Erythropoietin attenuates intestinal inflammation and promotes tissue regeneration

Erythropoietin for IBD

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Abstract

Background. The prevalence of inflammatory bowel disease (IBD) is increasing. Since patients usually need long-term treatment and suffer from reduced quality of life, there is a need to develop new therapeutic strategy. The aim of this study was to investigate the therapeutic potential of erythropoietin (EPO) for the treatment of inflammatory bowel disease (IBD). ***Methods.*** Murine colitis was induced by 3.0% Dextran Sulfate Sodium (DSS). Recombinant human EPO (rhEPO) was given to evaluate the anti-inflammatory and regenerative effects on intestinal inflammation. The effect of rhEPO on human colon epithelial cells was also evaluated. Immunohistochemical analysis of EPO receptor was performed in human IBD tissues. ***Results.*** While about 62% of control mice with severe colitis induced by 5-day DSS died, 85% of mice treated with rhEPO survived. Histological analysis confirmed that EPO treatment reduced the colonic inflammation. Furthermore, EPO treatment significantly downregulated the local expressions of IFN-γ, TNF-α and E-selectin in the colon, suggesting that the effect was associated with inhibiting local immune activation. In 4-day DSS-induced colitis model, rhEPO significantly improved the recovery of body weight loss compared to controls. Furthermore, PCNA expression was significantly upregulated in the colon tissue from mice treated with rhEPO compared to controls. In addition, rhEPO increased the growth of cultured human colon epithelial cells in a dose-dependent manner. Furthermore, EPO-receptor expression was confirmed in human IBD colon tissues. ***Conclusion.*** Three major functions of EPO, hematopoiesis, anti-inflammation and regeneration, may produce significant effects on intestinal

inflammation, therefore suggesting that rhEPO might be useful for IBD.

Key Words: Inflammatory bowel disease; erythropoietin; intestine; tissue regeneration; cytokine

Introduction

The incidence and prevalence of inflammatory bowel disease (IBD), primarily ulcerative colitis (UC) and Crohn's disease (CD), are increasing in many countries and regions [1]. IBD can be painful, and sometimes leads to life-threatening complications. The main symptoms are abdominal pain, vomiting, diarrhea, intestinal bleeding, and weight loss. Furthermore, anemia is the one of most frequent extraintestinal complication of IBD [2,3]. IBD develops mostly in young people and erodes quality of life (QOL) for a long time. The mainstay of treatment for IBD is medication including aminosalicylates, corticosteroids, and several immunosuppressants [4]. Furthermore, anti-tumor necrosis factor monoclonal antibodies have become one of standard treatments [4,5]. Although optimal treatment depends on disease condition and background of each patient, the first choice is to use anti-inflammatory agent. The treatment goal is to achieve remission, after which the patient is usually switched to a lighter drug with fewer side effects. In severe cases, patients may need surgical intervention, such as bowel resection, strictureplasty or a temporary or permanent colostomy or ileostomy [6,7]. Even after surgery has been performed, long-term treatment and follow-up are usually required to keep conditions and prevent relapse. However, long disease duration and treatment cause several problems, such as colitic cancer and side effects due to long-term use of medications [8,9]. Therefore, further new treatment strategy has been expected to improve their quality of life and prognosis.

Erythropoietin (EPO) is a cytokine that promotes proliferation and differentiation of erythroid precursor cells in bone marrow under conditions of hypoxia and anemia, and

increases production of red blood cells. Exogenous recombinant EPO is widely used for the treatment of anemia associated with chronic renal failure, and contributes to improvement of QOL in patients with renal failure [10]. Recently, several studies have shown that EPO had not only hematopoietic effect but also other various functions including anti-inflammation, angiogenesis, cell proliferation, and tissue repair [11-15]. Therefore, we hypothesized that these various non-hematopoietic effects as well as hematopoietic functions might exert significant effects on IBD. The aim of this study was to evaluate the potential of recombinant EPO as a new treatment for IBD.

Methods

Animals

Male BALB/c mice (8-12 weeks old) weighing 22-30g were obtained from CLEA Japan (Tokyo, Japan). All mice were maintained under specific pathogen-free conditions in the animal facility at Nara Medical University. All experiments were conducted under a protocol approved by our institutional review board.

