Decreased Expression of Retinoid X Receptors During Human and Azoxymethane-induced Colorectal Carcinogenesis in the Rat

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Abstract. *Background/Aim: The family of retinoid X receptors (RXRs) including RXRα, β and γ, is involved in regulating cell proliferation, differentiation, apoptosis and development. Materials and Methods: In order to characterize the role of RXRs during colorectal carcinogenesis, the expression of RXRs in human and azoxymethane (AOM)-induced rat colorectal tumors was profiled by immunohistochemistry. Results: Both human and rat normal colorectal epithelia and hyperplasia exhibited strong nuclear, but weak cytoplasmic staining for all three proteins. Expression of RXRα, β and γ was significantly reduced in rat carcinomas compared to high-grade dysplasia whether in aberrant crypt foci or in adenomas. All three proteins displayed dramatically reduced nuclear expression in both human adenomas and carcinomas. Reduced expression of RXRα and RXRγ seems more significant than RXRβ in both human and rat carcinomas. Conclusion: Reduced expression of RXRs is associated with colorectal carcinogenesis in both humans and AOM-treated rats.*

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Retinoids have been suggested to inhibit carcinogenesis, being effective not only in the treatment of pre-malignant lesions such as leukoplakia (1, 2), actinic keratosis (3) and cervical dysplasia (4), but also in inhibiting the development of skin cancer in patients with *xeroderma pigmentosum* (5) and secondary tumors in patients with primary liver cancer (6, 7). Both 13-cis-retinoic acid (13CRA) and vitamin A inhibited the development of rat colorectal aberrant crypt foci (ACF), pre-malignant lesions induced by either azoxymethane (AOM) or 1,2-dimethyl-hydrazine (DMH) and a high fat diet (8, 9). Conversely, vitamin A deficiency is associated with an increased incidence of colonic cancer in rats exposed to aflatoxin B1 and DMH.

The effects of retinoids are thought to be mediated through interactions with nuclear retinoid receptors, including retinoid acid receptors (RARs) and retinoid X receptors (RXRs). Each family has three subtypes: α, β and γ, that are encoded by different genes located on different chromosomes (10, 11), and have different amino- and carboxy-terminal domains. RARs and RXRs form heterodimers, which serve as ligand-activated transcriptional factors to regulate expression of a variety of genes that are involved in various biological activities. Alterations in their expression profiles may lead to aberrant responses to retinoid signaling and neoplastic transformation. Among these receptors, RXRs appear to play critical roles in modulating growth, apoptosis and tumor development. Targeted loss of the *RXRα* gene leads to embryonic lethality (12, 13), and conditional disruption of the RXRα in mouse prostate epithelium results in the development of prostate intraepithelial neoplasia (14). RXRα deletion in conjunction with expression of human papillomavirus 16 E6 and E7 oncoproteins is sufficient to induce cervical carcinogenesis in a mouse model (15). Furthermore, selective disruption of RXRα in mouse hepatocytes results in dysfunction of a variety of genes, including liver X receptor alpha, pregnane X receptor, farnesoid X receptor, constitutively activated receptor beta and peroxisome proliferator-activated receptor alpha that

heterodimerize with RXRα compromising various metabolic pathways (16). Recent studies also showed that reduced expression of RXRs was correlated with the development of skin (17), gastric (18), prostate (19), breast (20), pancreatic (21) and thyroid (22) tumors. More interestingly, Haugen *et al*. found that the cells expressing both RXRγ and RARβ showed significant growth inhibition in response to 9-CRA, while cells lacking RXRγ and RARβ expression were unresponsive (23). We and others have observed a decreased expression of RXRα protein and mRNA in AOM-induced rat colorectal ACF (24, 25) and increased RXRα expression in ACF treated by polyphenon E (PPE) and β-carotinoid. These studies suggest that RXRs may not only contribute to the progression of tumorigenesis but also play a significant role in prevention and treatment. However, there has been no detailed analysis of RXRα, RXRβ and RXRγ expression in colorectal tumors.

