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Cellular Retinoic Acid Bioavailability Determines Epithelial Integrity: Role of Retinoic Acid Receptor α Agonists in Colitis

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ABSTRACT

The epithelial barrier is determined primarily by intercellular tight junctions (TJs). We have demonstrated previously that all-*trans* retinoic acid (atRA) plays an important role in forming functional TJs through a specific retinoic acid receptor (RAR)/retinoid X receptor heterodimer in epithelial cells. However, the physiological relevance of retinoic acids (RAs) in maintaining the epithelial integrity remains to be examined. Here, we show that several types of RA, including atRA, promote the barrier function of epithelial TJs. Conversely, RA depletion in the cells by overexpressing CYP26s, cytochrome P450 enzymes specifically involved in the metabolic inactivation of RAs, induces an increase of permeability as measured by two differently sized tracer molecules, inulin and mannitol. This RA-mediated enhancement of barrier function is potentially associated with the increased expression of TJ-associated genes such as occludin, claudin-1, claudin-4, and zonula occludens-1. We also found that RARα is a preferential regulator of the epithelial barrier in vitro. Studies of murine experimental colitis, which is characterized by increased gut permeability, reveal that RARα stimulation significantly attenuates the loss of the epithelial barrier during colitis in vivo. Our results suggest that cellular RA bioavailability determines the epithelial integrity, because it is a critical regulator for barrier protection during mucosal injuries.

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Tight junction (TJ) is the apical-most intercellular structure in epithelial cells, playing a role in cell-cell adhesion, polarity, and the permeability barrier to paracellular transport of solutes across the cells (Tsukita et al., 2001; Matter et al., 2005). The TJ creates a semipermeable barrier, separating different organ compartments. Whereas the TJ is composed of a large number of protein components, including membrane proteins such as occludin and cytoplasmic scaffolding proteins such as zonula occludens-1 (ZO-1), it is now well accepted that the claudin family, which has been shown to contain more than 20 members, is the main constituent of the TJ, with tissue-specific distribution of its expression. The epithelial barrier is critical to maintain tissue homeostasis; however, only a few agents that can enhance or protect epithelial barrier function have been proposed (Prosser et al., 2004; Howe et al., 2005).

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Retinoic acid (RA) is a biologically active regulator that has a broad range of functions involving cell differentiation, proliferation, and apoptosis in various cell types (Durston et al., 1989; Osanai and Petkovich, 2005). The effects of endogenous RAs are achieved primarily by two types of RA isomers (all-*trans* RA [atRA] and 9-*cis* RA [9cRA]), which are synthesized from the retinalaldehyde precursor, a derivative of vitamin A, and are mediated by two classes of nuclear receptors, retinoic acid receptors (RARα, β, and γ) and their heterodimeric counterparts, retinoid X receptors (RXRα, β, and γ) (Kastner et al., 1995). RARs are activated both by atRA and 9cRA, but RXRs are selectively activated by 9cRA. Accumulated evidence supports the possibility that atRA is an obligatory component in the differentiation of epithelial cells that leads to the establishment of epithelial integrity, whereas only a very limited number of reports have documented the functions of atRA in TJ formation. One example is based on our observation that atRA plays an important role in the formation of functional TJs in F9 embryonal carcinoma cells through the involvement of specific RAR/

ABBREVIATIONS: TJ, tight junction; atRA, all-*trans* retinoic acid; RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; ZO-1, zonula occludens-1; 9cRA, 9-*cis* retinoic acid; MDCK, Madin-Darby canine kidney; TER, transepithelial electrical resistance; RT-PCR, reverse transcription-polymerase chain reaction; TNBS, 2,4,6-trinitrobenzene sulfonic acid; DMSO, dimethyl sulfoxide; Am80, 4[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carbamoyl]benzoic acid; Am580, 4(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphtamido)benzoic acid; FITC, fluorescein isothiocyanate; PPAR, peroxisome proliferator-activated receptor; FBS, fetal bovine serum.

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RXR heterodimer-mediated transcription machinery (Kubota et al., 2001). On the other hand, loss of expression of TJ-associated molecules such as occludin has been correlated with tumor development in carcinogenesis, and decreased and/or impaired TJ formation has been reported for various types of cancer (Tobioka et al., 2004; Osanai et al., 2006). It is interesting that disruptions of retinoid signaling through mutations in RARs and RXRs have been shown to be associated with the disorganization of epithelial architecture such as that observed in cancer tissues (de Thé, 1996). This evidence prompted us to examine the possible hypothesis that cellular RA bioavailability may play an important role in the establishment of functional TJs in epithelial cells.

Cellular RA bioavailability is regulated by the vitamin A nutritional status and the coordinated balance between RA synthesis and catabolism. RA metabolizing cytochrome P450 enzymes (CYP26s) are specifically involved in the metabolic inactivation of RA (White et al., 1997; Abu-Abed et al., 2001; Taimi et al., 2004; Osanai and Petkovich, 2005). A member of this family, CYP26A1, has been shown to be responsible for the catabolism of atRA, and CYP26C1 efficiently converts both atRA and 9cRA to biologically inactive polar metabolites. The expression of CYP26s plays a critical role in embryogenesis by restricting exposure to inappropriate concentrations of RA (Abu-Abed et al., 2001), and a state of atRA deficiency induced by enhanced expression of CYP26A1 is an important contributing factor for apoptotic resistance in certain types of cancer (Osanai and Petkovich, 2005). Although the functional roles of CYP26s remain to be studied, RA-catabolizing enzymes play an important role on limiting the bioavailability of RA to cells and efficiently caused an RA-depleted state in given cells. By overexpressing CYP26s, we generated RA depletion in cultured cells to examine whether RA was an important determinant for the epithelial barrier. Here, we show that RAs, which stimulate RARα, preferentially promote the epithelial barrier function of TJs, which is sufficient to result in attenuated loss of the barrier during experimental colitis in vivo, suggesting that RA bioavailability in the cell regulates the epithelial integrity.

Materials and Methods

Cell Line and Culture. Madin-Darby canine kidney (MDCK) cells were maintained in DMEM (Invitrogen, Tokyo, Japan) supplemented with 10% FBS (Sanko Jyunyaku, Tokyo, Japan), 10 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified 5% CO_2 atmosphere.

Expression Vector and Transfection. Full-length human CYP26A1 cDNA (the coding region is from nucleotide 26 to 1519 [CYP26A1$^{26–1519}$], GenBank accession number NM_000783) was amplified by reverse transcription (RT)-PCR from 1 μg of total RNA extracted from atRA-treated MCF7 cells for 24 h (White et al., 1997). We also amplified truncated forms of CYP26A1$^{26–1519}$, CYP26A1$^{26–1338}$, CYP26A1$^{26–976}$, and CYP26A1$^{396–1519}$ using site-specific primers. After subcloning into a TA-cloning vector (pCRII) using a TA cloning kit (Invitrogen), the digested approximately 1.5-, 1.3-, 0.9-, and 1.1-kilobase EcoRI fragments were ligated into the response plasmid pcDNA3.1(−) (Invitrogen). A CYP26C1 constitutive expression vector was also made by the insertion of full-length CYP26C1 cDNA in frame into pcDNA3.1(−). We verified the integrity of final constructs by direct sequence analysis. All primers used to make these constructs are available by request.

