ASC-associated inflammation promotes cecal tumorigenesis in aryl hydrocarbon receptor-deficient mice

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The aryl hydrocarbon receptor (AhR) plays a suppressive role in cecal carcinogenesis by CUL4B/AhR-mediated ubiquitylation and degradation of β-catenin, which is activated by xenobiotics and natural ligands. AhR-deficient (AhR−/−) mice develop cecal tumors with severe inflammation. To elucidate whether the tumors develop autonomously in AhR−/− mice due to impaired β-catenin degradation or in association with accelerated inflammation, we performed two kinds of experiments using germ-free (GF) AhR−/− mice and compound mutant mice lacking genes for AhR and apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), which plays an essential role in caspase-1 activation in inflammamomes. Both GF AhR−/− and AhR−/−ASC−/− mice showed considerably reduced tumor development compared with that in AhR−/− mice albeit in a ‘cancer-prone’ state with aberrant β-catenin accumulation. Blocking of the interleukin (IL)-1β signaling pathway by treatment with a caspase-1 inhibitor, YV AD, reduced cecal tumorigenesis in AhR−/− mice. Signal transducers and activators of transcription 3 (STAT3) activation was detected in the cecal epithelium of the AhR−/− mice due to enhanced IL-6 production. An inhibitor of the STAT3 signaling pathway, AG490 suppressed the tumor formation. ASC-mediated inflammation was also found to play a critical role in tumor development in Apc1099 mice, a mouse model of familial adenomatous polyposis. Collectively, these results revealed an important role of the bacteria-triggered or ASC-mediated inflammation signaling pathway in the intestinal tumorigenesis of mice and suggest a possible chemical therapeutic intervention, including AhR ligands and inhibitors of the inflammation pathway.

Introduction

The aryl hydrocarbon receptor (AhR), also known as the dioxin receptor, is a member of the basic helix-loop-helix/Per-AhR nuclear translocator-Sim homology superfamily and mediates a wide variety of pharmacological and toxicological effects, such as induction of drug-metabolizing enzymes, tumor promotion, teratogenesis, immunosuppression and wasting syndrome (1,2). Recently, it has been revealed that AhR is also involved in the normal development and homeostasis of multiple physiological processes, such as reproduction, innate immunity and tumor suppression (3). In addition to ligand-dependent transcriptional regulation, AhR regulates intracellular protein levels as a ligand-dependent E3 ubiquitin ligase of nuclear receptors such as estrogen and androgen receptors (4). We have recently shown that AhR−/− mice spontaneously develop colonic tubular adenocarcinomas especially in the cecum near the ileocecal junction, with abnormal accumulation of β-catenin (5). The tumor suppression mechanism involves AhR-mediated ubiquitylation and proteasomal degradation of β-catenin that is independent of the adenomatous polyposis coli (APC) degradation system, the canonical Wnt signaling pathway (6). This AhR function is activated by both xenobiotics and natural AhR ligands (5), such as indole derivatives, which are converted by intestinal microbes from dietary tryptophan and glucosinolates (7).

Although it is unknown till date why AhR−/− mice specifically develop cancers in the cecum, the host genetic predisposition to these cancers may be potentiated by microbial interaction or subsequent inflammation (8). Indeed, we and another group have observed severe inflammation in the intestines of AhR−/− mice (9) together with high levels of inflammatory cytokine expression (10). AhR−/− mice become hypersensitive to lipopolysaccharide (LPS)-induced septic shock (10,11), and the increased susceptibility to endotoxin is associated with elevated plasma concentrations of interleukin (IL)-1β, which is assumed to be a master mediator or initiator of inflammation (12). In this regard, it is notable that the processing of pro-IL-1β/pro-IL-18 to mature forms is accelerated by an activated inflammasome complex (13,14). Members of the nucleotide-binding oligomerization domain-like receptor family, including NLRP3, and the adaptor apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) (15) are critical components of the inflammasome, which activates caspase-1 in response to microbial and endogenous danger signals. Caspase-1 is synthesized as procaspase-1 that is proteolytically activated by cleavage of its COOH terminus. Activation of procaspase-1 depends on the oligomerization of procaspase-1 molecules via caspase recruitment domain interactions between proteins able to bind to the caspase recruitment domain of procaspase-1. AhR regulates plasminogen activator inhibitor-2 (Pai-2) gene expression through a mechanism involving nuclear factor-κB to suppress caspase-1 activation (10), resulting in negative regulation of IL-1β secretion (16).