In the present study, we profiled the expressions of these RXRs by immunohistochemistry in both human and AOM-induced rat colorectal pre-malignant and malignant lesions.

Materials and Methods

ACF and tumor tissues from AOM-treated F344 rats. ACF and tumors were from AOM-treated control rats in our serial studies on the effects of PPE on the formation of the colorectal ACF (24) and tumors (Hao X, unpublished data). The treatment of animals and characterization of ACF were described previously (24). To generate colorectal tumors, 25 F344 rats (Taconic Farms, Germantown, NY, USA) were given two weekly injections of AOM (Midwestern Research Institute, Kansas City, MO, USA) (15 mg/kg body weight), and then fed a 20% high-fat diet for 34 weeks. Colorectal tumors were counted and their dimensions were measured by a digital caliper. Tumors with a diameter more than 0.5 cm were divided into two: half was kept at −80˚C for biochemical studies and the other half was used for histopathological analysis as described previously (24).

Human colorectal cancer samples. Three different sets of human colorectal cancer tissue arrayed slides were purchased from Pantomics (San Francisco, CA, USA). Each arrayed slide contained two pieces of tissue from each sample. In total, there were 16 normal mucosa, 10 adenomas and 210 carcinomas with clinical characteristics including age, gender, histological differentiation and stage (TMN) but without prognostic follow-up data. Initial histopathological diagnoses were reconsidered after evaluation of the arrayed hematoxylin and eosin-stained slides using criteria described previously (26).

Immunohistochemistry. A standard avidin-biotin peroxidase complex (ABC) method was used as previously described (26). In brief, after dewaxing and rehydration, the slides were heated in a pressure cooker in sodium citrate buffer (0.01 M, pH 6.0) for 3 min after reaching full pressure. Endogenous peroxidase was quenched using 3% hydrogen peroxide in methanol. Sections were then blocked for 1 h at room temperature (RT) in PBS containing 3% normal goat serum. The sections were then immunostained overnight at RT with rabbit RXRs antibodies diluted in 10% goat serum (1:1000 for RXRα and 1:2000 for both RXRβ and -γ; Santa Cruz Biotechnology Inc. Santa Cruz, CA, USA). The sections were rinsed in PBS and incubated with a biotinylated goat anti-rabbit antibody and subsequently incubated in VECTORSTAIN ELITE ABC reagent for 30 min, using 3,3'-diaminobenzidine as the chromogen (Vector Laboratories, Burlingame, CA, USA). Sections were then counterstained for 2-3 min with hematoxylin (Sigma, St. Louis, MO, USA) and mounted with Permount. To confirm the specificity of the RXR antibodies, RXR-blocking peptides (Santa Cruz Biotechnology Inc.) were incubated with RXR antibodies at a similar concentration for 2 h at RT before being applied to the slides.

Evaluation of the staining. RXRs displayed both nuclear and cytoplasmic staining. Cytoplasmic staining of RXRs was consistently weak in both pre-malignant and malignant lesions and was excluded from further analysis. Positivity of nuclear staining of epithelial cells in the lesions was graded as: 0, negative or fewer than 1% positive cells; 1, ≤25% of cells; 2, >25-50% of cells; and 3, >50 % of cells. Nuclear staining intensity was graded as 0, negative; 1, weak; 2, intermediate; 3, strong staining. A final score between 0 and 9 was achieved by multiplying positivity and intensity. Scores of 7-9 were defined as: 3+, strong expression; scores of 4-6 as 2+, reduced expression; and scores of 0-3 were defined as 1+, markedly reduced expression (26).

Statistical analysis. Data on RXR expression in ACF, adenomas and carcinomas were analyzed by either Chi-square test or Fisher's exact test.

