Sulindac treatment alters collagen and matrixin expression in adenomas of ApcMin/+ mice

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Non-steroidal anti-inflammatory drugs (NSAIDs) have shown potential as chemopreventive agents against cancer formation, especially colorectal cancers. However, the mechanisms by which these drugs act are not fully understood. In this study, ApcMin/+ mice, a genetic model of human familial adenomatous polyposis, were treated with sulindac, and these mice demonstrated tumor reduction of >80%, consistent with previous reports. Gene microarray analyses of RNA from adenoma-derived dysplastic epithelial cells revealed that collagen genes, viz. Col1a2, Col5a2, Col6a2 and Col6a3, were upregulated, and matrixin matrix metalloproteases-7 (Mmp7) was downregulated, in sulindac-treated mice. Reverse transcription–polymerase chain reaction validated gene expression of the Col6a2 subunit of collagen VI and of Mmp7. Confocal microscopy and immunofluorescence showed that within the tumors of non-treated mice, collagen VI was present in low amounts, but was enhanced within the tumors of sulindac-treated mice. Collagens I and V demonstrated similar patterns, but were not as prominent as collagen VI. Mmp7 was found in ‘hot spot’ areas within the tumors of ApcMin/+ mice treated with the vehicle, but was greatly diminished in those mice treated with sulindac. Studies with ApcMin/+Mmp7−/− double-deficient mice demonstrated the reciprocal relationships of Mmp7 expression and the levels of these three collagens in vivo. The results of this study demonstrated that sulindac was effective in increasing the expression of different collagens and decreasing the expression of Mmp7, effects that may contribute to altered tumor burden in cancer patients undergoing NSAIDs treatments.

Introduction

The adenomatous polyposis coli multiple intestinal neoplasia (Apcrelated) mouse cancer model has been used successfully in delineating the genetics of colon cancer. This mouse line was developed by N-ethyl-N-nitrosourea mutagenesis altering the 15th exon of the Ap gene, thus resembling its familial adenomatous polyposis human counterpart (1,2). Patients with familial adenomatous polyposis inherit a defective Ap gene allele. Once the second allele becomes inactivated, multiple adenomas are formed in the large intestine, which have the potential of developing into malignant adenocarcinomas. Although familial adenomatous polyposis accounts for <10% of all colorectal cancers, it serves as a representative model to study this condition since most colorectal cancers also show alterations in expression of the Ap gene (3,4). Ap acts as a gatekeeper controlling the levels of β-catenin (5), and when this control mechanism becomes disrupted, stable β-catenin migrates to the nucleus and promotes the transcription of target genes that lead to uncontrolled proliferation and invasion.

Abbreviations: ECM, extracellular matrix; LCM, laser capture microdissection; Mmp7, matrix metalloproteases-7; NSAID, non-steroidal anti-inflammatory drug; PCR, polymerase chain reaction; PGE2, prostaglandin E2; RT, reverse transcription; WT, wild-type.

