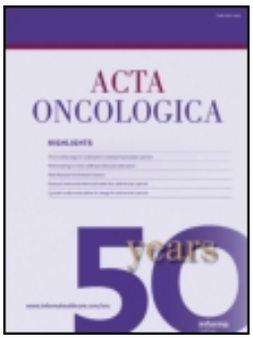

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ORIGINAL ARTICLE

Prostanoid receptor expression in colorectal cancer related to tumor stage, differentiation and progression

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Abstract

Introduction: Alterations in eicosanoid metabolism is well established in a variety of malignant tumors, particularly colorectal carcinoma. Recent studies in our laboratory have emphasized a role for EP subtype receptors in progression of colorectal cancer and disease specific mortality. Therefore, the aim of the present study was to extend our knowledge to include additional receptor expression (DP1, DP2, FP, IP, TP) for prostanoids (PGD_2, TXA_2, $PGF_{2\alpha}$, PGI_2) in relationship to tumor stage, differentiation and progression of colorectal cancer. *Material and methods*: Total RNA from 62 tumors and adjacent normal colon tissue (n = 48) was extracted. Quantification of receptor expression was performed by realtime PCR and related to the expression of an appropriate housekeeping gene (GAPDH). Tumors were assessed according to Dukes A-D (stage I-IV). *Results:* DP1, DP2, FP and IP receptor subtypes displayed significantly reduced overall expression in tumor tissue compared to normal colon tissue, while the TP receptor subtype showed significantly higher expression in tumor tissue. Overall expression of the prostanoid receptors in tumor tissue was not related to clinical indexes as tumor stage and tumor cell differentiation evaluated by multivariate analyses. Cultured colorectal cancer cell lines with low (HT-29) and high (HCA-7) intrinsic PGE_2 production at confluent state did not express DP1 and IP receptor subtypes, but displayed low expression of DP2, FP and TP receptor subtypes. *Conclusion:* The results in the present study indicate imbalanced expression of prostanoid receptors in colorectal cancer compared to normal colon tissue without clear cut relationship to disease progression. Therefore, future studies should be performed on defined cells within the tumor tissue compartment determining whether any prostanoid receptor(s) is useful as a molecular target in treatment or prevention of colorectal cancer.

Eicosanoids are involved in cell proliferation, adhesion, migration, differentiation, apoptosis, tumor invasiveness and angiogenesis [1–6]. Consequently, inhibition of cyclooxygenases (COX-1/COX-2) producing eicosanoids, by non-steroid anti-inflammatory drugs (NSAIDs), has confirmed to influence on tumor growth, progression and size-reduction of colon cancer [7–10]. Eicosanoids act as autocrine or paracrine mediators through binding to G-protein coupled receptors in the cell membrane altering cellular levels of cAMP with changed concentrations of Ca^{2+}- ions, affecting different signaling pathways in the cell [11].Thus, eicosanoids are closely related to the behavior of malignant tumors, where elevated levels of prostaglandin E_2 (PGE_2), PGD_2 and tromboxane A_2 (TXA_2) as well as reduced levels of $PGF_{2\alpha}$ and PGI_2 (prostacyclin) have been reported of in tumor tissue [12–14]. Normally, PGI_2 mediates increased vascular permeability and promotes inflammation. Opposing PGI_2 is TXA_2, a strong constrictor of vasculature and a potent stimulator of platelets aggregation. $PGF_{2\alpha}$ acts on smooth muscle cells whereas PGD_2 promotes production of cytokines and chemokines during antigenic challenges [15], which all may decide tumor behavior [16]. Accordingly, recent work in our laboratory, where COX and PGE_2 receptors (EP_{1-4}) as well as PPARγ were analyzed in colorectal tumors,

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displayed that increased expression of EP_2 receptor predicted poor survival following primary surgical operation [17]. Therefore, the present study was aimed to map remaining eicosanoid receptor expression in human colorectal cancer and corresponding normal colon tissue in relationship to tumor characteristics as histopathology (grade) and tumor progression (stage).