Results

RXRα, -β and -γ expression in normal rat colorectal mucosa, ACF, adenomas and carcinomas. Normal rat epithelial cells exhibited strong nuclear, but light cytoplasmic staining for all three proteins from the bottom to the top of the crypts (Table I and Figure 1A, E and H for RXRα, β and γ, respectively). Stromal tissues in the mucosa, including endothelial cells, fibroblasts and inflammatory cells, were also strongly stained, serving as internal positive controls. This staining pattern was totally inhibited by blocking peptides for RXRα (Figure 1B), β and γ, confirming the specificity of the antibodies.

As shown in Table I, all hyperplastic ACF displayed strong nuclear RXRα staining, whereas a lower expression was observed in 11.8% of ACF with low-grade dysplasia (2/17), 75.0% of ACF with high-grade dysplasia (9/12), 96.7% of adenomas with high-grade dysplasia (30/31) and 100% of adenocarcinomas (45/45). The ACF with high-grade dysplasia exhibited significantly reduced expression compared to those with low-grade dysplasia (p=0.0012). However, there was no statistical difference in RXRα expression between ACF and adenomas with high-grade dysplasia (p=0.07, Figure1C). Compared to adenomas and ACF with high-grade dysplasia, carcinomas had even lower or no expression of RXRα (both p<0.001, Figure 1D). Reduced expression of RXRβ was observed in 5 out of 13 ACF with high-grade dysplasia (38.5%, Figure 1F), 16 out of 31 adenomas with high-grade dysplasia (51.6%, Figure 1G), and 44 out of 45 carcinomas (97.8%, Figure 1G). In addition, expression of RXRβ was markedly reduced in 26 out of 45 carcinomas (57.8%). Similarly, reduced nuclear expression of RXRγ was seen in 4 out of 7 ACF with

Table I. *Retinoid X receptor (RXR) α, β and γ nuclear expression in azoxymethane-induced rat colorectal aberrant crypt foci (ACF), adenomas and carcinomas.*

	RXRα			RXRβ			RXRγ		
	3+	2+	1+	3+	2+	1+	3+	2+	1+
Normal mucosa	156	0	0	121	0	0	116	0	0
ACF									
Hyperplasia	6	0	0	6	0	0	8	0	0
Low-grade dysplasia	15	2	0	15	0	0	10	0	0
High-grade dysplasia	3	5	4	8	5	0	3	3	1
Adenoma with high-grade dysplasia	1	14	16	15	13	3	9	17	5
Carcinoma	0	2	43	1	18	26	2	5	38

The sample numbers stained for RXRα, -β and -γ differ due to the availability of the slides and lesions. Low- *vs*. high-grade dysplastic ACF: $p=0.0012$ for RXRα (Fisher's exact test, two-tailed); high-grade dysplastic ACF *vs*. high-grade dysplastic adenoma: $p=0.07$ for RXRα, $p=0.42$ for RXRβ and $p=0.65$ for RXRγ (Fisher's exact test, two-tailed); high-grade dysplastic ACF *vs*. carcinoma: $p<0.001$ for all three RXRs (Chi-square test). Adenoma *vs*. carcinoma: $p<0.001$ for RXRα, $p<0.0001$ for both RXRβ and RXRγ (Chi-square test). AOM-induced carcinoma: RXRα *vs*. RXRβ: $p=0.001$; RXRα *vs*. RXRγ: $p=0.16$; RXRβ *vs*. RXRγ: $p=0.007$ (Chi-square test).

high-grade dysplasia (57.1%, Figure 1I), 22 out of 31 adenomas with high-grade dysplasia (71.0%, Figure 1J), and 43 out of 45 carcinomas (95.6%, Figure 1J). Furthermore, a majority of carcinomas (38/45, 84.4%) displayed markedly reduced RXRγ expression. Both RXRβ and γ expression in carcinomas was significantly decreased compared with both ACF with high-grade dysplasia (both $p<0.001$) and adenomas with high-grade dysplasia (both $p<0.0001$). However, there were no statistical differences in RXRβ and γ expression between adenomas and ACF with high-grade dysplasia. As shown in Table I, a significantly higher proportion of rat carcinomas displayed markedly reduced expression for RXRα (43/45, 95.6%) and RXRγ (38/45, 84.4%) compared with RXRβ (26/45, 57.8%). RXRα and RXRγ were not found to differ in expression.