Several lines of evidence suggest that AhR plays an essential role in the development of IL-17-producing T-helper (Th17) cells (17–19), a new subset of CD4+ T cells that is involved in autoimmune diseases and the clearance of infectious agents (20). Differentiation of Th17 cells from naïve T cells, which are stimulated by transforming growth factor-β and IL-6, is normally enhanced by activated AhR and is severely impaired in AhR−/− mice (19). Interestingly, dietary AhR ligands such as 6-formylindolo[3,2-b]carbazole enhance Th17 cell differentiation in the presence of transforming growth factor-β and IL-6 from CD4+ naïve T cells (17), whereas kynurenine, the first breakdown product in the indoleamine 2,3-dioxygenase-dependent tryptophan degradation (21), activates AhR to stimulate Treg cell differentiation (22), suggesting that AhR participates in regulating the immune response by modulating Th17/Treg balance through different AhR ligands (23). It has recently been reported that AhR also controls survival and function of gut-residing innate lymphoid cells, which regulate the epithelial barrier function and inflammation (24). Here, we performed carcinogenesis experiments using germ-free (GF) AhR−/− and compound mutant mice lacking genes for both AhR and ASC, which plays a critical role in the inflammasome-dependent

**Abbreviations:** AhR, aryl hydrocarbon receptor; APC, adenomatous polyposis coli; BMDMs, bone marrow-derived macrophages; DKO, double knockout; GF, germ-free; IL, interleukin; JAK, Janus kinase; LPS, lipopolysaccharide; mRNA, messenger RNA; Pai-2, plasminogen activator inhibitor-2; PBS, phosphate-buffered saline; SPF, specific pathogen-free; STAT, signal transducers and activators of transcription; Th17, T-helper cells; WT, wild-type.

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activation of caspase-1. Both GF AhR−/− and the compound AhR−/−\(\times\)ASC−/− (double knockout [DKO]) mutant mice showed markedly reduced tumor development in the cecum compared with that in AhR−/− mice, albeit in a cancer-prone state with aberrant β-catenin accumulation, suggesting critical roles for microflora and resulting inflammation in cancer development. Similarly, deletion of the ASC gene also resulted in a remarkable decrease in intestinal carcinogenesis in ApcMin/+ mice, a mouse model of familial adenomatous polyposis (25). All other experimental data, including reduced carcinogenesis in AhR−/− mice and ApcMin/+ mice caused by consecutive injections of the caspase-1 inhibitor YYAVD and AhR−−dependent, cecal-specific signal transducers and activators of transcription 3 (STAT3) activation mediated by IL-6, revealed that aberrant accumulation of β-catenin together with inflammation promoted by the ASC-mediated pathway work cooperatively to develop cecal tumors in AhR−− and ApcMin/+ mice.

Materials and methods

Animal experiments and histological analysis

AhR−− or ApcMin/+ mice (5) were mated with ASC−− mice (15), and the double heterozygous offspring were interbred to generate AhR−−\(\times\)ASC−− or ApcMin/+\(\times\)ASC−− mice. These mice were back-crossed with C57BL/6J mice at least six times. The mice were bred and maintained under specific pathogen-free (SPF) conditions in the animal house of the Saitama Cancer Center. Pregenotyped by PCR using primers as follows: K05 (CGGGGCTGCCATGACGAC), K03 (TGTTGACATGTCGACTCTCTGGATG) and LacZ (CGGGGCTGCCATGACGAC) for AhR mutant mice; F25 (CCAGTAATGTTATTACGACACC), R40 (TGGTGACATGTCGACTCTCTGGATG) and F1 (TGAGGCTTTTTCAGACGGCTTC) for ASC mutant mice. GF mice were generated and housed in gnotobiotic isolators under a strict 12 h light cycle in the animal facility in CLEA, Japan. They were maintained by breeding AhR−− females with AhR−− males. Ac-YVAD-CMK (MERCK; 10 mg/kg body wt) (26), a caspase-1 inhibitor, or AG490 (MERCK, 20 mg/kg body wt) (27), a Janus kinase (JAK) inhibitor, were injected intraperitoneally in 28% dimethyl sulfoxide diluted with phosphate-buffered saline (PBS) for the indicated duration. Animal experiments were approved by the Saitama Cancer Center Animal Care and Use Committee. Immunohistochemistry was performed on 4–6 μm sequential paraffin or frozen sections. The anti-AhR antibody developed by R.Pollenz (clone 17-10) and the anti-β-catenin antibody (clone 14; BD Biosciences) were used for immunostaining. Slides were analyzed on a Leica DMR microscope. Image acquisition and processing were performed with Leica QWIN and Adobe Photoshop, respectively.

