Inducible nitric oxide synthase-dependent DNA damage in mouse model of inflammatory bowel disease

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Increased cancer risk occurs in inflammatory bowel disease (IBD) undergoing long-term chronic inflammation. To evaluate whether inducible nitric oxide synthase (iNOS)-dependent DNA damage plays a role in the carcinogenic process triggered by IBD, we prepared a mouse model of IBD induced by transfer of CD45RB^{high}CD4$^+$ T cells lacking regulatory T cells to female severe combined immuno-deficiency (SCID) mice. CD45RB^{high}CD4$^+$ T cells were isolated from mouse spleen after staining with fluorescein isothiocyanate (FITC)-conjugated anti-CD45RB monoclonal antibody, followed by anti-FITC-conjugated microbeads. This IBD mouse model showed that the bodyweight increased with aging to a lesser extent than non-treated controls, and that the intestine was shortened. Pathological findings of this mouse model, which showed severe inflammation in colon tissues, were similar to IBD patients. Double immuno-fluorescence technique revealed that both 8-nitroguanine and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) were formed mainly in epithelial cells of the IBD mouse model. 8-Nitroguanine was formed in most of 8-oxodG-immunoreactive nuclei of epithelial cells. iNOS, proliferating cell nuclear antigen and p53 protein were also expressed in the colon epithelium. These results indicate that nitrative DNA damage, as well as oxidative DNA damage, is induced in colon epithelial cells of the IBD mouse model followed by proliferation of these cells, which may contribute to colon carcinogenesis. (Cancer Sci 2005; 96: 157–163)

Ulcerative colitis and Crohn's disease are well known as chronic inflammatory diseases in the lower bowel, and share many clinical and pathological characteristics. These diseases are referred to as inflammatory bowel disease (IBD), which leads to long-term impairment of intestinal structure and function.[1] A large number of immunological abnormalities have been noted in patients with IBD.[2] It is well established that an increased cancer risk occurs in tissues undergoing chronic inflammation. Many cancers arise from sites of infection, chronic irritation and inflammation.[3] Epidemiological studies have suggested that the incidence of colorectal cancer in IBD is greater than the expected incidence in the general population.[4–6] The histological and molecular signatures suggest an inflammation-driven carcinogenesis process in IBD patients. This detailed mechanism is unclear, although a recent report has shown that NF-κB functions as a tumor promoter in inflammation-associated cancer.[7]

NF-κB can regulate genes like inducible nitric oxide synthase (iNOS).[8] Nitric oxide (NO) and reactive oxygen species (ROS) are considered to play the key role in inflammation-mediated carcinogenesis. ROS can induce the formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), which is an indicator of oxidative DNA damage.[9–12] The level of 8-oxodG has been found to significantly increase in IBD patients[13] and animal models.[14] NO can mediate the formation of 8-nitroguanine, a marker of nitrative DNA damage.[15,16] Akaike et al. reported that

8-nitroguanine is formed in ribonucleic acid by viral infection as a biomarker of inflammation.[17] In addition, 8-nitroguanine formed in DNA has been proposed to account for inflammation-associated carcinogenesis.[9,18–20] Therefore, the accumulation of 8-nitroguanine, as well as 8-oxodG formation, may play a key role in the initiation and/or promotion of IBD-mediated carcinogenesis. However, whether 8-nitroguanine is formed in the colonic epithelium in IBD has not yet been determined.

To evaluate whether nitrative DNA damage plays a role in the carcinogenic process triggered by IBD, we prepared a mouse model of IBD induced by transfer of CD45RB^{high}CD4$^+$ T cells lacking regulatory T cells to severe combined immunodeficiency (SCID) mice,[21,22] since mouse models of IBD can result from either excessive effector T cell function or deficient regulatory T cell function.[2] We performed a double immunofluorescent staining procedure to examine the formation of 8-nitroguanine and 8-oxodG in the colon tissues. We also examined the expression of iNOS by immunohistochemistry. To evaluate the proliferating activity of colonic epithelial cells and their response to DNA damage, we examined the expression of proliferating cell nuclear antigen (PCNA) and p53 in the colon tissues.