RXRα, -β and -γ expression in human normal colorectal mucosa, adenomas and carcinomas. Since AOM-induced rat colorectal tumors may not exactly simulate the process of human colorectal cancer development, we further characterized RXR expression in human colorectal tissues using arrayed slides containing 16 normal mucosal samples, 10 adenomas and 210 cancer samples. In human mucosal tissues, all three proteins displayed strong nuclear (3+) and weak cytoplasmic expressions in the epithelial cells from the bottom to the top of the crypts, as well as stromal cells, including smooth muscle cells, fibroblasts and endothelial cells (Figure 2A, D and 2G for RXRα, β and γ, respectively), which served as internal positive control. Eight out of 10 adenomas (Figure 2B) and 89.0% (186/210) of carcinomas (Figure 2C) exhibited decreased RXRα expression, and 4/10 of adenomas (Figure 2E) and 51.0% (107/210) of carcinomas (Figure 2F) showed reduced RXRβ nuclear staining. Furthermore, 7/10 of adenomas (Figure 2H) and 99% (208/210) of carcinomas (Figure 2I) exhibited reduced nuclear expression of RXRγ.

Since there was only one stage T1 case and two stage T4 cases, we analyzed the correlation between RXRs expressions and stages II and III. Expression of all three proteins was unrelated to gender, stage, histological differentiation and type (all $p>0.1$, data not shown). As seen in AOM-induced carcinomas, a significantly higher proportion of human carcinomas exhibited markedly reduced RXRα (121/210, 57.6%) and RXRγ (135/210, 64.3%) expression as compared with RXRβ (21/210, 10.0%) ($p<0.0001$, Table II). No differences in RXRα and RXRγ expression were observed. Seventeen percent of cancer tissues (36/210) were seen to be heterogeneous for RXRα expression in two different areas of the same cancer sample, with some cancer tissues showing stronger expression, while others displayed weaker expression. Interestingly, 9 out of 10 mucinous carcinomas exhibited strong RXRβ nuclear staining.

Discussion

The AOM-induced rat colon carcinogenesis model is known for its progression from ACF to adenomas and further to carcinomas. This not only mimics the adenoma–carcinoma sequence observed in human colonic carcinogenesis, but also shares many pathogenetic molecular events associated with the human situation (27, 28).

In the present study, we systemically analyzed the expressions of three RXR proteins in AOM-induced rat ACF, as well as in normal colorectal mucosa, adenomas and carcinomas from humans and AOM-treated rats. Previous studies showed that RXRβ is expressed in almost all tissues; RXRα is highly expressed in liver, spleen, kidney and skin; while RXRγ is mainly expressed in skeletal and heart muscle, skin and brain (10, 11). Herein we demonstrated that all three RXR proteins were strongly expressed in both human and rat