Material and methods

Patients

Tumor and large bowel tissue samples were collected from 62 unselected patients at primary operation for colorectal carcinoma between 2001 to 2004 at Sahlgrenska University Hospital (n = 14) and a regional county hospital (Uddevalla, n = 48) of Sweden. The group consisted of 50% males and 50% females with a median age of 73 years (range 40 to 91 years) at surgery. Disease specific mortality was as expected accounting for age and tumor stage. Tumors were histologically classified as Dukes A (10), Dukes B (26), Dukes C (16) and Dukes D (10), corresponding to tumor stage I-IV respectively. All patients underwent surgery as the only curative treatment and none received neoadjuvant radiochemotherapy according to individual patient judgments and local hospital routines.

Tumor tissue material

Tumor (n = 62) and normal large bowel tissue samples (n = 48) (down to the serosa layer) were collected during surgery and kept fresh frozen in liquid nitrogen and stored in $-70^{\circ}C$ until analysis. Certified pathologists staged all tumors. Tumor samples for RNA analysis contained around 70–80% tumor cells according to microscopic visual inspection.

Cell culture

Prostanoid receptor expression was also studied in two well-differentiated human colon adenocarcinoma cell lines with low (HT-29, ATCC) and high (HCA-7, Sigma-Aldrich, St.Lois, USA) intrinsic PGE_2 production. HCA-7 cells were chosen since these cells are reported to express all EP_{1-4} subtype receptors while HT-29 cells lack expression of EP_2 and EP_3 as confirmed. Cultured cells were maintained in monolayers in flasks (25 cm^2) from Corning in a humidified (95%) incubator at $37^{\circ}C$ with 5% CO_2. HT-29 cells were maintained in McCoy's 5A medium (ICN Biomedicals, Aurora, OH, USA) supplemented with 10% fetal calf serum (FCS). The split ratio was 1/12. HCA-7 cells were maintained in Dulbecco's modified Eagle's medium D6546 (DMEM) from Sigma-Aldrich with 10% FCS and a split ratio of 1/5. Penicillin, streptomycin and L-glutamine were added to the concentration of 100 U/ml, 100 μg/ml respectively 292 μg/ml (Bio Whittaker, Europe, Verviers, Belgium). Weekly medium changes (McCoy's 5A/DMEM + 2% FCS) were provided.

RNA extraction and cDNA synthesis

Total RNA from 48 tumors and adjacent normal colon tissue (29 patients) was extracted with GenElute™ Mammalian Total RNA Kit from Sigma. The remaining 14 tumors and adjacent tissue (14 patients) RNA was extracted with RNeasy® Fibrous Tissue Midi kit from Qiagen according to the protocol for Total RNA Isolation from Fibrous Tissue enclosed by the manufacturer. RNA from cultured cells (HT-29 and HCA-7) was extracted with RNeasy® Mini kit from Qiagen according to enclosed protocol. Purity and concentration of extracted RNA were checked and quantified by reading at 260 and 280 nm in a spectrophotometer (Pharmacia GeneQuant RNA/DNA Calculator). A quality control and concentration measurement of RNA was performed in the Agilent Technologies 2100 Bioanalyzer per manual for eukaryotic total RNA before cDNA synthesis. One μg of RNA was used in BD Advantage™ RT-for-PCR kit (BD Bioscience) according to the manufacturer's instruction. Sterile water substituted for RNA in negative controls. Samples without RT-polymerase were run to exclude genomic contamination.

Realtime PCR

Realtime PCR was performed in the LightCycler 1.5 with LightCycler FastStart DNA Master SYBR Green1 or FastStart DNA Master^{Plus} SYBR Green1 kit to analyze relative expression of the five prostanoid receptor genes in tumor tissue and normal colon tissue according to a standard protocol (Roche). Primers were added to a final concentration of 0.5 μM and 2/5 μl cDNA (SYBR Green I/PLUS) were added to each capillary (Table I). All samples were performed in duplicate and related to the expression of an appropriate housekeeping gene (GAPDH), as confirmed in previous work [17]. Reactions were optimized regarding $MgCl_2$ concentration, annealing temperature and primer concentration. The products were checked in the Bioanalyzer 2100 (Agilent Technologies) according to the protocol for DNA1000 for correct amplicon size. PCR-graded water was used as negative control in every reaction. Results were produced by use of

Table I. The primers used in LightCycler® realtime PCR from 5′ to 3′.