Induction of colitis and administration protocol of EPO

Dextran Sulfate Sodium (DSS; M.W. 36,000-50,000) was purchased from MP Biomedicals. (Irvine, CA, USA). Recombinant human EPO (rhEPO, Epoetin Beta) was purchased from Chugai Pharmaceutical Co. (Tokyo, Japan). To evaluate the anti-inflammatory and the regenerative effect of EPO, we used two ways of induction of colitis and administration protocol of rhEPO. In the first method, to evaluate the

anti-inflammatory effect, severe colitis was induced by addition of 3.0% DSS to drinking water for 5 days. In EPO group, rhEPO 150 IU/body (5000 IU/kg) was given daily as subcutaneous injection from day 1 to day 12. In control group, distilled water was given instead of rhEPO. Mice were monitored daily for loss of body weight and survival for 12 days. At 12 days or when mice had lost more than 25% of their initial body weight after DSS administration, they were sacrificed and their distal colon were removed for analysis. In the second method to evaluate the regenerative effect, relatively mild colitis was induced by addition of 3.0% DSS to drinking water for 4 days. The same dose of rhEPO was administered from day 4 to day 21. Mice were monitored daily for body weight for 21 days.

Real time RT-PCR analysis

Expressions of cytokines (IFN-γ and TNF-α), adhesion molecule (E-selectin) and T cell subsets ($CD4^+$ and $CD8^+$) were analyzed by quantitative real-time RT-PCR as previously described [16,17]. In brief, amplification and detection were done with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, California, USA) with the following profile: 10 min at 95 °C, and 40 cycles at 95 °C for 15 s and 60 °C for 1 min. All primers and probes were purchased from Applied Biosystems (Foster city, California, USA). Each gene expression of cytokines and adhesion molecule was normalized to β2-microgloblin before the fold change was calculated. The fold increase in each gene expression in the colon was calculated.

Cell proliferation analysis

Human colon epithelial cells, HT29 and Caco2 were used for cell proliferation assay. They were obtained from the American Type Culture Collection (ATCC). HT29 was grown in RPMI 1640 and Caco2 was in Dulbecco's modified eagle's medium (DMEM), which were supplemented with 10% heat-inactivated fetal bovine serum and incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. Cell proliferation effect of EPO was determined by MTS assay using the Cell-titer 96 aqueous one solution cell proliferation assay kit, according to the instruction manual (Promega Corporation, Madison, WI, USA). To evaluate the dose-dependent effects on growth potential for the human colon epithelial cells, five sets of groups (control IgG, rhEPO 5IU, 10IU, 20IU and 50IU) were selected. Medium was prepared for each experimental group and aliquoted (50 μL/well) into 3 wells per group. The colon epithelial cells were adjusted to a density of 1×10^4 cells/mL, and then 100 μL were aliquoted into each well and incubated for 7 days. Cell-titer 96 aqueous one solution was added to each well and incubated for an additional 1 hour. The absorbance at 490 nm was recorded with a 96-well plate reader. Each experiment was performed in triplicate and repeated at least thrice.

Immunohistochemistry

To ensure the expression of EPO receptor (EPO-R) in human intestine, we performed immunohistochemical analysis in surgically resected UC and CD colorectal tissues. Normal colon tissues were obtained from surgical specimens other than colorectal

cancer. Formalin-fixed, paraffin-embedded tissues were cut into 5-μm sections, deparaffinized, and rehydrated in a graded series of ethanol. Antigen retrieval was performed at 99 °C for 40 min, using a Target Retrieval Solution, pH 9.0 (DAKO, Tokyo, Japan). To block endogenous peroxidase, sections were immersed in 3% solution of hydrogen peroxide in absolute methanol for 10 min at room temperature and washed thrice in fresh PBS, each of 5 min duration. EPO-R (rabbit polyclonal antibody, H-194; Santa Cruz Biotechnology, CA, USA) diluted 1:50 with Antibody Diluent (DAKO) was added and incubated overnight at 4 °C. Sections were washed thrice in PBS, each of 5 min duration. Detection steps were done using a commercially available kit (Dako Envision™ System-HRP, DakoCytomation, Kyoto, Japan) according to the manufacturer's instructions. Hematoxylin was used as counter stain. Written informed consent was obtained from all patients according to our institutional guidelines.

Statistical analysis

All data were expressed as mean ± standard deviation. Statistical significance between two groups of parametric data was evaluated by using an unpaired Student's *t* test. The survival curve by the Kaplan–Meier method was analyzed by a log-rank test. *P* values less than 0.05 were considered significant.