Five micrograms of each plasmid was transfected into MDCK cells using FuGENE 6 reagent (Roche Diagnostics, Tokyo, Japan). G418 (Sigma, Tokyo, Japan)-resistant clones were expanded as a monoclonal population. Two weeks later, cell lines were selected to examine the strong expression of introduced gene by Northern blot analysis, and metabolic activities in each cell line were determined by measuring the metabolism of radiolabeled RA ([^{3}H]atRA and [^{3}H]9cRA; PerkinElmer Life and Analytical Sciences, Yokohama, Japan) (White et al., 1997). Because our preliminary experiments showed that observed phenotypes of the cells in the event of CYP26A1 overexpression were similar but not the same between at least three different independent clones, these cells were equally mixed to establish stably transfected cell lines to avoid possible clonal variation.

Assessment of Epithelial Permeability. We measured the transepithelial electrical resistance (TER) and paracellular flux. For TER analysis, cells were grown to confluence on Transwell membranes (pore size, 0.4 μm; Corning Life Sciences, Acton, MA) in the presence or absence of 100 nM atRA, 100 nM 9cRA, or 100 nM synthetic retinoids as a default concentration. TER was measured using an EVOM voltmeter with ENDOHM-12 (World Precision Instruments, Sarasota, FL). For calculation of resistance, the background TER of a blank filter was subtracted from the measured values, and each value was normalized by the area of the monolayer expressed in standard units of ohms per square centimeter. Paracellular tracer flux was measured with the radiolabeled molecules having two different molecular weights by using [^{14}C]inulin (molecular mass, 5 kDa) and [^{14}C]mannitol (molecular mass, 182 Da) (GE Healthcare, Tokyo, Japan). An epithelial monolayer treated with or without RA was exposed to [^{14}C]inulin or [^{14}C]mannitol in the apical compartments to adjust the radioactivity using 5×10^5 or 1×10^5 cpm/well, respectively. Samples were collected from the basolateral compartment (outer chamber) in a time-dependent manner, and the radioactivity of [^{14}C] was counted by scintillation counter (Beckman Coulter, Fullerton, CA). The paracellular flux was normalized by the value measured in blank Transwell filters. In some experiments, ketoconazole (0.5–10 μM, Sigma-Aldrich) was added 4 h before RA stimulation and then maintained throughout the experiments.

RT-PCR and Southern Blotting. After extraction of total RNA using TRIzol (Gibco BRL) reagent, total RNA (1 μg) was reverse-transcribed using MuLV reverse transcriptase (Applied Biosystems, Foster City, CA). For analysis of gene expression, the gene of interest was amplified from dilutions of cDNA using specific primers for between 15 and 40 cycles to define the optimal conditions for linearity to permit quantitative analysis of signal length. As a positive control, and to confirm that each RNA sample could yield equal amounts of product after reverse transcription reactions, amplification was also done using glyceraldehyde-3-phosphate dehydrogenase primers. Agarose gels were then transferred to Hybond-N+ membranes (Amersham Biosciences), and the blots were probed with the corresponding random-primed ^{32}P-labeled cDNA (Nippon Gene, Tokyo, Japan) with a specific activity of approximately 1×10^9 cpm/μg. For the densitometric analysis, signals in the blot were quantitated using Scion Image 1.62 (Scion Corporation, Frederick, MD).

Western Blot Analysis. Whole lysates (20 μg) extracted from the cells were run on 12% polyacrylamide gels containing SDS and electroblotted onto nitrocellulose filters. After blocking with 5% nonfat dry milk in phosphate-buffered saline, filters were immunoblotted with antibodies against occludin (Zymed Laboratories, San Francisco, CA), claudin-1 (Ishizaki et al., 2003), claudin-4 (Ishizaki et al., 2003), ZO-1 (Santa Cruz Biotechnology, Santa Cruz, CA), and β-actin (Santa Cruz Biotechnology) protein. The filters were washed extensively, reacted with corresponding peroxidase-labeled secondary antibodies, and again washed; finally, the immunoreactions were visualized by using an enhanced chemiluminescence system (Amersham Biosciences). Equal loading was demonstrated by β-actin expression.

Experimental Murine Colitis. 2,4,6-Trinitrobenzene sulfonic acid (TNBS; Sigma) colitis was induced as described previously (Morris et al., 1989). In brief, mice were sensitized by cutaneous administration of 1% TNBS on day 1 followed by intrarectal administration

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of 5 μl/g body weight of 2.5% TNBS solution on day 14. The control animals received a corresponding volume of vehicle (50% ethanol) alone. Some animals continued receiving intraperitoneal administrations of a vehicle [dimethyl sulfoxide (DMSO)], atRA (45 mg/m^2), or Am580 (3.75 mg/kg) every 2 days for 1 week. Animals received these agents as a single initial dose when TNBS was instilled intrarectally under general anesthesia. After treatment, intestinal permeability was assessed using mice that received oral gavage of FITC-dextran (4 kDa, 40 mg/kg; Sigma) 24 h before sacrificing animals. Upon death, blood was collected by cardiac puncture, and the serum was immediately analyzed for FITC-derived fluorescence (Wallac 1420; PerkinElmer Wallac, Gaithersburg, MD). For all mice, colon length, which was defined as the distance between the most distal portion of the cecum and most terminal aspect of the rectum, was measured, and histological evaluation was done to assess the histological grading of colitis (Dieleman et al., 1998). The maintenance and handling of animals were carried out using protocols approved by the Animal Care Facility of Sapporo Medical University.

Assessment of Bacterial Translocation. Spleens from mice were sonicated in phosphate-buffered saline, and small aliquots (50 μl) of tissue homogenates were inoculated onto the bacterial agar plates. Plates were incubated for at least 48 h at 37°C.

Statistical Analysis. All data represent the mean ± S.D. of at least three independent experiments, each in triplicate wells. Statistical differences were analyzed using the Mann-Whitney U test and were considered statistically significant when $p < 0.05$.