Primer		Annealingtemp. (C°)	Amplicon size (bp)	Primer conc. (μM)	$MgCl_2$ conc. (mM)
PGD_2 receptor DP1	f TGATGACCGTGCTCTTCACT r CCAAGGGTCCACAATTGAAA	55	158	0.5	PLUS
PGD_2 receptor DP2	f CCTCTGTGCCCAGAGCCCCACGATGTCGGC r CACGGCCAAGAAGTAGGTGAAGAAG	68	301	0.5	5
$PGF_{2\alpha}$ receptor FP	Hs_PTGFR_1_SG (Qiagen)	55	95	*	PLUS
PGI_2 receptor IP	f TGCTCCCTGCCTCTCACGAT r TGGCTTCTGCTTTGGACGAC	65	387	0.5	5
TXA_2 receptor TP	Hs_TBXA2R_1_SG (Qiagen)	60	99	*	2

(f = forward, r = reverse). * 2 μl primer were added according to the manufactures protocol. PLUS $MgCl_2$ included in the master mix and not added separately.

the relative standard curve method where the standard specimen was a colon tumor (Dukes C) resected at Sahlgrenska University Hospital. All samples were confirmed to be within the range of the standard curve. EP_{1-4} subtype receptor expression in cultured cells were measured as described elsewhere [17]. Standard samples displayed expression as expected and negative control samples showed no expression.

Statistics

Results are presented as relative gene expressions per GAPDH expression and presented as mean ± standard error of units obtained from the LightCycler® datafiles. The statistical testing was performed by non-parametric tests (Kruskal-Wallis and Mann-Whitney). Survival analysis was performed according to Kaplan-Meier and tested statistically with the log rank technique. Alive patients were censored in survival analyses. Multivariate regression analysis was performed according to standard procedures with disease specific mortality as dependent factor (Statview 5.0.1, SAS Institute Inc.). $P < 0.05$ was regarded statistically significant and $p < 0.10$ a trend to significance in two-sided tests.

Results

DP1 and IP receptor subtypes displayed clear cut significantly lower overall expression in tumor tissue compared to normal colon tissue, while the TP receptor subtype showed significantly higher expression in tumor tissue than in normal colon tissue, although a more differentiated pattern appeared when normal colon tissue was compared to Dukes A-D in a multigroup analysis (Figure 1, Table II). Expression of any subtype prostanoid receptor was not related to tumor stage (Tables II and III) or tumor cell differentiation in univariate analysis (Table IV). Multivariate regression analysis did not indicate any of the subtype receptors to be a significant predictor of survival (not shown), although disease specific survival in itself was related to tumor stage as expected (not shown). Both tumor tissue specimen and normal colon tissue were simultaneously obtained from 43 patients. Receptor expression analysis within patient group was performed to check the validity of our results from the cross-sectional analyses derived on the entire patient material (Table II). Results of receptor expression within patient group agreed with cross-sectional results for all receptor subtypes except for the TP receptor, which had a lower expression in tumor tissue in 74% (32 of 43) of patients in the within group analysis as compared to increased expression in the cross-sectional samples. FP subtype receptor expression was below the detection limit in 18% of tumors (11/62), while only two patients displayed the same phenomenon in normal colon tissue. Tumor stage, grade and patient survival did not correlate to unexpressed FP subtype receptor. However, none of these patients had highly differentiated tumors and 73% of these patients (8 of 11) were males.

Tumor cells (HT-29 and HCA-7) did not express DP1 and IP receptor subtypes, but displayed low

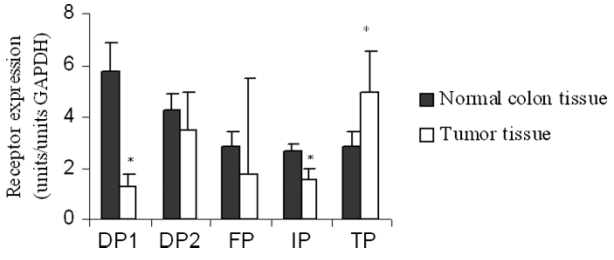

Figure 1. Prostanoid receptor expression in tumor tissue (n = 62) compared to normal colon tissue (n = 43). *$p < 0.05$ vs. normal colon tissue.