Results

Protective effect of EPO on DSS colitis mice

First, to evaluate the therapeutic efficacy of EPO on inflammatory bowel conditions, we employed a severe murine colitis model. In this model, DSS was given for 5 days and treatment was started on the same day. In naïve mice, hemoglobin level was 17.5 ± 0.6 g/dl (n = 7). It was significantly decreased on 7th day in control mice (n = 4, 15.6 ± 0.8 g/dl)($P < 0.01$). In sharp contrast, EPO treatment significantly increased the hemoglobin level on 7th day compared to control and naïve mice (n = 3, 18.5 ± 0.6 g/dl)($P < 0.01$). As expected, EPO treatment significantly improved the anemia in mice with colitis.

While about 62% of control mice died by the time of analysis, 85% of mice treated with rhEPO survived, indicating that EPO treatment significantly improved the survival rate ($P = 0.02$)(Figure 1A). Furthermore, rhEPO significantly prevented the body weight loss in this model (Figure 1B). Histological analysis demonstrated that EPO treatment reduced the colonic inflammation induced by DSS administration (Figure 2). On day 7 after starting DSS administration, considerable cellular infiltration was observed in control mice, while there was relatively mild inflammation seen in EPO-treated mice (Figure 2A and B). On day 12, mucosal thickness and massive cellular infiltration were identified in control mice, inflammation was almost resolved in EPO-treated mice (Figure 2C and D). Thus, data clearly indicated that EPO has a significant effect on inflammatory bowel conditions.

Effect of EPO on local immune activation and T cell infiltration

To elucidate the underlying mechanism, we evaluated the local intestinal immune activity. We examined the local expressions of several cytokines, chemokines and

adhesion molecules in the distal colon using quantitative real-time RT-PCR analysis. The expressions of several cytokine and adhesion molecule were upregulated by day 7 compared to naïve colon (Figure 3). EPO treatment suppressed the local expressions of several potent proinflammatory cytokines including IFN-γ and TNF-α compared to control on the analysis of day 7 (Figure 3A and B). In addition, a potent adhesion molecule, E-selectin, was also significantly downregulated by EPO treatment on day 7 (Figure 3C). Furthermore, we analyzed T cell infiltration into the inflamed intestine in this model. As a result, there were no differences in both $CD4^+$ and $CD8^+$ T cells between control and EPO-treated mice (Figure 4).

Regenerative effect of EPO on DSS colitis mice

Next, we evaluated the regenerative effect of EPO on inflammatory bowel conditions. To this end, we used another murine colitis model. In this model, DSS was given for four days, and the treatment was started on the fourth day after starting DSS administration. The recovery of body weight loss was significantly improved in mice treated with rhEPO compared to control (Figure 5A). Histological analysis further showed that there were severe cellular infiltration and tissue destruction in control mice (Figure 5B). In contrast, there were less cellular infiltration and relatively preserved tissue structure in EPO-treated mice (Figure 5B). Data suggested that EPO treatment might promote tissue regeneration of inflamed intestines. To confirm this possibility, we examined PCNA expression, as a marker of cellular proliferation, in distal colon by real-time PCR analysis. As a result, PCNA expression was significantly upregulated in

the colon tissue from mice treated with rhEPO compared to control and naïve colon (Figure 6A). Furthermore, we analyzed local immune status by real-time PCR. However, there were no differences in cytokine and adhesion molecule expressions between control and EPO-treated mice (Figure 6B).

Effect of EPO on proliferation of human colon epithelial cells

Toward clinical application, we then evaluated the effect of EPO on human intestine tissue. We performed cell proliferation analysis by MTS assay using two types of human colon epithelial cells. Data showed a dose-dependent increase in growth of colon epithelial cells at rhEPO doses ranging from 5 to 50 IU (Figure 6). Therefore, EPO is suggested to have the growth stimulation effect on human colon epithelial cells.

EPO-R expression in human IBD colon epithelium

Finally, we examined EPO-R expression in human UC and CD intestinal tissues as well as normal colon by immunohistochemical analysis (Figure 7). As a result, positive staining was observed mainly in basal cells in mucosal epithelium and enteric nerves. Importantly, EPO-R expression was confirmed in not only normal colon but also both UC and CD colon tissues.