For many years, non-steroidal anti-inflammatory drugs (NSAIDs), such as sulindac, have shown significant benefits as chemopreventive agents for colorectal cancers (6–8). However, sulindac used as a chemopreventive agent does not completely eliminate the risk of developing adenocarcinomas. The adenomas reappear once the treatment is halted (9) and side effects are considerable. As a result, a number of studies have been initiated in order to determine the mechanisms of sulindac and, in general, all NSAIDs, in regulating adenoma formation in order to design better and more specific treatment modalities. Elucidation of the mechanisms by which NSAIDs function has proven to be more complex than initially assumed, and a complete understanding of this process remains unresolved. COX-2 has been reported to be upregulated in several cancers, including colorectal (10) and prostate (11) cancers. Sulindac has been shown to not only inhibit the activity of COX-2 but also to reduce its expression (11). Other work has indicated that prostaglandin E2 (PGE2) stabilizes β-catenin and promotes cell proliferation (12). This partially explains why sulindac is so effective in decreasing hyperproliferation and is consistent with previous studies suggesting that inhibition of PGE2 contributes to the antitumor effect of sulindac in Apcrelated mice (13). Additionally, it has been shown that sulindac has a proapoptotic effect in studies utilizing HCT-15 and HT-29 cells in culture, even after addition of PGE2, Prostaglandin F2-alpha and prostaglandin I2 (14). The proliferation markers, proliferating cell nuclear antigen and Ki-67, are also suppressed in these cell lines after sulindac sulfide treatment (15). Some of the late effects of sulindac have been elucidated, and it has been demonstrated that sulindac causes a G0/G1 arrest in HT-29 cells after a 72 h treatment (16) and regulates the expression and activity of many cyclins (17). Most of the studies elucidating the functional mechanism of sulindac have been performed with cell lines, including the only known transcriptional study (18). In the current investigation, the transcriptional expression of adenomas from Apcrelated mice after sulindac treatment was analyzed after extracting RNA from adenoma epithelial cells selected by laser capture microdissection (LCM). This approach allowed for the analysis of a homogenous population of cells, increasing the sensitivity and selectivity of the study. A short period of sulindac treatment was used based on our data, and previous reports (19,20), aiming to capture the first genetic responses to sulindac in order to better elucidate the initiating mechanisms by which sulindac decreases tumor burden. The results of these studies are summarized herein.

Material and methods

Mice and tissue processing

The animal protocols used in this study were approved by the University of Notre Dame Institutional Animal Care and Use Committee. Male wild-type (WT), Apcrelated and matrix metalloproteases-7 (Mmp7)-deficient (Mmp7−/−) mice (5 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME). For some studies, Apcrelated mice were crossed with Mmp7−/− mice to generate Apcrelated/Mmp7−/− mice, which were further crossed to generate Apcrelated/Mmp7−/− mice. Twelve- and 20-week-old male Apcrelated and Apcrelated/Mmp7−/− mice in a C57BL/6 background were used for analyses. At 86 days of age, sulindac was administered (0.6 mg/mouse/day) to Apcrelated mice via gavage for 6 days. Tumor counting was performed in vehicle- and sulindac-treated 12-week-old Apcrelated mice and non-treated 12- and 20-week-old Apcrelated/Mmp7−/− mice blinded to the genotype and treatment. Intestines were opened longitudinally, cleaned, swiss rolled, fixed with periodate–lysine–paraformaldehyde and embedded in paraffin. For transcriptional studies, mice were treated for 2 days. The control group received only phosphate buffer (vehicle). After completion of treatment, mice were euthanized by anesthesia overdose and the intestines removed. The ileum of the intestines was divided in half, opened longitudinally and cleaned. Each intestinal ileum half was embedded in Tissue-Tek (Sakura Finetek, Torrance, CA). Cryosections (8 μm) were mounted on double-frosted slides (Fisher Scientific, Fair Lawn, NJ) and stored at −80°C.

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Laser capture microdissection

Cryosections (8 μm) were stained with the Histogene kit (Arcturus, Mountain View, CA). Epithelial cells from WT crypts and from ApcMin+/− tumors were captured using the PixCell IIE LCM system and CapSure HS caps (Arcturus). A 7.5 μm diameter laser spot was used with 60 mW of laser power for single-fire duration of 1.3 ms to select cells. Approximately 30,000 laser firings per sample were used, employing several slides. Five different biological replicates were used for ApcMin+/− mice treated with sulindac and six for ApcMin+/− mice treated with vehicle. Two additional technical replicates were used for the sulindac and the vehicle group. Individual animals were used for each biological replicate. Several tumors were sampled for each mouse.