Materials and Methods

Production of anti-8-nitroguanine antibody. As described previously, a polyclonal antibody against 8-nitroguanine was produced.[23] Briefly, the 8-nitroguanine-aldehyde-rabbit serum albumin conjugate was mixed with Freund's complete adjuvant and injected into a rabbit. We obtained the serum and purified the antibody by affinity chromatography. The specificity of this purified antibody was confirmed by a dot immunobinding assay and absorption test.[23]

Induction of IBD in SCID mice by adoptive transfer of CD45RB^{high}CD4$^+$ T cells. Female BALB/c and SCID mice (C.B-17) of the BALB/c background were obtained from CLEA JAPAN (Osaka, Japan), and used at >6 week of age. Mice were maintained at the Institute of Laboratory Animal of Life Science Center, Mie University. The animal experiments were conducted according to the guidelines of Mie University School of Medicine. Single cell suspensions of spleen from BALB/c mice were enriched for CD4$^+$ T cells by negative selection using a CD4 isolation kit (Miltenyi Biotec, Bergisch, Germany). CD45RB^{high}CD4$^+$ T cells were positively selected from the resultant CD4$^+$ T cell preparation using AutoMACS (Miltenyi Biotec) after staining with FITC-conjugated anti-CD45RB monoclonal antibody (mAb) 16A (PharMingen, San Diego, CA, USA) followed by anti-FITC-conjugated microbeads (Miltenyi Biotec), according to the manufacturer's instruction. For induction of

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IBD, four C.B-17 SCID mice were injected intraperitoneally with purified CD45RB^{high}CD4$^+$ T cells (4×10^5 cells/mouse) in phosphate-buffered saline as described previously.[21] Four non-treated C.B-17 SCID mice were used as the control. Mice were observed daily and weighed weekly. All mice were killed 12 weeks later. Under deep anesthesia, the mice were transcardially perfused with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Colon tissues were embedded in paraffin and cut into 5 μm thick sections. The sections were routinely stained with hematoxylin and eosin (HE) for histopathological examination, or used for immunohistochemical analyses.

Light microscopic immunohistochemistry. As described previously, 8-nitroguanine and 8-oxodG immunoreactivity in the colon tissues was assessed by double immunofluorescence labeling study.[24] Sections were incubated with rabbit polyclonal anti-8-nitroguanine antibody (2 μg/mL) and mouse anti-8-oxodG mAb (4 μg/mL; Japan Institute for the Control of Aging, Fukuroi, Japan) overnight. The sections were then incubated for 3 h with Alexa 488-labeled goat antibody against rabbit immunoglobulin G (IgG) and Alexa 594-labeled goat antibody against mouse IgG (1:400; Molecular Probes, Oregon, USA). The stained sections were examined under an inverted Laser Scan Microscope (LSM 410; Zeiss, Gottingen, Germany). In a certain experiment, to demonstrate 8-nitroguanine formation in DNA more clearly, tissue sections were pretreated with 10 μg/mL ribonuclease (RNase) at 37°C for 1 h according to the method described by Tsuruya *et al*.[25]

As described above, the expression of iNOS, PCNA and p53 were also assessed by double immunofluorescence labeling study with 8-nitroguanine. Anti-PCNA (1:200; Novocastra Laboratories Ltd, Newcastle-upon-Tyne, UK), anti-iNOS (1:1000; Sigma, St Louis, MO, USA) and anti p53 (1:100; Novocastra Laboratories Ltd) mAbs were used as primary antibodies.

Electron microscopic immunohistochemistry. The colon tissues were postfixed in 2% glutaraldehyde in 0.1 M phosphate buffer for 3 h, and sectioned by a microslicer (D.S.K., Kyoto, Japan) in 100 μm thickness for electron microscopic immunohistochemistry. Staining by the postembedding immunogold method was performed as described previously.[26] The sections were embedded in Epon 812, and 75 nm thick ultra-thin sections were cut. The ultra-thin sections were incubated with 0.3% H_2O_2 in Tris-buffered saline (TBS) for 7 min, rinsed in 0.1% solution of bovine serum albumin in TBS (BSA-TBS) for 10 min, and incubated with 2% normal goat serum for 30 min. The sections were incubated with anti-8-nitroguanine antibody for 6 h at room temperature, rinsed twice in BSA-TBS for 5 min, and incubated with goat antibody against rabbit IgG (diluted 1:40 in BSA-TBS) and coupled to colloidal gold (15 nm; Janssen Life Sci. Prod., Beerse, Belgium), anddiluted 1:40 in BSA-TBS for 3 h. The sections were counter-stained with uranyl acetate for 15 min and 1% lead citrate for 2 min. Control staining was made by omission of 8-nitroguanine antibody.

Results

Immunologic IBD mouse model. We isolated CD45RB^{high}CD4$^+$ T cells from CD4$^+$ T cells, obtained from mouse spleen, using FITC-conjugated anti-CD45RB mAb and anti-FITC-conjugated microbeads. The fluorescence intensity of CD45RB on CD4$^+$ T cells was widely distributed (Fig. 1a), whereas isolated CD45RB^{high}CD4$^+$ T cells showed a single peak (Fig. 1b). This result suggests that CD45RB^{high}CD4$^+$ T cells were successfully isolated from mouse spleen.