Cell culture and preparation of macrophages

Murine macrophages and murine monocytic cell line J774.1 were maintained in RPMI-1640 medium (Sigma–Aldrich) supplemented with 10% fetal calf serum and gentamycin under 5% CO2 at 37°C. Bone marrow cells were obtained from the femurs, and bone marrow–derived macrophages (BMDMs) used for the experiments were isolated by culturing bone marrow cells with 10 ng/ml granulocyte–macrophage colony-stimulating factor (Pepro Tech) for 8 days followed by washing the attached cells with PBS three times. To isolate peritoneal exudate macrophages, mice were injected intraperitoneally with 3 ml of 4% thioglycolate (Sigma). Four days after the injection, peritoneal cells were isolated, incubated for 3 h in plates and washed with PBS. The adherent cells were used for experiments. To determine the effect of cytokines on STAT3 activity, cells were incubated with murine recombinant IL-1β or IL-6 (R&D), or IL-18 (MBL).

Reverse transcriptase–PCR

RNA was extracted from tissue samples or cultured cells using Isogen (Nippon gene). Reverse transcription was performed using RNA PCR kit (Takara Bio), according to the manufacturer’s instructions. PCR analyses were performed with ExTag DNA polymerase (Takara Bio) using a GeneAmp PCR system 9700 (Applied Biosystems). The PCR products were visualized by electrophoresis on 2% agarose gels. Quantitative PCR analysis was performed on a Light-Cycler FastStart DNA Masterplus SYBR Green I (Roche). The PCR products were evaluated by a melting curve analysis, following the manufacturer’s instructions. Sequences of the PCR primers used are given in the Supplementary Table 1, available at Carcinogenesis Online.

Immunohistochemistry

Tissue samples were prepared by homogenization in sodium dodecyl sulfate lysis buffer supplemented with 1 mM NaF and 1 mM NaVO3, followed by boiling. The protein samples were mixed with 100 mM dihiothreitol and bromophenol blue before loading on polyacrylamide gels of appropriate concentrations, followed by transfer to nitrocellulose membranes. The membranes were incubated in blocking solution containing 3% gelatin dissolved in Tris-buffered saline. The antibody probes used were as follows: anti-AhR (Novus); anti-β-catenin (Santa Cruz Biotechnology), antiphospho-STAT3 (Cell Signaling), anti-β-actin (Sigma–Aldrich) and anti-β-catenin (BD Biosciences). The membrane was incubated with secondary antibodies conjugated with horseradish peroxidase and detected using the Immobilon Western Chemiluminescence horseradish peroxidase substrate (Millipore) followed by visualization with LAS-1000 (Fuji Film). An in vitro processing assay for caspase-1 activation was performed in a cell-free system, as described previously (15). IL-1β secreted by macrophages into culture medium was detected with anti-IL-1β (Santa Cruz).

Cytokine enzyme-linked immunosorbent assays

Plasma cytokine concentrations were determined using an enzyme-linked immunosorbent assay kit (Invitrogen) 2 h after intraperitoneal injection of LPS (20 mg/kg of body wt) (Sigma). Cytokines secreted from the cecum were determined in the supernatant after the tissues were cultured for 24 h.

Results

No tumor development in GF AhR−− mice

We first performed the carcinogenesis experiments using the GF AhR−− mice to elucidate the association between cecal cancer development and accelerated inflammation mediated by microflora in AhR−− mice. Because we maintained GF mice by breeding AhR−− males with AhR−− female, experiments were performed using AhR−− mice as control. We have already observed that AhR−− mice do not develop tumor (5) and that AhR−− mice show similar phenotype to wild-type (WT) mice. We observed reduced AhR expression in the intestine of GF AhR−− mice in comparison with SPF AhR−− mice by immunohistochemical staining (Figure 1A) and western blot analysis, which revealed a 40% reduction quantitated by densitometrical analysis using ImageJ software (Figure 1B) consistent with the previous finding that AhR is induced in macrophages by LPS treatment (10). Thus, the results reveal that both production of natural AhR ligands, which we showed previously (5), and AhR expression are dependent on the presence of intestinal microflora. Furthermore, we observed pronounced accumulation of β-catenin in the intestine of both AhR−− and AhR−− mice under the GF conditions, whereas β-catenin accumulation was only observed in AhR−−, but not in AhR−− mice under SPF conditions because of ubiquitination-dependent degradation by the expressed AhR (Figure 1C).