Gene expression and statistical analysis

Total RNA was isolated using the PicoPure RNA Isolation kit (Arcturus). Samples were processed through two rounds of amplification using the RiboAmp RNA Amplification kit (Arcturus). In the second round of amplification, the Enzo RNA Transcript Labeling kit (Enzo Biochem, Farmingdale, NY) was used for synthesis of biotin-labeled complementary RNA. Labeled complementary RNA from each sample (15 μg) was individually hybridized to a GeneChip® 430A Mouse Expression Array (Affymetrix, Santa Clara, CA) and analyzed.

Calculation of expression measurements and statistical analysis were performed with the Bioconductor software package (http://www.bioconductor.org). Gene expression values were computed with gcma (21). Genes were excluded from further analysis if the expression value was below an accepted threshold or varied little across the samples. Genes were tested for differential expression between the vehicle ApcMin+/−-treated mice samples and the sulindac-treated samples using a modified t-test as described previously (22), implemented in the limma package. The method of Benjamini and Hochberg (23) was used to control the false discovery rate with an adjusted P value cutoff of 0.05. GeneSpring (Silicon Genetics, Santa Clara, CA) was used for visualization.

Real-time reverse transcription–polymerase chain reaction validation

RNA was obtained from cells selected by LCM. Concentration and quality of the samples were assessed using the spectrophotometric method (24). Real-time RT–PCR was performed to validate Col6a2 transcriptional differences and results were consistent with the microarray data (Figure 2b). Mmp7 showed a statistically questionable differential expression in the gene array data, but the real-time RT–PCR validation results (Figure 2c) confirming the microarray results (P = 0.0070), including the WT group (data not shown). The heat map of the hierarchical clustering based on ECM-related genes (Figure 2a) shows very clearly how sulindac increases (red) in a similar pattern the expression of the

Immunohistochemistry and confocal microscopy

For sulindac- and vehicle-treated ApcMin+/− mice, cryosections (8 μm) were fixed for 10 min in acetone, followed by immunohistochemical staining. For non-treated ApcMin+/+ and ApcMin+/+Mmp7+/−/− mice, intestines were fixed with periodate-lysine–paraformaldehyde and embedded in paraffin. Sections (3 μm) were cut for hematoxylin and eosin staining and (4 μm) for immunostaining. Collagens type I, V, and VI were identified using polyclonal rabbit anti-human antibody specific for each collagen (Abcam, Cambridge, MA), as the primary antibody followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA). The slides were then developed in 3, 3’-diaminobenzidine and a hematoxylin QS counterstain was applied (Vector Laboratories, Burlington, CA). For immunofluorescence, Alexa Fluor 647-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR) was used as the secondary antibody, and Prolong Gold with 4’,6-diamidino-2-phenylindole for nuclear staining was applied before coverslipping. Mmp7 was identified using a polyclonal rabbit anti-human Mmp7 antibody (Abcam), followed by the secondary antibody Alexa Fluor 647-conjugated antibody used to identify the collagens.

Stain quantification

Images of tumors were acquired at a magnification of ×200 using a Nikon Eclipse TE2000U microscope (Melville, NY). MicroPublisher 5.0RTV color camera (QImaging, Burnaby, BC, Canada) and Photometrics Cascade 512B fluorescence camera (Tucson, AZ). The images were then analyzed using MetaMorph 7.0r3 software (Molecular Devices, Sunnyvale, CA). The extent of collagen VI and Mmp7 expression in tumors was determined as percent positive area per analyzed tumor area.

Results

Quantification of tumor number in ApcMin+/− mice after sulindac treatment and Mmp7 deletion

ApcMin+/− mice administered sulindac for 6 days showed a dramatic decrease in polyp number (Figure 1a), which was consistent with previous reports using piroxicam (19) and sulindac (20), where the maximum therapeutic effect was attained after 6 days of treatment. A decrease of 81.2% in tumor numbers was observed in the sulindac-treated ApcMin+/− mice compared with vehicle-treated ApcMin+/− mice. A similar reduction in intestinal tumors was observed in ApcMin+/−/Mmp7−/− mice at 12 and 20 weeks of age (Figure 1b). This is a significant decrease compared with non-treated ApcMin+/− mice and is consistent with what has been reported in the literature (25).