Mice transferred with CD45RB^{high}CD4$^+$ T cells showed an increase in bodyweight with aging to a lesser extent than non-treated controls (Fig. 2). Bodyweight of the mice transferred with CD45RB^{high}CD4$^+$ T cells was significantly lower than that of non-treated controls at 12 weeks ($P < 0.05$). Seven weeks
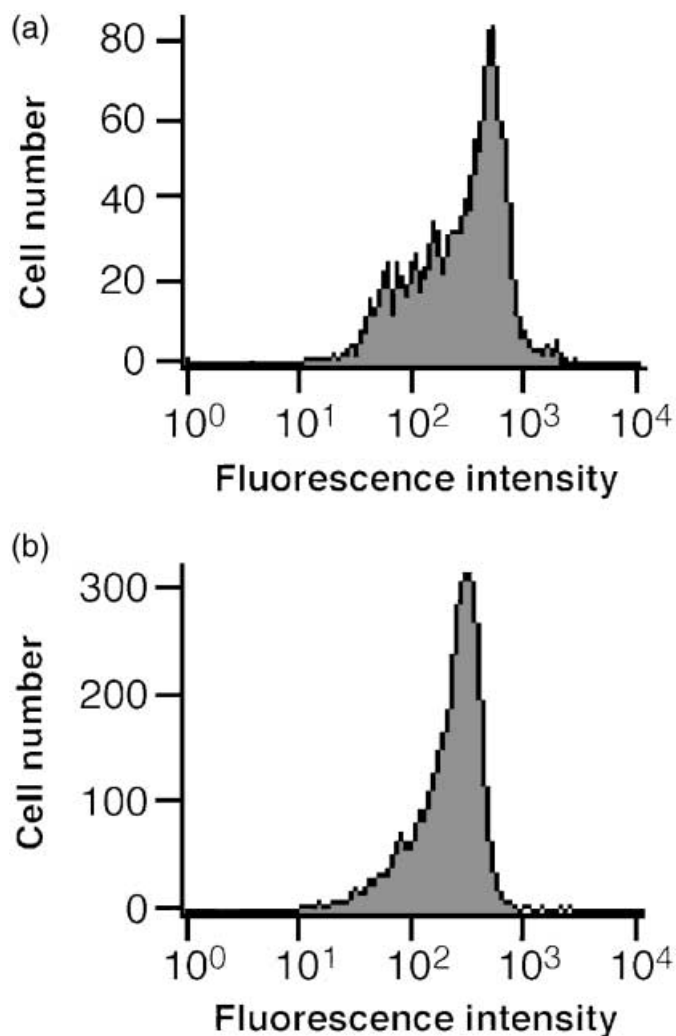

Fig. 1. Flow cytometric analysis of CD45RB on total CD4$^+$ T cells and isolated CD45RB^{high}CD4$^+$ T cells. CD45RB^{high}CD4$^+$ T cells were positively selected from CD4$^+$ T cells obtained from BALB/c mice spleen after staining with fluorescein isothiocyanate (FITC)-conjugated anti-CD45RB monoclonal antibody, followed by anti-FITC-conjugated microbeads. (a) CD45RB on CD4$^+$ T cells (before isolation), and (b) isolated CD45RB^{high}CD4$^+$ T cells. The horizontal axis shows the fluorescence intensity, and the vertical axis shows the cell number.

after CD45RB^{high}CD4$^+$ T cell transfer, mice developed clinical signs of colitis, including diarrhea and weight loss. This IBD mouse model showed that the intestine was shortened compared with non-treated controls.

Histopathological analysis of the IBD mouse model. The colon tissues of mice injected by CD45RB^{high}CD4$^+$ T cells showed chronic inflammatory response (Fig. 3). Open ulcers, which were characterized by sloughing of epithelial cells and increased transmigration of leukocytes in the lamina propria, was observed in the colon tissues (Fig. 3a). Regenerated epithelial cells with mild dysplasia and goblet cell depression, besides remarkable subepithelial fibrosis, were observed in the epithelium (Fig. 3b). Some inflammatory cells infiltrated into the epithelial cell layer (Fig. 3c). Many various inflammatory cells, such as neutrophils, macrophages, plasma cells (Fig. 3d) and lymphocytes (Fig. 3e), were permeated lamina propria.