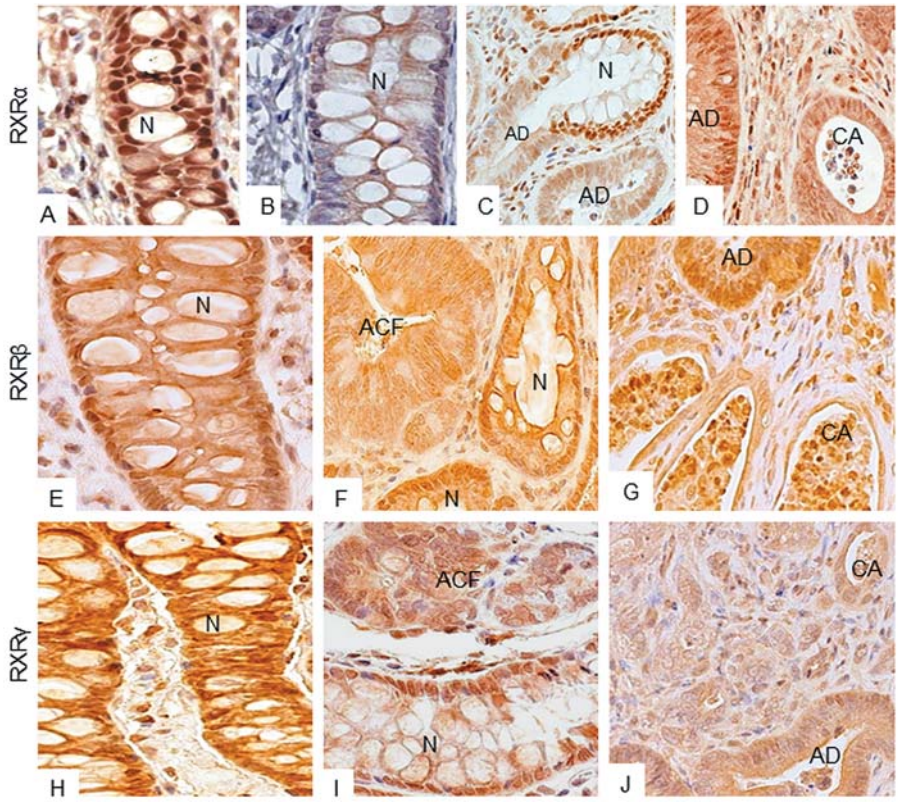

Figure 1. *Expression of retinoid X receptor (RXR) α, β and γ in colorectal tissues from azoxymethane-treated rats. Normal mucosa (N) displaying strong nuclear staining (3+) in epithelial cells from the bottom to the top of the crypts and stromal cells, including fibroblasts, endothelial cells and inflammatory cells for RXRα (A), RXRβ (E) and RXRγ (H). Normal mucosa displayed negative RXRα staining blocked by RXRα blocking peptide (B). Reduced RXRα nuclear expression is apparent in adenoma (AD) compared to strong nuclear expression (3+) in adjacent normal epithelial cells (N) which served as an internal positive control (C). Aberrant crypt foci (ACF) with dysplasia exhibited reduced nuclear expression (2+) compared with adjacent normal mucosa (3+) for both RXRβ (F) and RXRγ (I). Carcinoma (CA) showed markedly reduced nuclear RXRα (+, D), RXRβ (1+, G) and RXRγ (1+, J) expression compared to associated adenoma (AD) (2+).*

normal colorectal mucosa. We further observed that expression of RXRα, β and γ decreased progressively from AOM-induced colorectal ACF and adenomas with high-grade dysplasia to carcinomas. In human colorectal adenomas and carcinomas, we observed similar trends for the expression profiles of all three proteins, but their expression patterns were unrelated to histological grade. These data suggest that decreased RXRα, β and γ expression is associated with the development of both AOM-induced rat and human colorectal carcinogenesis. Progressive decreases in RXRα, β and γ mRNA or protein expression have been reported in human skin, gastric and prostatic pre-malignant and malignant lesions (17-19, 29). Together with our current study, these results imply that decreased expression of RXRα, β and γ may be early common events in the development of different types of cancer.

Differential expression of RXRs has been observed in different types of cancer tissues. In prostatic cancer, decreased RXRβ mRNA or protein expression was more prominent than decreases in RXRα or RXRγ (29, 30). In

Table II. *Retinoid X receptor (RXR) α, β and γ expression in human colorectal normal mucosa, adenomas and carcinomas.*

	Staining score			
	N	3+	2+	1+
Normal mucosa				
RXRα	16	16		
RXRβ	16	16		
RXRγ	16	16		
Adenoma				
RXRα	10	2	5	3
RXRβ	10	6	4	0
RXRγ	10	3	2	5
Carcinoma				
RXRα	210	26	63	121
RXRβ	210	103	86	21
RXRγ	210	2	73	135

Chi-square test for RXR expression in adenocarcinoma: RXRα *vs.* RXRβ: $p<0.001$; RXRβ *vs.* RXRγ: $p<0.001$; RXRα *vs.* RXRγ in markedly reduced expression: $p>0.05$. Numbers indicate number of cases.