Next, we examined whether GF AhR−− and GF AhR−− mice develop tumors in the cecum. Interestingly, no tumors developed in GF mice till they were 25 weeks of age regardless of their genotypes, judging from macroscopic observation and histopathological examination (Supplementary Figure 1, available at Carcinogenesis Online), whereas all of AhR−− mice kept under SPF or conventional conditions developed adenomas or adencarcinomas at that age (5). In addition, close microscopic examination revealed a markedly severe inflammation in the intestines of SPF AhR−− mice, consistent with previous results (9,10) but not in those of GF AhR−− mice (Figure 1D). These results suggest that microbial interaction or subsequent infection is required for cecal tumor development in AhR−− mice.

Functional cross-talk between AhR and ASC

It is well known that ASC plays an essential role in inflammatory responses during host defense against infectious diseases and is involved in the release of IL-1β/IL-18 through caspase-1 activation in inflammasomes (13,15). In contrast, AhR enhances Pai-2 expression to inhibit caspase-1 activity (10), resulting in an anti-inflammatory function. To reveal the functional cross-talk between ASC-mediated activation and AhR-mediated suppression of caspase-1 in vivo and to
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investigate the involvement of ASC-mediated inflammation in cecal carcinogenesis of \( \text{AhR}^{-/-} \) mice, we generated \( \text{AhR}^{-/-}\cdot\text{ASC}^{-/-} \) (DKO) mice with the same genetic background (Supplementary Figure 2A, available at Carcinogenesis Online).

AhR messenger RNA (mRNA) induction was clearly observed in macrophages from WT and \( \text{ASC}^{-/-} \) mice following LPS treatment (Supplementary Figure 2B, available at Carcinogenesis Online), consistent with AhR upregulation in the presence of intestinal microflora (Figure 1A and B). As expected, AhR expression was completely lost in macrophages isolated from either \( \text{AhR}^{-/-} \) or DKO mice (Supplementary Figure 2B, available at Carcinogenesis Online). ASC mRNA expression levels in \( \text{AhR}^{-/-} \) mice were similar to those of WT mice, whereas no ASC mRNA expression was observed in \( \text{ASC}^{-/-} \) and DKO mice. In addition, no significant change in caspase-1 mRNA expression was observed among mice regardless of their genotypes (Supplementary Figure 2B, available at Carcinogenesis Online).

Pai-2 mRNA expression was markedly induced in response to LPS treatment in BMDMs from WT and \( \text{ASC}^{-/-} \) mice, whereas only minimal levels of Pai-2 mRNA expression were observed following LPS treatment of \( \text{AhR}^{-/-} \) and DKO BMDMs (Figure 2A). In contrast, IL-1\( \beta \) mRNA expression was similarly enhanced by LPS treatment in BMDMs in these genotypes (Figure 2A).

We next analyzed the \textit{in vitro} activation of caspase-1 and secretion of IL-1\( \beta \) in macrophages isolated from WT, \( \text{AhR}^{-/-} \) and DKO mice. In the LPS-primed macrophages of WT mice, procaspase-1 (p45) was...
evidently processed to a mature form of caspase-1 (p10). The activated form of caspase-1 was markedly increased in $AhR^{−/−}$ macrophages, whereas it was hardly detectable in the DKO macrophages (Figure 2B). This alteration in the caspase-1 processing was confirmed by time-course studies (Supplementary Figure 2C, available at Carcinogenesis Online). Consistent with these observations, essentially no matured form of IL-1β (p17) was secreted from the DKO macrophages, whereas its secretion was even increased from $AhR^{−/−}$ macrophages treated with LPS compared with that from WT macrophages (Figure 2C). These results clearly indicate that AhR suppressed ASC-mediated processing of IL-1β by inhibiting caspase-1 activity.