Accumulation of 8-nitroguanine, 8-oxodG and expression of iNOS in colon tissues of the IBD mouse model. In the CD45RB^{high}CD4$^+$ T
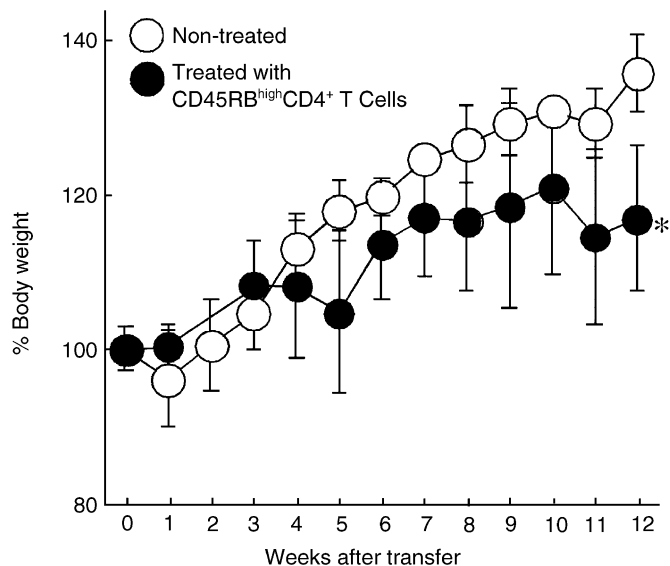
Fig. 2. Change in bodyweight of the mice in the experiment duration. Bodyweight of the mice transferred with CD45RB^{high}CD4^{+} T cells (●) was significantly lower than that of non-treated controls (○) at 12 weeks ($n = 4$, *$P < 0.05$).

cell-transferred mouse, significant 8-nitroguanine accumulation was induced in the nuclei and the cytoplasm of epithelial cells, and was also present in infiltrated cells supposed to be inflammatory cells in lamina propria (Fig. 4a,d). 8-Nitroguanine was formed in most of 8-oxodG-immunoreactive nuclei of epithelial cells and infiltrated cells (Fig. 4b,c). When the sections were pretreated with RNase, 8-nitroguanine immunoreactivity was more clearly observed in the nuclei of epithelial cells (Fig. 4g). This result suggests that 8-nitroguanine was formed in genomic DNA. iNOS was expressed in the cytoplasm of epithelial cells and infiltrated cells in the lamina propria of the IBD mouse model (Fig. 4e). iNOS was expressed mainly in 8-nitroguanine immunoreactive epithelial cells (Fig. 4f). However, no or little 8-nitroguanine, 8-oxodG and iNOS were observed in non-treated control mice (Fig. 4h–j).

Electron microscopic immunohistochemistry further confirmed that 8-nitroguanine was accumulated mainly in the nucleus of the colon epithelial cells (Fig. 5). Immunogold particles were spread in the cytoplasm, and weak immunoreactivity was observed in mitochondria. No immunoreactivity was observed when 8-nitroguanine antibody was omitted (data not shown).