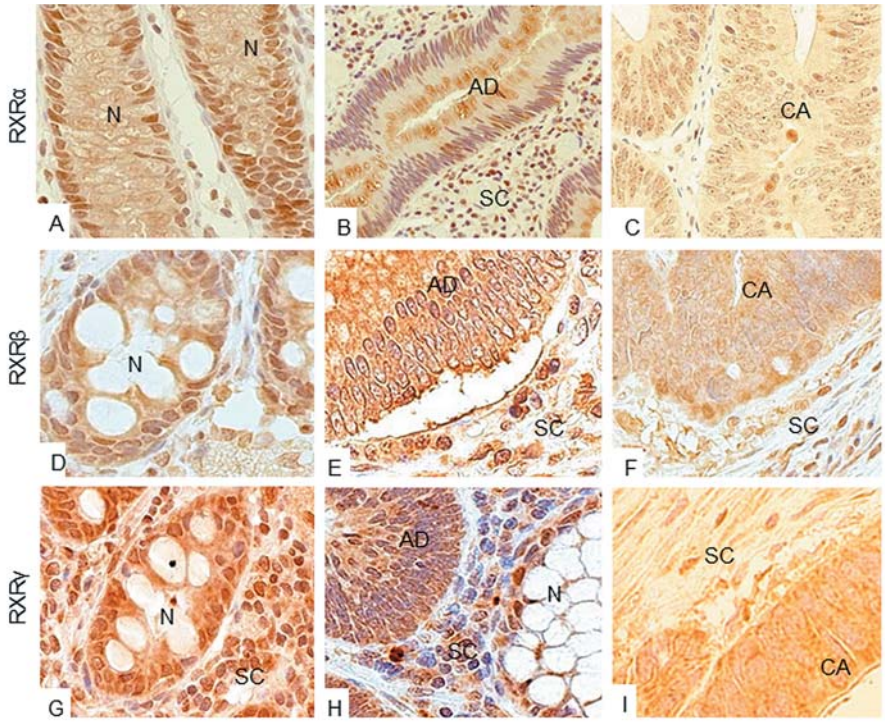

Figure 2. *Expression of retinoid X receptor (RXR) α, β and γ in human normal mucosa, adenomas and carcinomas. In normal mucosa, RXRα (A), RXRβ (D) and RXRγ (G) showed strong nuclear (3+) and weak cytoplasmic staining. In adenomas, RXRα (B), RXRβ (E) and RXRγ (H) displayed reduced nuclear but weak cytoplasmic staining compared with stromal cells (SC) and normal mucosa (N). In carcinomas, RXRα (C), RXRβ (F) and RXRγ (I) exhibited markedly reduced nuclear but weak cytoplasmic staining compared to stromal cells (SC).*

contrast, we found that a significantly higher proportion of both human and AOM-induced rat cancer tissues showed markedly reduced nuclear expression of RXRα and RXRγ compared to RXRβ. This suggests that reduced expression of RXRα and RXRγ may play a more important role than reduced expression of RXRβ during the development of colorectal cancer.

The mechanisms responsible for reduced expression of the three RXRs during colorectal carcinogenesis are unclear. Since expression of all three RXR decreased consistently during progression from pre-malignant to malignant lesions, and the fact that RXRα expression can be modulated by dietary factors such as PPE (24) and fat (31), it seems unlikely that alterations in gene structure rather than changes in transcriptional regulation may determine the expression levels of the three RXRs. Further work is needed to clarify how these three RXRs are down-regulated during the development of colorectal cancer.

In conclusion, we demonstrated for the first time that decreased expression of RXRα, β and γ occurs early during both human and AOM-induced rat colorectal carcinogenesis and that RXRα and RXRγ may selectively play more important roles than RXRβ during the development of colorectal cancer.

Conflicts of Interest

None.

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