We next performed an in vivo experimental endotoxin shock induced by LPS (Supplementary Figure 2D, available at Carcinogenesis Online) and observed that although $AhR^{−/−}$ mice had a significantly shorter lifespan than WT mice as previously reported (10), DKO mice became even more resistant to septic shock than WT mice. Correspondingly, plasma concentrations of the inflammatory cytokines, IL-1β (Figure 2D), IL-6 (Figure 2E) and IL-18 (Supplementary Figure 2E, available at Carcinogenesis Online) were increased in $AhR^{−/−}$ mice, whereas DKO mice showed low levels of IL-6 secretion and even lower levels of IL-1β than those in WT mice. These results indicate a functional association between ASC- and AhR-directed pathways, which regulate the IL-1β-dependent inflammatory response in vivo.

**Reduced cecal carcinogenesis in DKO mice**

To investigate whether cecal carcinogenesis observed in $AhR^{−/−}$ mice is autonomously developed due to lack of ligand-dependent β-catenin degradation by AhR or involves inflammation due to lack of anti-inflammatory function of AhR as well, we performed experimental carcinogenesis using DKO mice. No cecal tumor development was observed in WT or ASC−/− mice (Figure 3A). When the tumor incidence in DKO mice was compared with that of $AhR^{−/−}$ mice (5) (Figure 3A), DKO mice showed no tumor development until 30 weeks of age. Cecal tumor development was markedly delayed in DKO mice in comparison with $AhR^{−/−}$ mice. The cecal cancers that developed later in DKO mice were tubular adenocarcinomas (Supplementary Figure 3A, available at Carcinogenesis Online), as observed in $AhR^{−/−}$ mice (5), with varying degrees of malignancy. Immunohistochemical staining of these cells showed concomitant overexpression of β-catenin and c-myc, a target gene of the β-catenin/TCF4 pathway (Supplementary Figure 3A, available at Carcinogenesis Online).

To elucidate why DKO mice were more resistant to cecal carcinogenesis than the $AhR^{−/−}$ mice, β-catenin levels were monitored in the cecum of DKO mice at 14 weeks of age, when tumors were already observed in the cecum of $AhR^{−/−}$ mice. Although tumors and morphologically aberrant epithelia had not developed, abnormal β-catenin accumulation was observed in the cecum of 14-week-old DKO mice by immunohistochemical staining and western blot analysis revealed approximate 3-fold increase compared with WT cecum (Figure 3B). These results suggest that the intestinal epithelia of DKO mice are in a cancer-prone state with aberrant β-catenin accumulation, similar to the intestinal epithelia of $AhR^{−/−}$ (5) and GF $AhR^{−/−}$ mice (Figure 1C).

Next, we investigated tissue-specific IL-1β expression in the cecum near the ileocecal region in mice with different genotypes. IL-1β protein levels expressed in $AhR^{−/−}$ mice were significantly higher than those expressed in WT mice ($P = 0.006$, WT versus $AhR^{−/−}$), whereas its expression levels were lower (approximate to that of WT mice) in ASC−/− and DKO mice (Supplementary Figure 3B, available at Carcinogenesis Online). IL-18 levels were also elevated in $AhR^{−/−}$ mice (Supplementary Figure 3C, available at Carcinogenesis Online). Furthermore, IL-6 expression, which was upregulated in response to IL-1β, changed in a manner similar to the IL-1β expression, depending on AhR and ASC genotypes ($P = 0.0004$, WT versus $AhR^{−/−}$; $P = 0.094$, WT versus DKO; $P = 0.026$, $AhR^{−/−}$ versus DKO) (Supplementary Figure 3D, available at Carcinogenesis Online). Antitumor effects of IL-18 have been reported in experimental tumor models (28,29). Recent studies also showed that IL-18 production is critically involved in protection against colorectal tumorigenesis.

**Fig. 3.** Reduced cecal carcinogenesis in compound DKO mice. (A) Time course of cecal tumorigenesis in $AhR^{−/−}$ (blue circles), DKO (red circles) and WT (green circles) mice. No cecal tumor development was observed in
Based on these results, it has been suggested that the enhanced expression of inflammatory cytokines, such as IL-1β and IL-6, is involved in the cecal carcinogenesis in AhR−/− mice, in addition to aberrant β-catenin accumulation. In support of these observations, intraperitoneal injections of YVAD, an IL-1β-converting enzyme inhibitor II, administered to AhR−/− mice once a week from 5 to 18 weeks of age (Figure 3C) exerted significant inhibitory effects on both growth (Figure 3D; \(P = 0.0041\), control versus YVAD groups) and histological grade of tumor atypia (Figure 3E; \(P = 0.04\), control versus YVAD groups) compared with the sham-treated AhR−/− mice.