Expression of PCNA and p53 in colon tissues of the IBD mouse model. In the IBD mouse model, intense PCNA was expressed mainly in the nuclei of many glandular epithelial cells, particularly in the lower part of the glands, whereas 8-nitroguanine localized
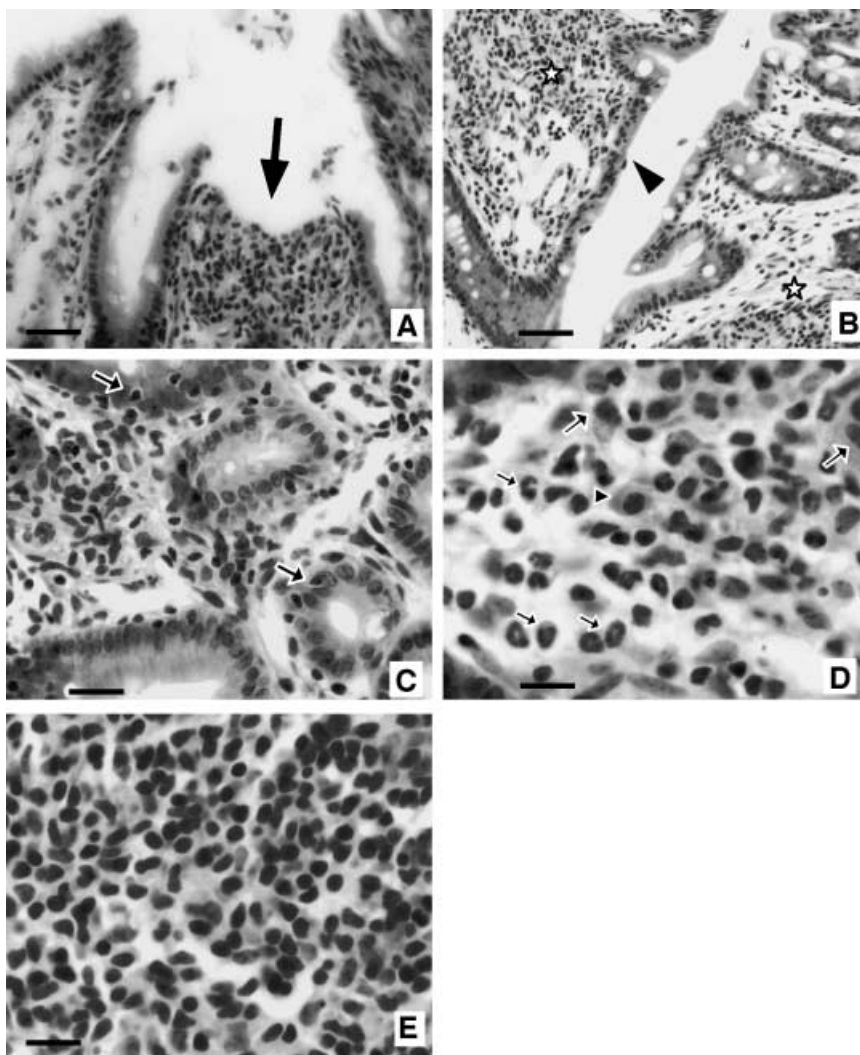
Fig. 3. Pathological findings in the colon of CD45RB^{high}CD4^{+} T cell-transferred mice (hematoxylin–eosin staining). (a,b) Low power view of colon mucosa. (a) Open ulcer (arrow), which is characterized by sloughing of epithelial cells and leukocyte infiltration in lamina propria is observed. (b) Regenerated epithelial cells with mild dysplasia and goblet cell depression are prominent (arrowhead). Marked or moderate inflammatory cell infiltration and fibrosis are apparent in subepithelial tissue (asterisk). (c) Inflammatory cell infiltration in epithelial cell layer (arrow). (d,e) High power view of infiltrated inflammatory cells in lamina propria. (d) Many neutrophils (small arrow), plasma cells (arrowhead), macrophages (large arrow), and (e) lymphocytes permeate in lamina propria layer. Bars: b, 50 μm; a,c, 25 μm; d,e, 10 μm.
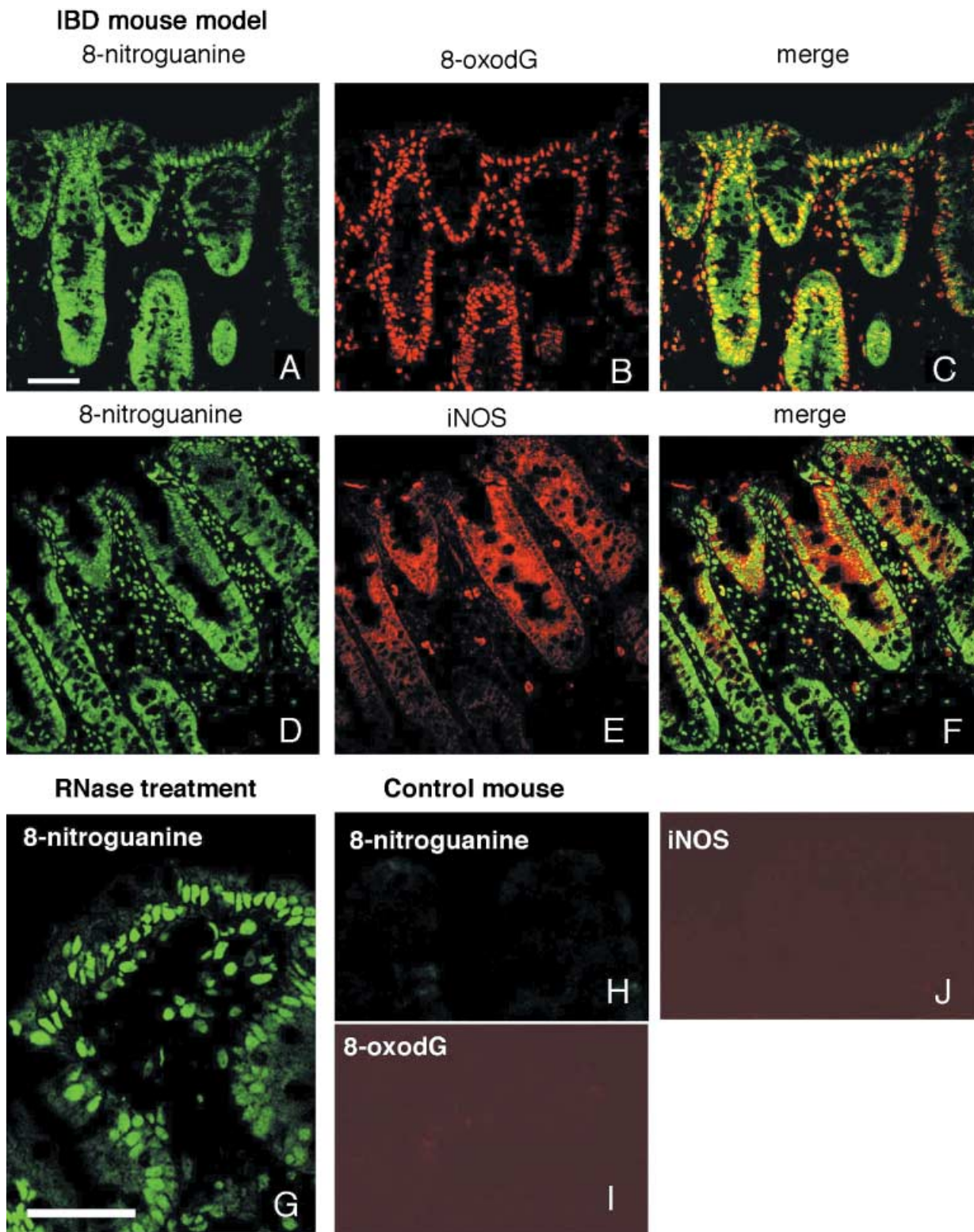