Gene expression

In order to detect early transcriptional changes occurring in the adenomas after 2 days of sulindac treatment, LCM was used to only select dysplastic epithelial cells. Two days of treatment was selected based on our observations and previous studies (19,20) where it was shown that the maximum effect of sulindac is achieved rapidly at 6 days and observable initiating changes are seen even after 2 days of treatment. Three WT biological replicates were included as reference, but it was not the intention of this study to report expression differences between WT and the ApcMin+/− mouse, since it has been reported previously (26). Twenty-five genes with significant differential expression were observed in treated mice compared with the control group. The list includes an extracellular matrix (ECM) cluster (Figure 2a) with four procollagen genes upregulated after sulindac treatment; collagen type I, alpha 2 (Col1a2); collagen type V, alpha 2 (Col5a2); collagen type VI, alpha 2 (Col6a2) and collagen type VI, alpha 3 (Col6a3). Real-time RT–PCR was performed to validate Col6a2 transcriptional differences and results were consistent with the microarray data (Figure 2b). Mmp7 showed a statistically questionable differential expression in the gene array data, but the real-time RT–PCR validation results (Figure 2c) confirming the microarray results (P = 0.0070), including the WT group (data not shown). The heat map of the hierarchical clustering based on ECM-related genes (Figure 2a) shows very clearly how sulindac increases (red) in a similar pattern the expression of the...
Mmp7 is decreased in tumors of ApcMin/+ mice after 2 days of sulindac treatment

A decrease of Mmp7 at the messenger RNA level was determined by real-time RT–PCR and correlated with the downregulation observed in the gene microarray data. To further analyze Mmp7 antigen and to determine its localization, immunofluorescence stains were performed and quantified (Figure 4). The localization of Mmp7, within the tumors of the control group, was variable. Mmp7 was found in some cells within the tumor in a ‘hot spot’ arrangement (Figure 4a). The Mmp7 detected in the tumors of sulindac-treated mice was similar to background levels (Figure 4b). The percent positive area clearly showed a significant reduction (2.28 ± 0.56%, n = 11 tumors per six mice versus 7.45 ± 1.2%, n = 10 tumors per four mice, P < 0.0001) after 2 days of sulindac treatment (Figure 4c).

Sulindac does not affect local inflammation in the intestinal tumors

Sulindac, as a NSAID, decreases inflammation through the inhibition of COX-1 and COX-2. CD45 immunostains were performed (data not shown) to determine if the increase in collagen could be related to a local decrease in inflammation. Although a small decrease in inflammation was observed after sulindac treatment, it was not significant and the variation among the different tumors, even within the same animal, was considerable (data not shown).

Collagens type I and VI but not V are increased within the tumors of mice lacking Mmp7

Since several metalloproteinases are capable of degrading different components of the ECM, including all types of collagen, the presence of collagens type I, V and VI was determined in intestinal tumors of ApcMin/+ /Mmp7−/− mice and compared with the levels found in intestinal tumors of ApcMin/+ mice to determine if these collagenases are responsive to changes in the expression of Mmp7, in vivo, in the absence of sulindac treatment. The immunofluorescence stains from paraffin-embedded sections showed that there was a discrete increase in collagen expression in tumors of ApcMin/+ /Mmp7−/− mice compared with ApcMin/+ mice (Figure 5 upper panels). After determining the percent area positive for collagen, it was found (Figure 5 lower panel) that collagens type I and VI were significantly increased in the ApcMin/+ /Mmp7−/− group (12.3 ± 1.74%, n = 11 tumors per six mice versus 8.8 ± 0.78%, n = 27 tumors per six mice, P = 0.039 for collagen 1 and 19.2 ± 1.59%, n = 12 tumors per six mice versus 14.5 ± 1.25%, n = 20 tumors per six mice, P = 0.028 for collagen VI) but the change for collagen type V did not reach significance.