To confirm that the ASC-dependent inflammatory pathway is also involved in small intestinal tumorigenesis in ApcMin/+ mice, we generated mice with compound mutations in the Apc and ASC and found a remarkable reduction in spontaneous development of small intestinal polyps compared with those in ApcMin/+ mice (Figure 4A). Remarkably, decreased polyp formation was clearly observed in the ApcMin/+ASC−/− mutant mice in an ASC allele dose-dependent manner. Consistent with the results obtained from ApcMin/+ASC−/− mice, polyp formation in ApcMin/+ mice was remarkably inhibited following the intraperitoneal injections of YVAD (Figure 4B). Thus, these results suggest that the ASC-associated inflammatory pathway markedly promotes intestinal carcinogenesis in mice with aberrant accumulation of β-catenin caused by genetic deficiency in APC and AhR.

AhR−/−-dependent, cecal-specific STAT3 activation

Members of the STAT family of proteins are central in determining whether immune responses in the tumor microenvironment promote or inhibit cancer development (31), and because levels of IL-6, known to activate STAT3, were elevated in AhR−/− mice, we investigated the activation of these proteins in the intestines of the AhR−/− animals. Among the five STAT proteins examined, STAT3 hyperactivation (phosphorylation of Y705) was particularly observed in the normal cecal tissues of 8-week-old AhR−/− mice (Figure 5A, left) and later observed in 23-week-old DKO mice (Figure 5A, right). Furthermore, STAT3 was activated most prominently in the cecum, which is consistent with the site of tumor development in AhR−/− mice (5), and weak STAT3 activation was observed in the upper part of the small intestine (duodenum) and in the lower part of the colon (rectum) (Figure 5B).

Immunohistochemical analyses also revealed specific staining of activated STAT3 in the cecum of AhR−/− mice. STAT3 activation was observed only in stromal cells (red arrowheads) in 8-week-old mice, whereas it was detectable in stroma and epithelial cell nuclei (blue arrowheads) at 19 weeks of age (Figure 5C).Further immunofluorescence analysis revealed that STAT3 was activated in a subset of CD45-positive cells (Supplementary Figure 4A, available at Carcinogenesis Online).

Using isolated peritoneal macrophages from WT and AhR−/− mice, we studied the effects of IL-1β, IL-6, and IL-18 on STAT3 activation (phosphorylation of Y705). As shown in Figure 5D, IL-6 was involved but neither IL-1β nor IL-18 was particularly involved in STAT3 activation, and the STAT3 was activated in an IL-6-dose-dependent manner (Supplementary Figure 4B, available at Carcinogenesis Online).

### Supplementary Figure 4B

**Figure 4.** ASC-mediated inflammation is also involved in tumorigenesis in ApcMin/+ASC−/− mice. (A) Spontaneous polyp formation in the small intestines in ApcMin/+ASC−/− (open circles), ApcMin/+ASC−/− (closed triangle) and ApcMin/+ASC−/− (closed circles) mice. Five mice were used in each group. The numbers of polyps indicate the mean values developed in five animals. (B) The caspase-1 inhibitor YVAD suppressed polyp formation in the small intestines. An intraperitoneal injection of YVAD (10 mg/kg) was administered to ApcMin/+ mice once a week from 5 to 15 weeks of age. The number of polyps in each animal is shown as a circle (control, n = 10; YVAD, n = 7; \(*P = 1.07 \times 10^{-10}\) ). (C) AG490 suppressed polyp formation in the small intestines. Intraperitoneal injection of AG490 (20 mg/kg) was administered to ApcMin/+ mice at an interval of 5 days from 5 to 15 weeks of age. The number of polyps developed in each animal is shown as a circle (control, n = 10; YVAD, n = 7; \(*P = 0.004\) ). Results were obtained in two independent experiments.
Inflammation and tumorigenesis in AhR KO mice