Fig. 4. Formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), 8-nitroguanine and inducible nitric oxide synthase (iNOS) in the mouse model of inflammatory bowel disease. (a,d) 8-Nitroguanine is accumulated in the nuclei and the cytoplasm of epithelial cells. 8-Nitroguanine is also present in infiltrated cells. (b) 8-oxodG formation is observed in the nuclei of epithelial cells and the infiltrated cells in lamina propria, (c) 8-oxodG and 8-nitroguanine co-localized in the nuclei of most epithelial cells and infiltrated cells, (e) iNOS is expressed in the cytoplasm of epithelial cells and infiltrated cells in lamina propria, and (f) iNOS and 8-nitroguanine colocalized in the cytoplasm of many epithelial cells and some infiltrated cells. The colon tissue sections were pretreated with ribonuclease and then immunohistochemical analysis was performed. (g) 8-Nitroguanine immunoreactivity is more clearly observed in the nuclei of epithelial cells. (h) Little or no immunoreactivity of 8-nitroguanine, (i) 8-oxodG, and (j) iNOS was observed in control group mice. Scale bars: 50 μm.
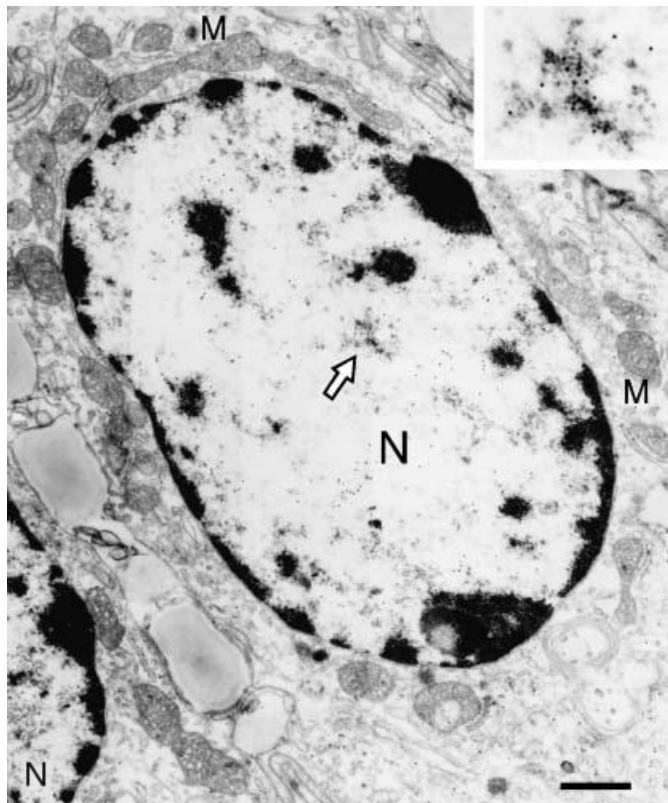
Fig. 5. Immunoelectron micrograph of 8-nitroguanine immunoreactivity in colon epithelial cell of the mouse model of inflammatory bowel disease. 8-Nitroguanine is accumulated mainly in the nucleus (N) of the colon epithelial cells. Immunogold particles were spread in the cytoplasm, and weak immunoreactivity was observed in mitochondria (M). *Inset*, the magnification of the area indicated by an arrow. Scale bar: 1 μm.