Results

atRA Regulates Barrier Function of TJ. Numerous reports have revealed that MDCK cells are an excellent tool for studying epithelial permeability because they have higher TER, abundantly express TJ-associated genes such as *claudin-1*, *claudin-4*, and *occludin*, and bear well-developed TJ strands (Furuse et al., 2001). To deplete the cells of atRA, we established an MDCK cell line, constitutively overexpressing CYP26A1, which is specifically involved in the metabolic inactivation of atRA, designated MDCK^{CYP26A1}. Cells transfected with an empty vector were used as a control (MDCK^{Vec}). We first examined the effects of atRA on MDCK cells. Treatment with atRA significantly increased TER in MDCK^{Vec} cells in a time-dependent manner; however, MDCK^{CYP26A1} cells showed basal levels of TER after atRA treatment in the range of 10 nM to 1 μM for 3 days (Fig. 1A). TER reached the maximum in control cells after 5 days in the presence of ~1 μM atRA (Fig. 1A). Unexpectedly, higher concentrations of atRA (>1 μM) increased TER even in MDCK^{CYP26A1} cells, suggesting that CYP26A1 enzymatic activity could not completely catabolize atRA contained in the media. We next measured the paracellular flux in both cell lines (Fig. 1B). Treatment with atRA also affected paracellular flux, significantly inhibiting the flux of inulin (5 kDa) but not of mannitol (182 Da). Breakdown of atRA mediated by CYP26A1 increased the permeability as measured both by inulin and mannitol. This observation suggested that a pharmacological dose of atRA regulated paracellular transport of solutes with high molecular weight but that depletion of a physiological dose of atRA induced a leaky epithelial monolayer even for solutes with small molecular weight.

Direct Relationship Between atRA Bioavailability and Epithelial Barrier Function. Ketoconazole, a broad-spectrum inhibitor of CYP26s, was next used to establish whether the effect of CYP26A1 on cells was mediated by the metabolism of atRA (Osanai and Petkovich, 2005). Ketoconazole alone did not significantly affect the values of TER and flux in MDCK^{Vec} cells, whereas treatment of MDCK^{CYP26A1} cells with ketoconazole partially increased the TER (Fig. 2A) and conversely decreased the permeability to inulin and mannitol (Fig. 2B). Ketoconazole treatment did not fully restore the TJ function in MDCK^{CYP26A1} cells to the level seen in control cells, suggesting that the period of time required for clonal expansion in RA-free conditions (due to CYP26A1 overexpression) may have limited the ability of these cells to respond to various stimuli presented in the media.

To confirm the functional relationship between the atRA bioavailability and barrier function of epithelial cells, we made various deletion mutants of CYP26A1 (Fig. 2C) with different catabolic activities of atRA (Fig. 2D). It is interesting that the cells transfected with deletion mutants of CYP26A1 showed significant abrogation in decreasing TER (Fig. 2E) and increasing paracellular flux (Fig. 2F) mediated by metabolic inactivation of atRA contained in the media, which was clearly associated with the catabolic activities of atRA. These data support our novel concept that atRA bioavailability in the cells is an important contributing factor for determining the epithelial barrier of TJs.

RARα Is an Alternative Regulator to Barrier Function. To use the substrate specificities of CYP26A1 and C1, we next investigated the role of 9cRA in barrier function. 9cRA could enhance the barrier function, and the cells with forced expression of CYP26C1 showed significant inhibition of the increasing effects of TER mediated by both atRA and 9cRA (Fig. 3A). Pretreatment with ketoconazole partially abrogated this CYP26C1-mediated effect (Fig. 3B). TER recovered ~59% in CYP26A1-expressing cells (Fig. 2A) versus ~30% in CYP26C1-expressing cells (Fig. 3B) after treatment with 5 μM ketoconazole compared with the cells without ketoconazole treatment ($p < 0.05$), which is consistent with

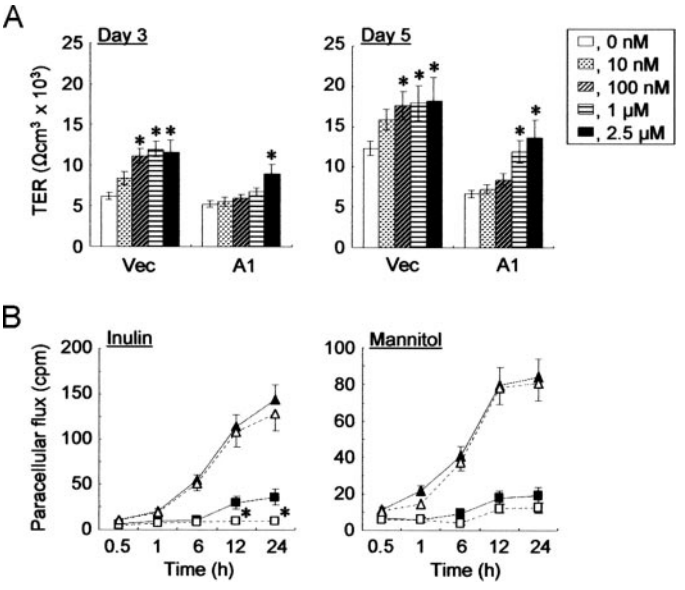

Fig. 1. Barrier function of TJ is regulated by atRA. A, TER was examined after 3 and 5 days in the presence or absence of various concentrations of atRA in MDCK^{Vec} (Vec) and MDCK^{CYP26A1} cells (A1). B, paracellular flux was measured in a time-dependent manner in the presence (open symbol and broken line) or absence (filled symbol and solid line) of 100 nM atRA for 3 days in MDCK^{Vec} (squares) and MDCK^{CYP26A1} cells (triangles), using two different radiolabeled molecules as tracers. *, $p < 0.05$ versus cells without atRA treatment.

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the previous observation that CYP26C1 was less sensitive than CYP26A1 to the inhibitory effects of ketoconazole (Taimi et al., 2004). CYP26A1 overexpression in MDCK cells did not have any effect on the 9cRA-mediated increase of TER (Fig. 3C), suggesting that RXRs activated by 9cRA were also involved in the regulation of the epithelial barrier.

We next used various types of synthetic retinoids to specifically stimulate and inhibit RAR and RXR subtypes to confirm the involvement of RARs and RXRs in the regulation of barrier function. RAR and RXR antagonists partially abrogated atRA- and 9cRA-mediated changes of permeability (Fig. 3, D and E), suggesting that both RARs and RXRs were involved in the regulation of epithelial permeability. The synthetic compounds Am80 and Am580, which are resistant to metabolism by CYP26s, were reported to stimulate RARα selectively as efficiently as atRA (Luu et al., 2001). In the dose range from 2.5 to 100 nM, a clear dose-dependence of enhancement was found even in the CYP26A1-overexpressing MDCK cells that were treated with Am80 and Am580 (Fig. 3, D and E, and data not shown). Although we could not exclude the participation of the definite RXR subtypes responsible for the modulation of barrier function due to the lack of specific agonists or antagonists for RXRs, the RARα agonists were, at lease in part, clearly involved in the regulation of barrier function.

