1. Le Bitoux MA, Stamenkovic I (2008) Tumor-host interactions: the role of inflammation. Histochem Cell Biol 130: 1079–1090.

2. Gupta RA, Dubois RN (2004) Colorectal cancer prevention and treatment: inhibition of cyclooxygenase-2. Nat Rev Cancer 1: 11–21.

3. Greenhough A, Smarrt HJ, Moore AE, Roberts HR, Williams AC, et al. (2009) The COX-2/PGE2 pathway: key roles in the hallmarks of cancer and adaptation to the tumor microenvironment. Carcinogenesis 30: 377–386.

4. Michaud DS (2004) Epidemiology of pancreatic cancer. Minerva Chir 59: 99–111.

5. Farrow B, Albo D, Berger DH (2008) The role of the tumor microenvironment in the progression of pancreatic cancer. J Surg Res 149: 319–326.

6. Colby JK, Klein RD, McArthur MJ, Conti CJ, Kaguchi K, et al. (2008) Progressive metaplastic and dysplastic changes in mouse pancreas induced by cyclooxygenase-2 overexpression. Neoplasia 10: 762–796.

7. Funahashi H, Satake M, Dawson D, Huynh NA, Reber HA, et al. (2007) Delayed progression of pancreatic intraepithelial neoplasia in a conditional Kras(G12D) mouse model by a selective cyclooxygenase-2 inhibitor. Cancer Res 67: 7060–7071.

8. Muller-Decker K, Furstenberger G, Aumuller N, Kucher D, Pohl-Arnold A, et al. (2006) Preinvasive duct-derived neoplasms in pancreas of keratin 5-promoter cyclooxygenase-2 transgenic mice. Gastroenterology 130: 2165–2176.

9. Bertagnolli MM, Eagle CJ, Zauber AG, Redston M, Solomon SD, et al. (2006) Morphogenesis by reconstituted basement membrane gel in three-dimensional cultures of rat aorta: a comparative study of angiogenesis in matrigel, collagen, fibrin, and plasma clot. In Vitro Cell Dev Biol 42: 119–126.

10. Corraliza IM, Campo ME, Soler G, Modolell M (1994) Determination of arginase activity in macrophages: a micromethod. J Immunol Methods 174: 231–235.

11. Ben-Sasson SA, Sherman Y, Gavriliu Y (1995) Identification of dying cells in situ staining. Methods Cell Biol 46: 29–39.

12. Kim KJ, Li B, Winer J, Armanini M, Gillett N, et al. (1993) Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. Nature 362: 841–844.

13. Rodriguez PC, Ochoa AC (2008) Arginine regulation by myeloid derived suppressor cells and tolerance in cancer: mechanisms and therapeutic perspectives. Immunol Rev 222: 180–191.

14. Jackson PG, Evans SR (2000) Intraperitoneal macrophages and tumor immunity: A review. J Surg Oncol 75: 146–154.

15. Murphy SM, Goldschmidt RA, Rao LN, Anunirait M, Buchmann T, et al. (1989) The influence of surgical trauma on experimental metastasis. Cancer 64: 2035–2044.

16. Seed JP, Brown JR, Freemantle CN, Papworth JL, Colville-Nash PR, et al. (1997) The inhibition of colon-26 adenocarcinoma development and angiogenesis by topical diclofenac in 2.5% hyaluronan. Cancer Res 57: 1625–1629.

17. Johnson JL, Lindskog M, Ponthan F, Petersen I, Ellman L, et al. (2004) Cyclooxygenase-2 is expressed in neuroblastoma, and nonsteroidal anti-inflammatory drugs induce apoptosis and inhibit tumor growth in vivo. Cancer Res 64: 7210–7215.

18. Davies NM, Anderson KE (1997) Clinical pharmacokinetics of diclofenac. Therapeutic insights and pitfalls. Clin Pharmacokinet 33: 104–213.

19. Lash JS, Spatzlani RW, Wagenaar E, Bijnen JH, Schinkel AH (2010) Hepatic clearance of reactive glucuronide metabolites of diclofenac in the mouse is dependent on multiple ATP-binding cassette efflux transporters. Mol Pharmacol 77: 607–609.

20. von Rahden BH, Stein HJ, Puhringer F, Koch I, Langer R, et al. (2005) Coexpression of cyclooxygenases COX-1, COX-2 and vascular endothelial growth factors (VEGF-A, VEGF-C) in esophageal adenocarcinoma. Cancer Res 65: 5039–5044.

21. Hartven F, Thumol S (1966) Peritoneal fluid volume and the oestrus cycle in mice. Nature 210: 1121–1125.

22. Nachtsheim R, Dudley B, McNeil PL, Howeschild TR (2006) The peritoneal cavity is a distinct compartment of angiogenic molecular mediators. J Surg Res 134: 28–35.

23. Rudge JS, Holsh J, Hylton D, Russell M, Jiang S, et al. (2007) Inaugural Article: VEGF Trap complex formation measures production rates of VEGF, providing a biomarker for predicting efficacious angiogenic blockade. Proc Natl Acad Sci U S A 104: 18363–18370.

24. Caso G, McNurlan MA, McMillan ND, Erermin O, Garlick P (2008) Tumour cell growth in culture: dependence on arginine. Clin Sci (Lond) 107: 371–379.

25. Gonzalez GG, Byus CV (1991) Effect of dietary arginine restriction upon leukocyte arginase activity in mice. J Nutr 121: 231–235.