more widely in the mucosa. PCNA was expressed partially in the nuclei of 8-nitroguanine-immunoreactive epithelial cells (Fig. 6a–c). In the IBD mouse model, significant p53 was accumulated in the nuclei of regenerated epithelial cells. p53 was also expressed in some infiltrated cells. p53 expression was overlapped with 8-nitroguanine (Fig. 6d–f). The four mice of IBD model that we used by transfer of CD45RB^{high}CD4^{+} T cells showed the similar results of histopathological analysis and immunohistostaining. No or little expression of PCNA and p53 was observed in non-treated control mice (data not shown).

Discussion

To examine the mechanism of carcinogenesis mediated by IBD, we produced an IBD mouse model by transfer of CD45RB^{high}CD4^{+} T cells to SCID mice. Flow cytometry revealed that CD45RB^{high}CD4^{+} T cells were successfully isolated from mouse spleen. The intestine was shortened and the inflammatory response was observed in CD45RB^{high}CD4^{+} T cell-transferred mice, similarly to the previous report.[22] Our present pathological findings of this mouse model were similar to those seen in IBD patients.[27,28] We confirmed that this mouse model and human IBD shared the findings of the chronic inflammatory response in mucosal lesion, ulceration and the appearance of regenerative epithelial cells with dysplasia. Therefore, this model is suitable

for investigation on inflammation-associated carcinogenesis in IBD patients. Recently, several animal models for colitis-related colon carcinogenesis have been reported. Colonic neoplasms developed in mice treated with a genotoxic colonic carcinogen, azoxymethane, followed by dextran sodium sulfate (DSS).[29] A colitis-associated neoplasia model in p53 deficient mice, by treatment with DSS, showed 100% incidence of neoplasias.[30] IBD mice, deficient for the *mdr1a* gene, spontaneously developed colitis.[31] Our IBD mouse model is more consistent with the pathogenesis of human IBD than other models, since our model was induced by immunological abnormality-mediated inflammation, which is independent of chemicals and genetic deficiency.

In the present study, we have demonstrated that inflammation can promote the formation of 8-nitroguanine as well as 8-oxodG in the colon epithelium of the IBD mouse model. The formation of 8-nitroguanine and 8-oxodG was strongly observed in the epithelial cells and slightly in stromal inflammatory cells. Both the light and electron microscopic immunohistochemistry revealed that 8-nitroguanine was formed in the nuclei and cytoplasm. In the colon tissue pretreated with RNase, 8-nitroguanine formation was more clearly observed in the nuclei of epithelial cells. These results suggest that 8-nitroguanine is formed mainly in genomic DNA. iNOS was highly observed in the inflamed epithelium. Relevantly recent studies have shown that iNOS is expressed in the epithelial cells in colitis patients.[11,32,33] Our results and these reports suggest that the colon epithelium, as well as inflammatory cells, is an important source of NO. Overproduction of NO plays a crucial role in an enormous variety of pathological processes including cancer.[34] In addition to macrophages[35] and neutrophils,[36] the colonic epithelial cells themselves have the ability to produce superoxide anion ($O_2^{\cdot-}$).[37–40] $O_2^{\cdot-}$ is dismutated to H_2O_2, which induces metal-dependent 8-oxodG formation.[10] NO reacts with $O_2^{\cdot-}$ to produce $ONOO^-$, which can induce 8-oxodG,[41] and 8-nitroguanine formation.[16,42] The formation of 8-oxodG is known to cause $G \rightarrow T$ transversion.[43,44] 8-Nitroguanine undergoes spontaneous depurination, leading to the formation of apurinic sites in DNA,[45] which may induce base substitution, such as $G \rightarrow T$ transversion.[46] Therefore, 8-nitroguanine, formed via NO generation particularly from the epithelial cells, is a putative mutagenic DNA lesion and may contribute to IBD carcinogenesis in addition to 8-oxodG.[47]

In the mouse model of IBD, dysplasia and PCNA expression were observed in colon epithelium, suggesting that IBD promotes cell proliferation via inflammation-mediated DNA damage. PCNA-labeling indices was significantly higher in high-grade than in low-grade dysplasia in IBD patients.[48] Therefore, colitis-mediated DNA damage, followed by epithelial cell proliferation, may promote colonic carcinogenesis. p53 is important in the cellular response to DNA damage.[49] Several studies have demonstrated the *p53* tumor suppressor gene expression during the procession to ulcerative colitis-associated carcinoma.[50,51] Recently, it has been reported that a high concentration of NO can induce p53 protein accumulation.[52,53] Our results are noteworthy, in that high levels of 8-nitroguanine and p53 were accumulated in the same inflamed epithelium. Therefore, 8-nitroguanine formation and resulting p53 accumulation may promote IBD-associated colonic carcinogenesis.