We further examined whether atRA attenuated the epithelial damages induced by various stimuli, such as hyperthermia and oxidative stress mediated by H_2O_2. We exposed these insults to the epithelial monolayer and showed that atRA was partially but significantly able to attenuate the disruption of barrier properties of MDCK monolayer induced by these stimuli (Fig. 4, A and B). We observed similar effect on epithelial monolayer by using Am580 (data not shown), suggesting a possible feasibility of RARα-mediated therapy for diseases that are characterized by epithelial hyperpermeability.

TJ-Associated Genes Are Regulated by Cellular atRA Bioavailability. We next examined whether changes

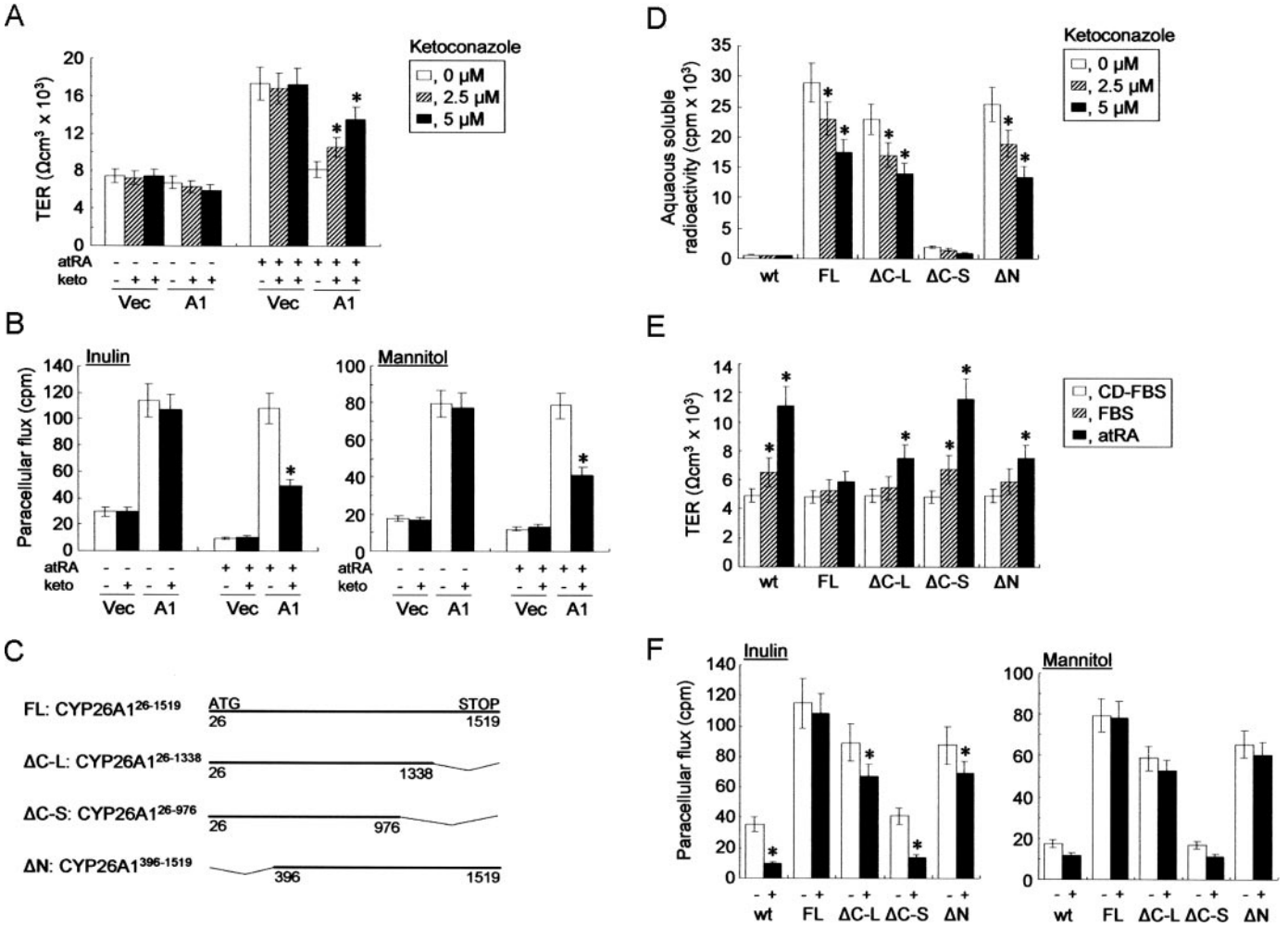

Fig. 2. Cellular RA bioavailability regulates epithelial barrier function. A and B, effects of ketoconazole on TER (A) and paracellular flux (B) in MDCK^{Vec} (Vec) and MDCK^{CYP26A1} (A1) cells after culture with or without 100 nM atRA for 3 days. C, four deletion mutants of CYP26A1 vector. D, metabolic activities of MDCK cells transfected with each CYP26A1 construct with various deletions using [^{3}H]atRA as a substrate. CYP26A1-mediated catabolic activity converted radioactive atRA to water-soluble metabolites and was suppressed by pretreatment with ketoconazole. E and F, TER (E) and paracellular permeability (F) was examined in MDCK cells transfected with each deletion mutant of CYP26A1 treated with (+) or without (−) 100 nM atRA for 3 days. In measuring TER, before treatment with atRA, the cells were changed to a medium supplemented with 10% charcoal-dextran FBS (CD-FBS), allowing us to exclude the possible effects of physiological concentrations of atRA contained in the medium. The samples for paracellular flux were collected after 24 h in the presence of [^{14}C]inulin or [^{14}C]mannitol (B and F). *, $p < 0.05$ versus cells without ketoconazole (A, B, and D) or atRA (E and F) treatment.

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in barrier function regulated by RA bioavailability in the cell were accompanied by gene-expression alterations of TJ-associated genes. We first examined the quantitativeness of our method and found that the threshold cycling parameter for saturation of band intensity showed a clear inverse proportion to the template (Fig. 5A), indicating that our assay was sensitive to examine the quantification of mRNA expression. Due to the limited availability of the canine cDNA sequence of TJ-associated genes, we analyzed genes known to be cloned such as *occludin*, *claudin-1*, *claudin-4*, and *ZO-1* (Fig. 5B). Treatment with atRA significantly up-regulated these genes in a time-dependent manner, and CYP26A1-mediated depletion of atRA partially abrogated this induction. Although the basal levels of gene expression were different between MDCK^{Vec} and MDCK^{CYP26A1} cells, there was evidence that normal FBS contained a physiological dose of atRA, which was sufficient for gene-expression of the TJ-associated genes and functions of TJ (Fig. 2E). In addition, the TJ protein kinetics in the event of atRA treatment was consistent with the gene-expression alterations of TJ-associated genes (Fig. 5C).