Discussion

Although the frequency of IBD is increasing worldwide, its etiology is still not fully

elucidated [1-5]. Previous studies have suggested several potential mechanisms including genetic predisposition, environmental factors and dysregulated immune response. Clinical and pathological characteristic of IBD is chronic, aberrant and repetitive inflammation. Since IBD often develops in young adulthood, patients usually need long-term treatment with anti-inflammatory drug and suffer from reduced quality of life. Therefore, there is an urgent need to develop new therapeutic strategy.

EPO is an essential cytokine that binds and activates EPO-R on the surface of erythroid progenitor cells, thereby promoting erythropoiesis in bone marrow under conditions of hypoxia and anemia. In clinical practice, rhEPO is widely used for the treatment of anemia associated with chronic renal failure [10]. Besides its hematopoietic effects, EPO has been shown to exert various functions such as anti-inflammation, anti-apoptosis, angiogenesis, cytoprotection, cell proliferation and tissue repair [11-15]. Therefore, we hypothesized that diverse functions of EPO might induce therapeutic effects on complex pathological conditions in IBD. First, we have examined in vivo efficacy of EPO in murine IBD model. And we found that rhEPO had a significant effect on murine colitis mimicking human IBD, as demonstrated by mouse survival and histological analysis. Furthermore, our data suggested that the effect of EPO might be associated with the inhibition of local immune activation including downregulation of several potent proinflammatory cytokines and adhesion molecules. Our data are consistent with previous reports indicating effect of rhEPO in different colitis models [18,19]. Therefore, the anti-inflammatory effect of EPO probably functions on intestinal inflammation.

Several previous studies have shown that EPO has efficacy to promote the proliferation, migration, differentiation and regeneration in various types of cells and tissues [11-13,20,21]. Therefore, we next focused on the proliferative and regenerative effects of EPO on inflamed intestine. We found that rhEPO treatment improved the body weight recovery in mice with established colitis. Furthermore, the efficacy was associated with upregulation of PCNA local expression in the murine intestine. Our further analysis indicated that rhEPO promoted the proliferative growth of cultured human colon epithelial cells. Taken together, rhEPO is potentially effective for tissue regeneration of the inflamed intestines in human IBD. This regenerative effect may be critically important for patients with IBD, since bowel inflammation can be repetitive and chronic. If the regeneration of inflamed intestines is sufficient enough, unfavorable long-term medication or invasive surgical intervention may be avoided in many patients.

It has been reported that the EPO-R is widely distributed in non-erythroid tissues such as the brain, heart, kidney, neural cells, smooth muscle cells, vascular endothelium, retina, pancreatic islets and gastric epithelial cells [14,15,22-32]. In this study, we also confirmed EPO-R expression in human normal colon tissues by immunohistochemical analysis. Importantly, it was also expressed in the intestinal tissues of both UC and CD colon. These clinical data further suggested that EPO has a therapeutic potential for the treatment of human IBD. However, there are fundamental differences in the intestinal microbial environment between experimental colitis especially under specific-pathogen-free conditions and human IBD. The intestinal microbiota is known

to play significant roles in the process of immunological and nonimmunological responses in humans. Therefore, careful evaluation and interpretation will be required to reach a definitive conclusion on clinical efficacy of rhEPO for the treatment of human IBD.

In conclusion, our data have strongly suggested that rhEPO may be potentially useful through both hematological and non-hematological effects on intestinal inflammation. Three major functions of EPO, including hematopoiesis, anti-inflammation and regeneration may produce additive or even synergistic effects on human IBD. Furthermore, other mechanisms such as antiapoptosis and angiogenesis may be involved [19]. Some clinical studies have already shown the safety and efficacy of rhEPO for the treatment of anemia in IBD patients, although the number of participants in each study is relatively small [33-36]. In addition, several long-acting rhEPO have become clinically available. Such agents may be desirable for the treatment of IBD with long disease duration. Therefore, further clinical study to address non-hematological effects of rhEPO is warranted.

Acknowledgments

This study was supported by the Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (S. Nakamura) and Grant from The Kidney Foundation, Japan (JKFB07-17, 08-6, 09-10)(M. Sho).

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Figure Legends

Figure 1. Protective effect of erythropoietin on experimental colitis. Mice were given dextran sulfate sodium (DSS) for 5 days in the drinking water to induce severe colitis. (A) EPO treatment significantly improved the survival rate compared to control ($n = 13$ of each group; $p < 0.05$). (B) EPO treatment significantly prevented the body weight loss compared to control ($^{*}p < 0.05$).