Discussion

We have previously demonstrated that AhR plays a tumor suppressor role in the intestine as an ubiquitin E3 ligase of β-catenin, and that this pathway functions independently of and cooperatively with the APC ubiquitylation system (5). Recent findings have demonstrated that AhR also has an anti-inflammatory function by inhibiting caspase-1 activity in the inflammasome through transcriptional activation of Pai-2 (10) and stimulating differentiation of Treg cells from CD4+CD25+ naïve T cells (17,19). Our experimental data showed remarkable activation of caspase-1 in cultured peritoneal macrophages derived from AhR−/− mice. Production of IL-1β in macrophages is suppressed by ectopic expression of Pai-2 (10), which is downregulated in AhR−/− macrophages. Maturation of IL-1β from the precursor is mediated by caspase-1, a component of the inflammasome. Pai-2 is known to be an inhibitor of urokinase-type plasminogen activator as well as an inhibitor of retinoblastoma protein degradation by interaction with these molecules (32). Then, the signaling pathway, which leads to inhibition of caspase-1 activity, is proposed to involve AhR/nuclear factor-κB signaling. Pai-2 expression is a crucial determinant for promotion of cecal carcinogenesis in AhR−/− mice in association with abnormal β-catenin accumulation.

Given the importance of ASC functioning as a component of the inflammasome complex for activation of caspase-1 activity (13,14), we generated DKO mice to investigate how intestinal carcinogenesis is associated with the pro-inflammatory state in AhR−/− mice. Remarkably, DKO mice exhibited clearly subdued inflammatory phenotype, cecal tumor development was markedly retarded and tumors occur exclusively because of impaired AhR-dependent β-catenin degradation activity, we might observe tumor formation in GF mice due to their abnormal β-catenin accumulation. Given that GF AhR−/− mice do not develop tumors up to 25 weeks of age, these mice are in a cancer-prone state with accumulated β-catenin, but need some additional cues involving the presence of intestinal microbes, which may cause inflammation and lead to carcinogenesis.

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Our experimental evidence using GF mice indicates that natural AhR ligand production (5) and AhR expression are dependent on the presence of intestinal microbes, even though responsible bacteria remain to be specified. If the autonomous development of cecal tumors occurs exclusively because of impaired AhR-dependent β-catenin degradation activity, we might observe tumor formation in GF AhR−/− mice due to their abnormal β-catenin accumulation. Given that GF AhR−/− mice do not develop tumors up to 25 weeks of age, these mice are in a cancer-prone state with accumulated β-catenin, but need some additional cues involving the presence of intestinal microbes, which may cause inflammation and lead to carcinogenesis.

Fig. 5. Cecal-specific activation of STAT3 in AhR−/− mice. (A) Activation of STAT3 in the cecum of mice at 8 weeks (left) and 23 weeks of age (right). (B) Tissue lysates were prepared from AhR−/− mice at 8 weeks of age and analyzed for STAT3 activation. Organs from two mice of each group were analyzed. The results are representative of three independent experiments. (C) Hematoxylin and eosin and immunohistochemical staining of phosphoSTAT3(Y705) in the cecum of 8- or 19-week-old AhR−/− mice. Normal rabbit IgG was used as a negative control. Positive cells in the stroma are indicated by red arrowheads, and those in the epithelium are indicated by blue arrowheads. The results are representative of three independent experiments. Bars, 20 μm in upper panel and 50 μm in lower panel. (D) Peritoneal macrophages prepared from WT or AhR−/− mice were incubated with 10 ng/ml IL-1β or with 10 ng/ml IL-6 or with 10 ng/ml IL-18 for 60 min. Cell lysates were analyzed for STAT3 activation. (E and F) The JAK inhibitor AG490 suppressed tumor development. (E) Tumor size was estimated based on ImageJ, as shown by circles. Mean values are indicated by bars. (*P = 0.0016, control versus AG490 groups). (F) Histological grade of atypia is as indicated. (P = 0.0015, control versus AG490 groups). About 7-10 mice/group were analyzed in three independent experiments.

available at Carcinogenesis Online, shows a clear upregulation of genes responsible for pro-inflammatory and pro-carcinogenic mediators, such as IL-1β, IL-6, COX2, tumor necrosis factor-α and IL-22. In addition, the expression levels of genes related to cancer development, such as MMP2, MMP9, CDKN1A and HIF-1α, were markedly increased in AhR−/− mice. In contrast, IL-17 levels, a cytokine secreted by Th17 cells, decreased markedly in AhR−/− mice, consistent with previous reports (18,19). Thus, loss of AhR-associated anti-inflammatory functions, including suppression of ASC-dependent caspase-1 activation through Pai-2 expression was a crucial determinant for promotion of cecal carcinogenesis in AhR−/− mice in association with abnormal β-catenin accumulation.