Treatment with small interfering RNA targeted to *occludin* did not change the epithelial permeability in MDCK monolayer (data not shown), which was consistent with the evidence that the claudin family is the main constituent for TJ, rather than occludin (Tsukita et al., 2001; Matter et al., 2005). However, it is agreeable that increased expression of TJ-associated genes potentially associated with the RA-mediated enhancement of barrier function, because of our previous study demonstrating the direct mechanistic link between atRA-mediated inductions of TJ-associated genes and the formation of functional TJs in F9 cells (Kubota et al., 2001).

RA-Mediated Enhancement of Epithelial Barrier Protects Mice from Colitis. Because RARα is an important determinant for epithelial barrier in vitro, we used TNBS-induced murine experimental colitis to examine the clinical relevance of RARα agonists. We first measured the body weight, because weight loss is a reliable method to determine the severity of TNBS colitis (Morris et al., 1989). Control mice lost body weight rapidly and failed to regain the weight during the course of the 7-day experiment, whereas atRA-

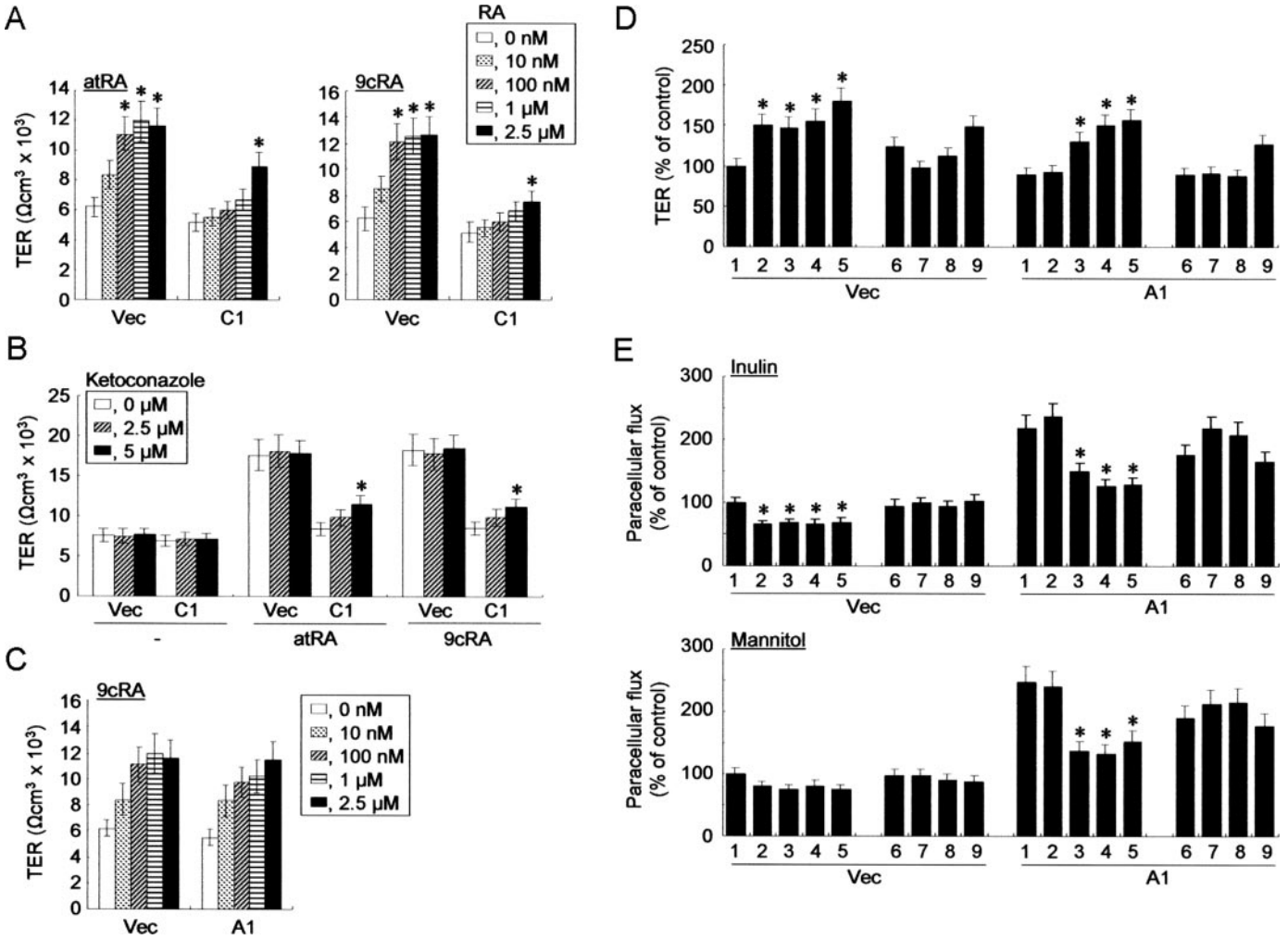

Fig. 3. Preferential interaction between RARα and epithelial barrier function. A to C, TER was examined after treatment with 100 nM atRA or 100 nM 9cRA for 3 days, or various concentrations of ketoconazole in MDCK^{Vec} (Vec), MDCK^{CYP26A1} (A1), and MDCK^{CYP26C1} (C1) cells. D and E, effects of various synthetic retinoids on TER (D) and paracellular flux (E) in MDCK^{Vec} (Vec) and MDCK^{CYP26A1} (A1) cells after culture with or without 100 nM synthetic retinoids for 3 days. The samples for paracellular flux were collected after 24 h in the presence of tracers. Lane 1, vehicle (DMSO); lane 2, atRA; lane 3, 9cRA; lane 4, Am80 (RARα agonist); lane 5, Am580 (RARα agonist); lane 6, atRA+LE135 (RARα+β antagonist); lane 7, atRA+HX531 (pan-antagonist of RARs and RXRs); lane 8, 9cRA+HX531; lane 9, 9cRA+PA452 (RXR pan-antagonist). *, $p < 0.05$ versus cells without RA treatment.

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and Am580-treated animals did not show significant weight loss (Fig. 6A). Likewise, the length of the colon, which shortens as a result of more severe inflammation, was significantly different between the control and RA-treated groups, in which animals treated with the vehicle showed significant shortening of the colon, whereas those treated with atRA and Am580 did not display severe shortening (Fig. 6B). In addition, as assessed by 4-kDa FITC-dextran, treatment with atRA and Am580 significantly abrogated the increase in intestinal permeability observed in mice with vehicle treatment (Fig. 6C). Furthermore, bacterial translocation, a measure of intestinal barrier integrity, was found in the spleens of TNBS-treated animals and was correlated with the permeability of FITC-dextran (Fig. 6D). Am580 was more effective for these parameters than atRA. Histologically, the colons were characterized by extensive ulceration with severe mucosal inflammation, multifocal dropouts of entire crypts in all parts of the colon, and extensive edema in vehicle-treated mice; however, mucosal injuries were markedly attenuated by the treatment with atRA and Am580, with slight amounts of inflammatory infiltrates, mucosal surface erosion, and various degrees of regenerative changes without crypt depletion (Fig. 6E). Histological grading of colitis clearly demonstrated these differences between mice treated with RA and untreated control animals (Fig. 6F), suggesting that RA served an important barrier-protective mechanism for the severity of colitis by regulating epithelial integrity.