**Fig. 2.** (a) Supervised hierarchical condition tree of the ECM cluster. The samples were clustered based on the expression of the ECM genes. The four collagen genes showed a similar expression pattern. Each sample was color adjusted based on the expression of the gene relative to the median. Green samples are downregulated and red samples are upregulated with respect to the median expression. (b) Real-time RT–PCR of Col6a2 and (c) Mmp7. Three replicates were used for the vehicle group and four for the sulindac group. The results were obtained using the ΔΔCt method with Rpl 19 used as the housekeeping gene.
(16.6 ± 1.96%, n = 11 tumors per six mice versus 15.3 ± 1.17%, n = 18 tumors per six mice, P = 0.56). These results suggest that collagens type I and VI are indeed in vivo substrates of Mmp7 and the lack of this enzyme would be consistent with the enhanced deposition of these collagens in intestinal tumors after sulindac treatment.

Discussion

This study demonstrated that 2 days of sulindac treatment leads to the differential expression of several ECM-related genes in the ApcMin/+ colorectal cancer mouse model that may partially account for tumor burden reduction. It was also demonstrated that collagens I and VI are increased in ApcMin/+Mmp7−/− mice compared with ApcMin/+ mice, which suggests that they may act as Mmp7 substrates in vivo.

ECM remodeling is a common event during tumor formation, where Mmps are important participants in regulating invasion of tumor cells. Mmps 2, 9 (27) and 7 are elevated in colon and rectal tumor tissue and Mmp7 has also been implicated in liver metastasis of colorectal cancer (28,29). Studies have demonstrated that sulindac can inhibit Mmp2 production directly or through inhibition of COX-2 regulating its activation (30,31). In the current study, it was shown that Mmp7, a matrix metalloprotease regulated by β-catenin, which has been reported to be expressed in cancer cells (32), is diminished in intestinal adenomas after sulindac treatment.

Type IV collagen is the most common collagen in the basement membrane of the intestine and degradation of type IV collagen by tumors leads to invasion. While collagen type IV did not appear to be altered by sulindac treatment, messenger RNA of some subunits of collagens type I, V and VI had increased expression in the tumor tissue after sulindac treatment. Functional collagens type I, V and VI within the tumor showed a similar type of trend. The relationship between an increase in collagen and tumor burden reduction is unclear. It is thought that loss of collagen, a major connective tissue component (33), would result in loss of adhesion and structural organization (34) that would allow the tumor cells to grow and move with limited restriction. Diminished Mmp expression/activation or enhanced collagen deposition would inhibit cells from invading into the surrounding tissue (35). Collagen type V is able to modulate the expression of apoptotic genes in breast cancer cells (36). Proapoptotic genes like Bcl-x and Bad, as well as some caspases, are upregulated when
collagen V is used as a substrate. It is reported that soluble collagen VI is able to reduce Bax expression in serum-starved fibroblasts, which results in a decrease of apoptosis (37). Additionally, collagen I subunits were identified as biomarkers for invasion, metastasis and carcinogenesis in gastric carcinoma (38). On the other hand, it was observed in microarray experiments of melanomas paired with melanocytes that the Col6a1 gene, together with STAT2 and CD9, were among the strongest downregulated transcripts in the study (39). Also using microarrays, Col6a3 was found to be downregulated in neuroblastomas (40), and functional collagen VI was completely lost in most spontaneous fibrosarcomas (41), arguing that a lack of these ECM proteins might be responsible for tumorigenicity, invasiveness and metastasis. Further studies are needed to fully determine the role of the different collagens in colon cancer.