Our experimental evidence using GF mice indicates that natural AhR ligand production (5) and AhR expression are dependent on the presence of intestinal microbes, even though responsible bacteria remain to be specified. If the autonomous development of cecal tumors occurs exclusively because of impaired AhR-dependent β-catenin degradation activity, we might observe tumor formation in GF AhR−/− mice due to their abnormal β-catenin accumulation. Given that GF AhR−/− mice do not develop tumors up to 25 weeks of age, these mice are in a cancer-prone state with accumulated β-catenin, but need some additional cues involving the presence of intestinal microbes, which may cause inflammation and lead to carcinogenesis.
AhR plays a dual role as a tumor suppressor of β-catenin E3 ubiquitination and in intestinal anti-inflammatory activity. AhR-dependent expression of Pai-2, which is a crucial inhibitor of ASC-mediated inflammation, is responsible for suppressing cecal carcinogenesis. Moreover, AhR may be involved in cecal tumor suppression by modulating the Th17/Treg balance through different AhR ligands as well as negatively regulates the IL-6 synthesis in combination with STAT1.

Collectively, we conclude that AhR-dependent cecal tumor suppression. AhR plays a dual role as a tumor suppressor of β-catenin E3 ubiquitination and in intestinal anti-inflammatory activity. AhR-dependent expression of Pai-2, which is a crucial inhibitor of ASC-mediated inflammation, is responsible for suppressing cecal carcinogenesis. Moreover, AhR may be involved in cecal tumor suppression by modulating the Th17/Treg balance through different AhR ligands as well as negatively regulates the IL-6 synthesis in combination with STAT1.

Fig. 6. A model of AhR-dependent cecal tumor suppression. AhR plays a dual role as a tumor suppressor of β-catenin E3 ubiquitination and in intestinal anti-inflammatory activity. AhR-dependent expression of Pai-2, which is a crucial inhibitor of ASC-mediated inflammation, is responsible for suppressing cecal carcinogenesis. Moreover, AhR may be involved in cecal tumor suppression by modulating the Th17/Treg balance through different AhR ligands as well as negatively regulates the IL-6 synthesis in combination with STAT1.

cancer-prone state was consistent with the results obtained from GF AhR−/− mice. Interestingly, intestinal carcinogenesis in ApcMin/+ mice was also significantly reduced by deletion of ASC. This reduction in tumor development is probably not due to decreased carcinogenesis in either AhR+/−ASC−/− or ApcMin/+ASC−/− mice, but due to retarded tumor growth and proliferation because these double mutant mice showed delayed tumor development, as reported previously in ApcMin/+ MyD88−/− mice (36, 37). These results suggest that AhR plays dual tumor suppressor roles; it acts as a regulator of both β-catenin degradation and anti-inflammation in the intestine (Figure 6). When taken together with the results of carcinogenesis experiments using the caspase-1 inhibitor YVAD, inflammatory conditions are a prerequisite for tumor development in both AhR−/− and ApcMin/+ mice.

Persistently activated STAT3 and, to some extent, STAT5 increase tumor cell proliferation and invasion while suppressing anti-tumor immunity (38–40). STAT3, but not other STAT isoforms, was activated particularly in the cecum of AhR−/− mice and also in DKO mice. This specific STAT3 activation in the ileocecal junction most probably resulted from the interaction of an AhR−/− mouse intestine hypersensitive to inflammation with commensal bacteria colonizing in this region. High levels of IL-6 production in the cecum of mice and small intestine of AhR−/− mice. Moreover, ApcMin/+ mice in association with inhibition of STAT3 activation, suggesting that the JAK–STAT3 signal pathway is involved in spontaneous intestinal carcinogenesis in both types of the mutant mice. Collectively, we conclude that AhR−/− mice is a useful model to elucidate the molecular mechanisms of inflammation-associated intestinal carcinogenesis, and further investigation into the host–microbes interaction will be needed to provide a new strategy for chemoprevention and chemotherapy of intestinal cancer using AhR ligands.

Supplementary material

Supplementary Table 1 and Figures 1–4 can be found at http://carcin.oxfordjournals.org/

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References


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