Discussion

Here, we demonstrated that several types of RA, including RARα stimulants, promoted the epithelial barrier function in vitro and could reduce the breakdown of the gastrointestinal barrier in vivo. In addition, metabolic depletion of RAs in the cells was clearly proportional to the epithelial barrier dysfunction, suggesting that the cellular RA bioavailability regulates the epithelial barrier accompanied by gene-expression alterations of TJ-associated genes. Whereas the establishment and regulation of epithelial permeability is the end result of a cascade of events triggered by cell-cell interactions, our present study provides significant evidence that cellular RA bioavailability is an important contributing factor for determining the epithelial integrity.

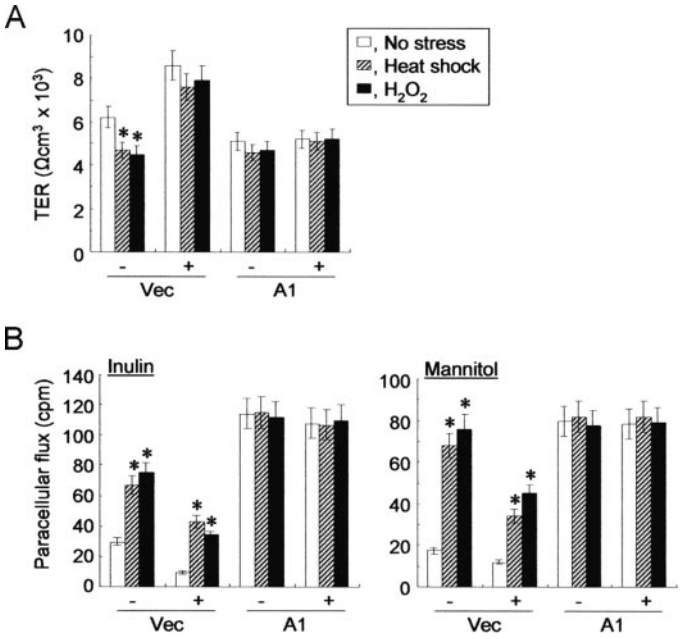

Fig. 4. atRA attenuates the epithelial hyperpermeability induced by hyperthermia and H_2O_2. A and B, effects of atRA on TER (A) and paracellular flux (B) in $MDCK^{Vec}$ (Vec) and $MDCK^{CYP26A1}$ (A1) cells after culture with (+) or without (−) 100 nM atRA for 3 days. These cells were also treated with cell stressors including heat shock at 42°C for 1 h and 10 μM H_2O_2 for 24 h. *, $p < 0.05$ versus cells without cell stressor.

This study focused on mechanisms of the mucosal barrier protection during intestinal inflammation. There seems to be a logical gap between in vitro experiments carried out with MDCK cells and the in vivo study on the experimental colitis in mice. Accumulated evidence has demonstrated that MDCK cell represents an excellent model to study general properties of epithelial barriers; however, there is still a point of controversy whether the MDCK data are applicable to the intestinal epithelium. Our preliminary experiment suggested that the intestinal cell lines such as T84 did not show significant increase of barrier function after the treatment with atRA. Although we cannot explain this specific underlying mechanism(s), one possibility is based on the cellular property showing that T84 cells abundantly express endogenous CYP26A1 mRNA, which is highly inducible by the treatment with atRA (our unpublished observation). Endogenous CYP26A1 activity in T84 cells enables us to show the evidence of whether the exogenously applied atRA would modulate the epithelial permeability. In addition, we should note here that the commercially available intestinal cell lines are originated from cancer tissues that have lost the physiological intestinal barrier function in the carcinogenesis. This is also supported by the fact that the cancer cells can hardly be said to faithfully mimic normal biology, in addition to the fact that their genomes are notoriously diverse and poorly characterized. One would thus be likely to accept that MDCK cells were appropriate for this study rather than cancer-derived intestinal cells.

Intestinal mucosa has crucial functions for regulating intestinal homeostasis by strictly separating the subepithelial compartment from potentially noxious luminal compounds. Intestinal barrier dysfunction initiated by various etiologies is a main contributing factor in several pathological conditions involving the gastrointestinal tract. Increased TJ permeability provides a major site for both infection and establishment of inflammatory responses in the gut. Bacterial translocation is, for example, the passage of viable bacteria and nonviable bacteria such as endotoxins and/or bacterial DNA are believed to be made through the intestinal lumen to extraintestinal sites via a paracellular pathway between the epithelial cells, even before massive disruption of the epithelial TJ-based barrier (Van Leeuwen et al., 1994). Our present study with TNBS colitis revealed a potentially central role for RA as an endogenous regulator of inflammatory colitis, providing a significant insight into the molecular details linking retinoid action to the pathology of diseases with gut hyperpermeability.

Given the complexity of the cell signaling events regulating epithelial permeability (Gordon et al., 2005; Prasad et al., 2005), it is not surprising that RAs modulate various sets of genes, including *occludin* and *claudins*. Whereas a previous report has demonstrated that the human occludin promoter is down-regulated by inflammatory cytokines such as tumor

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necrosis factor α and interferon-γ, which may be an important mechanism in gastrointestinal diseases accompanied by barrier defects (Mankertz et al., 2000), this is the report demonstrating that RARα stimulation preferentially could enhance the expression of TJ-associated genes such as *occludin*. This mechanism is partially explained by our preliminary study of the occludin promoter using approximately 2.5-kilobase upstream from the transcription initiation site, suggesting that RARα agonists stimulate *occludin* expression at the transcriptional level (our unpublished results). We cannot conclude from our present study whether the RA effect is direct on the epithelial integrity, whereas our observation may support the plausible hypothesis that strategies that increase RA bioavailability in epithelial cells may be promising to ameliorate intestinal morbidity in many intestinal disorders in which elevated levels of cytokines result in gut barrier dysfunction. This is consistent with epidemiological studies showing that vitamin A supplementation significantly reduces the childhood mortality caused by the persistent diarrhea with inflammatory bowel diseases (Sommer et al., 1986; McCullough et al., 1999).