Table II. Transcript expression of PG receptors in normal colon tissue and colorectal cancers grouped according to Dukes A-D stage.

			Tumor Tissue					
PG	Receptor	Normal colon tissue (43)	Dukes A (10)	Dukes B (26)	Dukes C (16)	Dukes D (10)	Statistics K-W among all p<	K-W within Dukes p<
PGD_2	DP1	5.73±1.09	0.29±0.23	0.98±0.29	0.58±0.23	3.86±3.04	<0.0001	ns
PGD_2	DP2	4.26±0.59	3.93±1.75	1.30±0.24	7.01±5.29	3.10±1.88	<0.0001	0.07
$PGF_{2\alpha}$	FP	2.88±0.53	1.26±0.67	2.37±0.99	1.76±0.73	0.73±0.27	<0.003	ns
PGI_2	IP	2.63±0.26	1.32±0.40	1.67±0.61	2.03±0.95	0.94±0.24	<0.0001	ns
TXA_2	TP	2.84±0.55	9.72±7.94	3.45±1.46	3.49±1.54	6.44±3.57	<0.02	0.07

Kruskal-Wallis test (K-W).
Mean±SEM.
Results are units per units GAPDH.

expression of DP2, FP and TP receptor subtypes (Table V). Also, EP_{1-4} subtype receptor expression varied considerably between HT-29 and HCA-7 cell cultures (Table V).

Discussion

The first reports of beneficial effects of NSAIDs in colorectal cancer patients were published several years ago [7,18]. Still, the molecular basis of why and how NSAIDs inhibit tumor progression is unclear. Most reports have focused on PGE_2, the major product of COX-2, and have left remaining products of COX without detailed considerations in colorectal cancer. In our earlier report we quantified PGE_2 receptor expression in human colorectal tumor tissue in comparison to expression in adjacent normal colon tissue. The results revealed that high expression of EP_2 receptor subtype predicted reduced disease specific survival [17]. However, overall changes in expression of any other EP subtype receptor could not be related to tumor progression or tumor differentiation [17]. Therefore, we have now focused on additional receptors (DP1, DP2, FP, IP, TP) for prostanoids (PGD_2, TXA_2, $PGF_{2\alpha}$, PGI_2) produced by cyclooxygenases (COX) in order to get a more complete evaluation, since reports on the expression of prostanoid receptors in colorectal cancer are sparse or lacking in the literature.

Our results showed reduced expression in four of five prostanoid subtype receptors in Dukes A-D tumors compared to normal colon tissue, although these findings were overall most consistent for DPI and IP expression, while TP receptor expression was increased in tumor tissue (Figure 1). These findings are in part a sign of imbalanced eicosanoid receptor expression in colorectal cancer tissue probably affecting tumor progression, as earlier reported for EP_{2-4} subtype receptors [17]. However, clear cut connections or mathematical correlations to tumor stage, differentiation and progression were not observed, as found for particularly EP_2 and COX-2 expression in tumor tissue predicting reduced disease specific survival [17,19]. Therefore, one may anticipate more complex relationships for prostanoids in tumor carcinogenesis and progression, since altered eicosanoid homeostasis in tumor tissue is well recognized and appears a global tumor phenomenon [12–14], which may decide metastatic spread [16], affecting tumor angiogenesis, cell proliferation, apoptosis and immune reactions [20]. However, an obvious limitation to present approach with overall tissue measurements is the risk to oversee specific alterations within or between defined cell types as tumor, endothelial- and migrating immune cells. Our recent evaluation have suggested that mathematical modeling of growth factor proteins to explain tumor progression defines different explanations accounting for prostanoids and tissue production [19]. Thus, it is likely that prostanoids are important factors to define colorectal cancer progression, although it is presently not possible to present a simple and unified model.

Each prostanoid ligand and corresponding receptor has certain functions in cells and tissues; PGD_2/DP1-2 are involved in the immune response, $PGF_{2\alpha}$/FP affect smooth muscle cells while PGI_2/IP and TXA_2/TP involve vascularity control in tissues [15,21,22]; functions that are all changed in tumor

Table III. Transcript expression of prostanoid receptors in tumor tissue during progression of colorectal carcinoma.