To address whether the increase at the protein level of collagens I, V and VI is related to downregulation of Mmp7, ApcMin+/Mmp7-/- mice, shown to develop 58% fewer and 20% smaller tumors than ApcMin+ mice (25), were used. It has been determined in in vitro studies that collagens I and VI are Mmp7 substrates (42), and through immunofluorescence stains the individual levels of collagens type I, V and VI in intestinal tumors of ApcMin+/Mmp7-/- and ApcMin+ mice were determined in this study. All collagens were increased in the tumors of ApcMin+/Mmp7-/- mice, but only collagen type I and VI reached significance. Analyzing differences between sulindac treatment and Mmp7 deficiency, it was noted that the collagen type I increase was very similar under both conditions (40.3 versus 39.4%, respectively), concluding that observed increase in functional collagen type I after sulindac treatment is consistent with the finding that Mmp7 is decreased and probably not related to collagen subunit gene Col1a2 upregulation after sulindac treatment. Collagens type V and VI were more enhanced in sulindac-treated ApcMin+ than non-treated ApcMin+/Mmp7-/- mice (14.9 versus 8.9% and 47.0 versus 32.7%). In the case of collagen type V, the increase at the protein level was most probably not related to Mmp7 since no significant increase of collagen type V was found in ApcMin+/Mmp7-/- mice. Finally, collagen type VI, which showed the most striking difference in localization within the tumors of sulindac-treated mice, appeared to be affected by Mmp7-mediated degradation as well as sulindac treatment.

It is believed that sulindac decreases Mmp7 expression by inhibiting COX-2 and therefore PGE2 production. In the absence of sulindac, PGE2 is produced by COX-2. When PGE2 binds to its receptor EP2, it triggers a Wnt-like response (12). As a result, β-catenin shuttles to the nucleus where it promotes the transcription of several target genes, among them Mmp7. When sulindac is present, its active metabolite, sulindac sulfide, reverses this effect by blocking PGE2 production. The expression of Col1a2, Col5a2, Col6a2 and Col6a3 is increased through an unknown mechanism perhaps even involving the oxidated metabolite, sulindac sulfone. The upregulation of these genes translates into an increase at the protein level for collagens V and VI. However, the increase in collagen I is probably related to decreased proteolysis due to Mmp7 downregulation. Collagen type VI has the most dramatic change since sulindac causes an upregulation of two of its three genes that translates into higher protein levels and lower degradation levels by Mmp7.

In conclusion, it is proposed that sulindac increases functional collagens V and VI by increasing their gene expression. Additionally, sulindac indirectly increases collagens I and VI by reducing their proteolytic degradation through a Cox-2-dependent downregulation of Mmp7. It is proposed that the effect of sulindac on intestinal tumor burden reduction is linked to ECM remodeling and that different collagens and proteases, especially collagen type VI and Mmp7, are major participants in this process.

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Fig. 4. Mmp7 immunofluorescence stains (a and b). Intestines from vehicle-treated (a) and sulindac-treated (b) ApcMin+/mice were stained for Mmp7 (red). The vehicle-treated group showed considerably more Mmp7 in the tumors when compared with the sulindac-treated group, where Mmp7 levels are similar to background levels. 4',6-Diamidino-2-phenylindole was used for nuclear stain. Original magnification ×200. (c) Extent of tumor-associated Mmp7 in ApcMin+/mice treated with vehicle or sulindac. The Student’s t-test showed that the difference between both groups was significant.
Fig. 5. Collagen immunofluorescence stains. Paraffin sections of 20-week-old Apc\(^{Min+}\) (a–c) and Apc\(^{Min+}/Mmp7\(^{-/-}\) (d–f) mice were stained for collagen I (a and d), V (b and e) and VI (c and f). Images show areas with tumor tissue. Stain is pseudocolored yellow, magenta and green for collagens I, V and VI, respectively. 4’-6-Diamidino-2-phenylindole was used for nuclear stain. Original magnification ×200. The extent of tumor-associated collagen in Apc\(^{Min+}\) and Apc\(^{Min+}/Mmp7\(^{-/-}\) mice is shown in the lower panel. The Student’s t-test showed that the difference between both groups was significant for collagens I and VI but not for collagen V.

References

Sulindac treatment and collagen expression in tumors

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