Previous reports have shown the functional RA deficiency in a variety of tissues in pathological settings and demonstrated that RA depletion drives the opening of paracellular pathways in epithelial cells, as observed in cancer cells. Our results suggest that impaired RA signaling caused by RA depletion leads to the disruption of functional TJs. It is thus interesting to speculate that strategies designed to enhance RA signals and/or blockade RA catabolism may be of therapeutic value in intestinal diseases that have increased gut permeability. However, in short-term responses to infection, increased permeability may have a protective function in the gut (Grencis and Bancroft, 2004). Better understanding of the mechanisms that regulate epithelial integrity may provide valuable insights into various intestinal disorders (McCullough et al., 1999). It is interesting that altered gut permeability may not be simply due to TJ-associated gene-expression alterations but also to deregulated phosphorylation, disruption of distribution, and vacuolization of TJ proteins (Clayburgh et al., 2004; Fasano and Shea-Donohue, 2005). In addition, TJ proteins have controversial effects on the cells showing that overexpression of a certain type of claudin in MDCK cells paradoxically increases paracellular permeability (Furuse et al., 2001). This evidence suggests

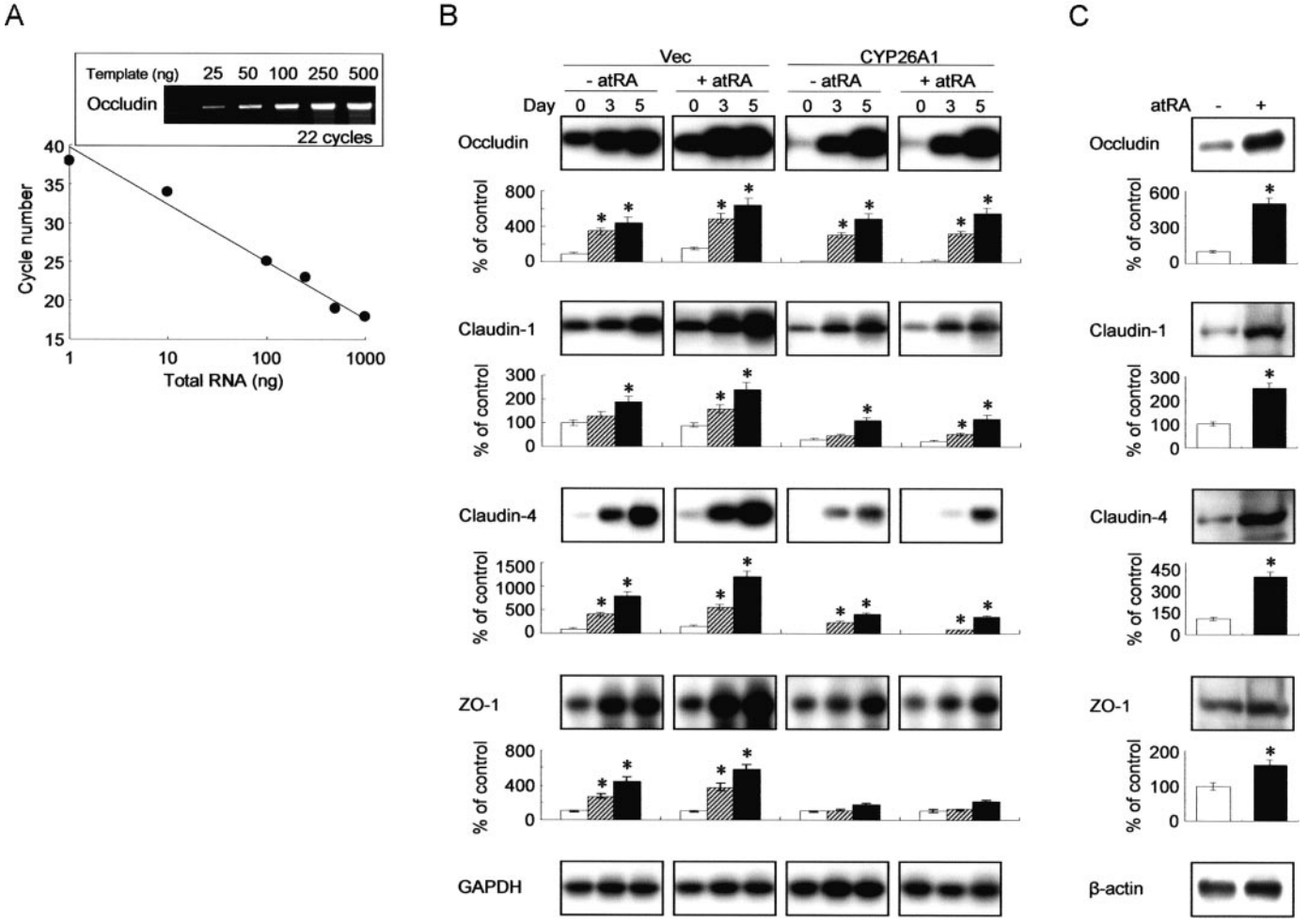

Fig. 5. Cellular atRA bioavailability associates with the expression of TJ-associated genes. A, threshold cycling parameter to saturate band intensity to the various amounts of RNA template. When we amplified cDNA reverse-transcribed from various amounts of known concentrations of RNA, a clear correlation was observed between the amount of the template and signal intensity after PCR reaction. B, semiquantitative RT-PCR-Southern blot analysis to examine the changes of TJ-associated genes in MDCK^{Vec} (Vec) and MDCK^{CYP26A1} (CYP26A1) cells after culture with or without 100 nM atRA for up to 5 days. C, Western blot analysis of TJ proteins in MDCK^{Vec} cells after culture with or without 100 nM atRA for 3 days. $*$, $p < 0.05$ versus cells without RA treatment.

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that the functional TJ is governed by complex regulation in response to a wide variety of pathological insults, and future work may reveal more detailed mechanisms explaining how RAs maintain epithelial integrity and potentially attenuate mucosal damage.

Vitamin A is especially critical for gut immunity, and its deficiency results in deregulated production of cytokines such as interferon-γ and impaired antibody responses (Cantorna et al., 1994). Because the intestinal mucosa is an enriched source of cytokines that are secreted from various immunological cell types, the role of the immune system is likely to generate an aggressive physiological response to the imbalance induced by the barrier dysfunction because intestinal epithelial cells interface directly with various potentially harmful antigens (Strober et al., 2003). A number of studies have shown that RA attenuates inflammation-related epithelial damage in mouse colon (Atreya et al., 2000; Gordon et al., 2005). Although it is impossible to conclude whether such attenuation is due to nonspecific anti-inflammatory effects of RA or whether it is determined by a direct protective action of RA on mucosal epithelium, we clearly demonstrated that RARα stimulation was able to attenuate the disruption of barrier properties of MDCK monolayer induced by the pathological insults such as oxidative stress and hyperthermia, providing a logical connection between in vitro experiments and the in vivo study. It is therefore noteworthy to expect that high RA bioavailability in the intestinal microenvironment preferentially affects gut epithelium, resulting in a reduced incidence of life-threatening epithelial hyperpermeability of the gut.