In conclusion, 8-nitroguanine may not only be a promising biomarker for inflammation, but also a useful indicator for the risk of inflammation-mediated carcinogenesis.

Acknowledgments
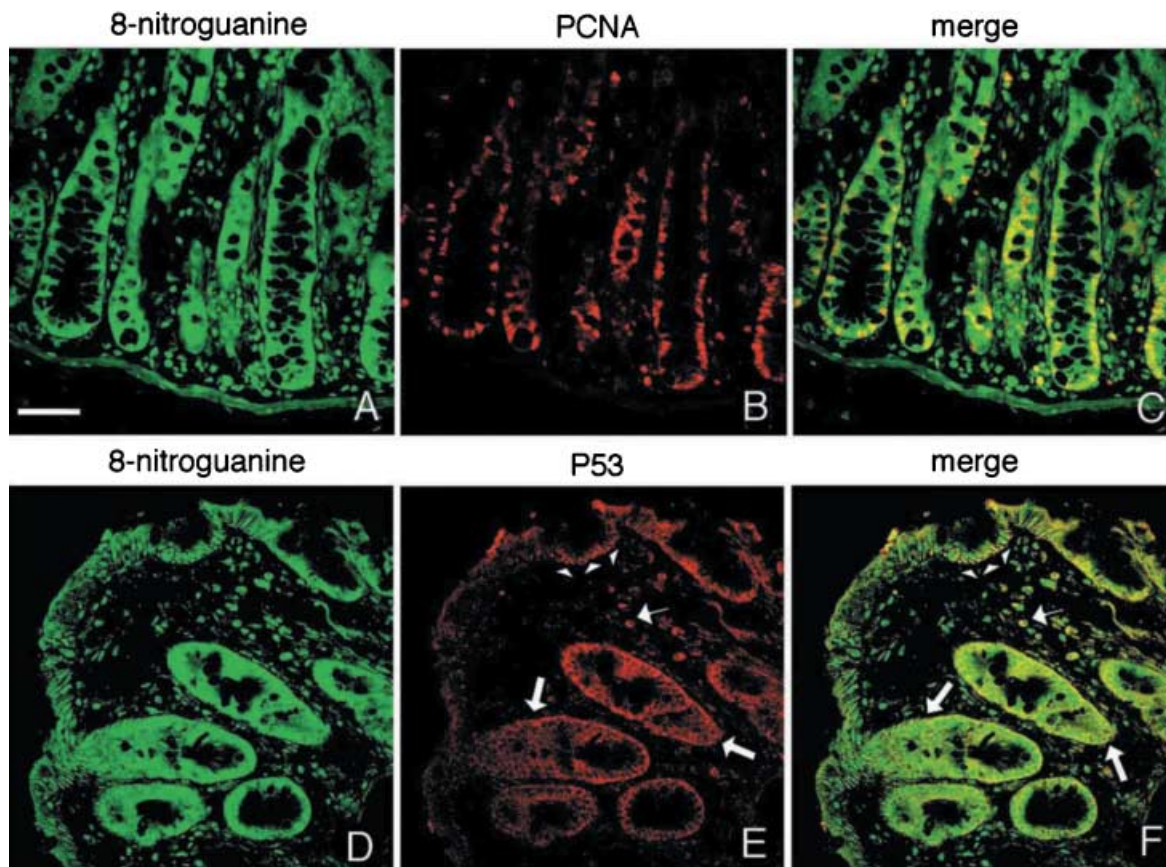
Fig. 6. Proliferating cell nuclear antigen (PCNA) and p53 expression in the mouse model of inflammatory bowel disease. (a–c) Double immunostaining for 8-nitroguanine and PCNA. (a,d) 8-Nitroguanine localizes widely in the mucosa, (b) intense PCNA is expressed in the nuclei of many glandular epithelial cells and infiltrated cells in lamina propria, (c) PCNA is expressed partially in the nuclei of 8-nitroguanine-immunoreactive epithelial cells, (d–f) double immunostaining for 8-nitroguanine and p53, (e) significant p53 expression is observed in the nuclei of regenerated epithelial cells (arrowhead) and some infiltrated cells (small arrow). p53 is also induced in the cytoplasm of many epithelial cells in lower portions of the gland (large arrow). (f) p53 is expressed in most of 8-nitroguanine-immunoreactive epithelial cells. Scale bar: 50 μm.

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