Receptor	Dukes A+B (36)	Dukes C+D (26)	Mann-Whitney p <
DP1	0.87±0.22	1.88±1.18	ns
DP2	2.03±0.54	5.51±3.31	ns
FP	2.06±0.74	1.36±0.47	ns
IP	1.58±0.45	1.61±0.59	ns
TP	5.19±2.42	4.62±1.65	ns

Mean±SEM.
Units per units GAPDH.
Events of tumor progression are reflected by grouping tumors to Dukes A+B.
versus Dukes C+D.

Table IV. Transcript expression of prostanoids receptors in tumor tissue from colorectal cancers of various differentiation.

PG	Receptor	High (5)	Medium (42)	Low (10)	Kruskal-Wallis p <
PGD_2	DP1	0.54±0.13	1.62±0.75	0.48±0.12	ns
PGD_2	DP2	1.10±0.23	4.43±2.09	1.91±0.81	ns
$PGF_{2\alpha}$	FP	1.16±0.31	2.08±0.68	0.73±0.27	ns
PGI_2	IP	1.85±0.93	1.69±0.51	1.26±0.45	ns
TXA_2	TP	1.30±0.28	6.60±2.25	1.83±0.42	ns

Mean±SEM.
Units per units GAPDH.

tissue. The FP receptor may activate potentially oncogenic pathways such as the β-catenin transcription [23] and is sometimes up-regulated in adenocarcinomas promoting neoplastic epithelial cell proliferation [24]. TXA_2 is involved in angiogenesis and subsequent development of tumor metastases, while PGI_2 displayed anti-cancerogenic effects in a murine cell model [4,25]. The DP receptor has been linked to inflammation which may represent a pre-stage to cancer, where DP1 shows anti-inflammatory effects and DP2 pro-inflammatory actions [26], although PGD_2 displayed anti-proliferative activity in-vitro [27]. Interestingly, established cultured human colon carcinoma cell lines (HT-29, HCA-7), with low (HT-29) and high (HCA-7) intrinsic PGE_2 production, expressed pro-cancerogenic receptor subtypes only as DP2, FP and TP, which are principally in agreement with reduced overall prostanoid levels in tumor tissue. Confusingly, our result on prostanoid receptor expression in HCA-7 and HT-29 cells did not entirely agree with results reported by others [28], where Hawcroft et al. observed expression of DP1 in HT29 cells only without any expression of DP2 in any of five tumor cell lines including HCA-7; results apposite to ours (Table V). This may be a question of detection limits in levels of transcripts or eventually indicates how sensitive receptor expression may be to environmental factors defined by the cell culture procedures.

In conclusion, imbalanced prostanoid receptor expression was observed in colorectal cancer without simple correlations to tumor stage, differentiation and progression. Further studies of eicosanoid receptor profiles in defined cells within colorectal tumors, obtained by fresh frozen microdissected material, are thus necessary in order to determine whether any prostanoid receptor(s) is useful as a molecular target in treatment or prevention of colorectal cancer.

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Table V. Estimates of prostanoid receptor expression in two established colorectal cancer cell lines with high (HCA) and low (HT-29) PGE_2 production. Results are provided relative to expression levels in colon cancer tissue and as raw measures obtained by Light Cycler and Taqman determinations as indicated.

	HCA-7[a]		HT-29[b]	
Receptors:	Relative to colon cancer	Direct measurement	Relative to colon cancer	Direct measurement
DP1 receptor	0	0[c]	0	0[c]
DP2 receptor	4%	0.34[c]	7%	1.3[c]
FP receptor	1%	0.04[c]	1%	0.1[c]
IP receptor	0	0[c]	0	0[c]
TP receptor	4%	0.45[c]	3%	0.9[c]
EP_1 receptor	2%	0.1[d]	433%	519[e]
EP_2 receptor	720%	31[d]	0	0[e]
EP_3 receptor	14%	0.2[d]	0	0[e]
EP_4 receptor	742%	4[d]	86%	85[e]

a: PGE_2 concentration in cell medium at confluence 120–130 pg/ml.
b: PGE_2 concentration in cell medium at confluence <2.5 pg/ml.
c: Light Cycler units/units GAPDH.
d: Taqman Relative gene expression versus a Dukes c colon cancer.
e: mol/mol GAPDH.

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