It is clear that the peroxisome proliferator-activated receptor γ (PPARγ) ligands such as n-3 polyunsaturated fatty acids play an anti-inflammatory role for modulating mucosal immunity in an animal model of inflammatory bowel disease (Su et al., 1999; Rogler, 2006) but also that RAR activation may compete with RXR, decreasing the activation of PPARγ/RXR heterodimer. Although this potential competition and paradoxical therapeutic effects of RAR and PPARγ activation after vitamin A and n-3 polyunsaturated fatty acids supplementation should be addressed in the future study, one possible explanation is that the ligand-mediated gene expression seems to be made depending on the cell type, pharmacokinetics of the ligands, relative abundance of nuclear receptors, extent of the competition or cross-talk among nuclear transcription factors, and the modulating role of coactivators and corepressors on ligand-dependent transcription. In addition, it is unlikely that only a limited amount of transcriptional machinery and signaling is available in a given tissue. Whether definite nuclear receptor(s) responsible for maintaining the intestinal microenvironment remains to be exam-

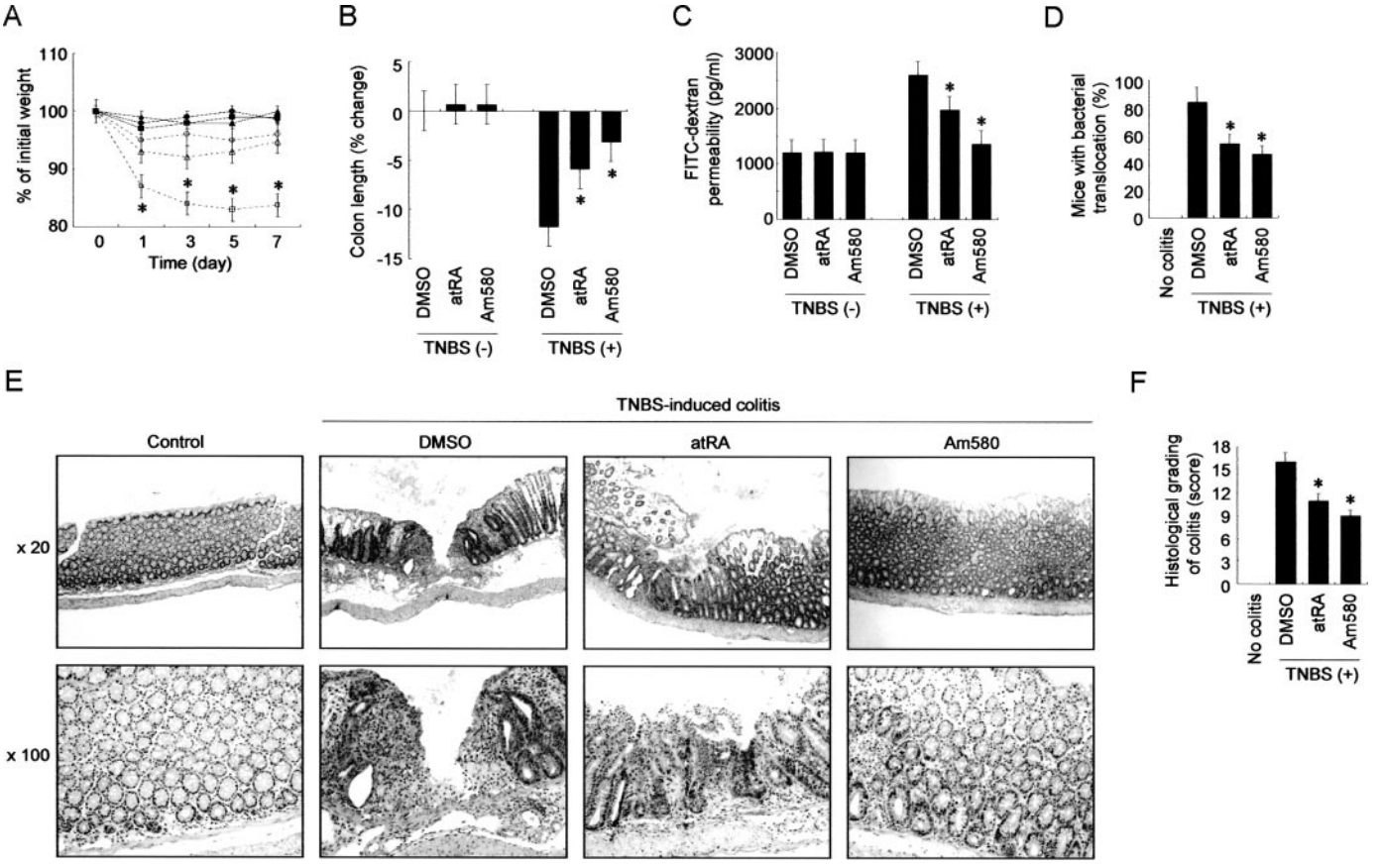

Fig. 6. RARα stimulation attenuates the loss of intestinal barrier during TNBS-induced colitis. A, changes of body weight after the induction of TNBS colitis. Compared with the control group (no colitis; solid line and filled symbols, $n = 3$ in each group), treatment group (broken line and open symbol) with vehicle (square, $n = 6$) displayed a more severe clinical course with significant loss of body weight. atRA (triangle, $n = 8$) and Am580 (circle, $n = 8$)-treated groups, however, did not show significant body weight loss over the course of the experiment. B, colon length relative to control animals 7 days after treatment. C, quantification of serum FITC-dextran as a measure of intestinal barrier function. D, incidence of bacterial translocation to the spleen. E, histological sections of mouse colon treated with vehicle (DMSO), atRA, and Am580 compared with the control animals (no colitis). Treatment with atRA and Am580 significantly protects colon from deep ulceration and severe mucosal inflammation. F, histological grading of colitis assessed by the scoring method. $*$, $p < 0.05$ versus mice without RA treatment.

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ined, RARα is, at least in part, an important determinant for the epithelial integrity.

Morphological examinations using immunohistochemistry insufficiently represented the observed differences of barrier functions between control and RA-treated MDCK cells in animals with colitis as well. This observation supports a concept proposed previously that barrier integrity can be significantly compromised with no observable changes in morphological architecture (Karhausen et al., 2004). Another example is presented by the observation that bacterial colonization of the proximal gut leads to a leaky intestine even without apparent mucosal damage (Asfaha et al., 2001). Alternatively, a possible explanation has emerged from the accumulated evidence that MDCK cells display highly differentiated cellular phenotypes, expressing abundant endogenous TJ-associated proteins in cell-cell junctions (Furuse et al., 2001).

Our present study indicates that RA provides barrier-protective elements to the epithelial cell in inflammatory mucosal injuries. Studies along this line will help us to understand the pathogenesis of diseases characterized by the disruption of epithelial integrity. We believe that a certain type of RA is a promising agent for modulating the TJ function in various diseases with gastrointestinal hyperpermeability.

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