Figure 2. Protective effect of erythropoietin on colon histology. Representative histological appearances of distal colon at day 7 and 12 by hematoxylin and eosin staining from control mice (A and C) and EPO-treated mice (B and D). EPO treatment markedly reduced colonic inflammation with mucosal thickness and infiltration of inflammatory cells compared to control (original magnification, x100).

Figure 3. Local expressions of cytokine and adhesion molecule. EPO treatment had a tendency to downregulate the local expression of IFN-γ (A) and TNF-α (B) compared to controls on the analysis of day 7. E-selectin was significantly downregulated by the treatment on day 7 (C)($n = 5$-10 of each group; $^{*}p < 0.05$).

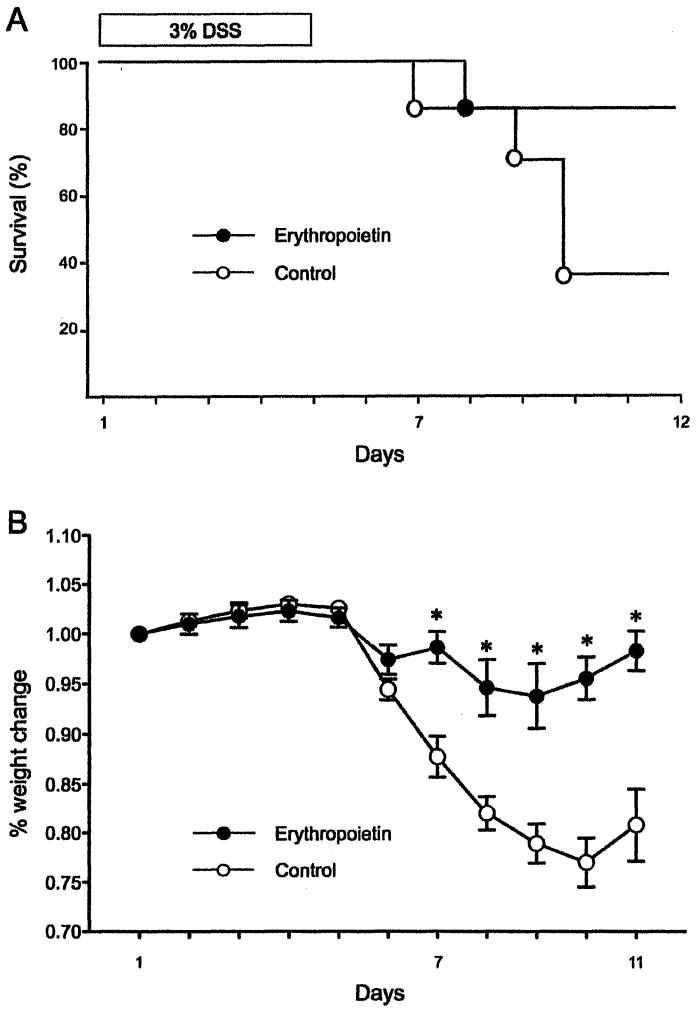
Figure 4. T cell infiltration into the inflamed intestine. There were no differences in local expressions of CD4 and CD8 T cell subsets between control and EPO treatment.

Figure 5. Regenerative effect of erythropoietin. Mice were given dextran sulfate sodium (DSS) for 4 days in the drinking water to induce colitis. And the treatment was started on 4th day after DSS administration. (A) Body weight change. EPO treatment significantly improved the recovery of body weight loss compared to control ($n = 12$ of each group; $^{*}p < 0.05$). (B) Representative histological appearances of distal colon at day 21 by hematoxylin and eosin staining from control and EPO-treated mice (original magnification, x100). (C) PCNA expression in the inflamed colon. PCNA expression was up-regulated in the DSS-induced inflamed colon treated with EPO compared to naïve and control colon ($n = 10$ of each group; $^{*}p < 0.05$). (D) Local expressions of cytokine and adhesion molecule. There were no differences in the local expressions of IFN-γ, TNF-α, and E-selectin between control and EPO treatment ($n = 10$ of each

group).

Figure 6. Cell proliferation analysis. EPO treatment inhibited proliferation of human colon epithelial cells in a dose-dependent manner ($^{*}p < 0.05$).

Figure 7. EPO-R expression in human intestinal tissues. EPO-R expression was found in human intestinal tissues form patients with normal control (A), ulcerative colitis (B), and Crohn's disease (C)(original magnification, x100).



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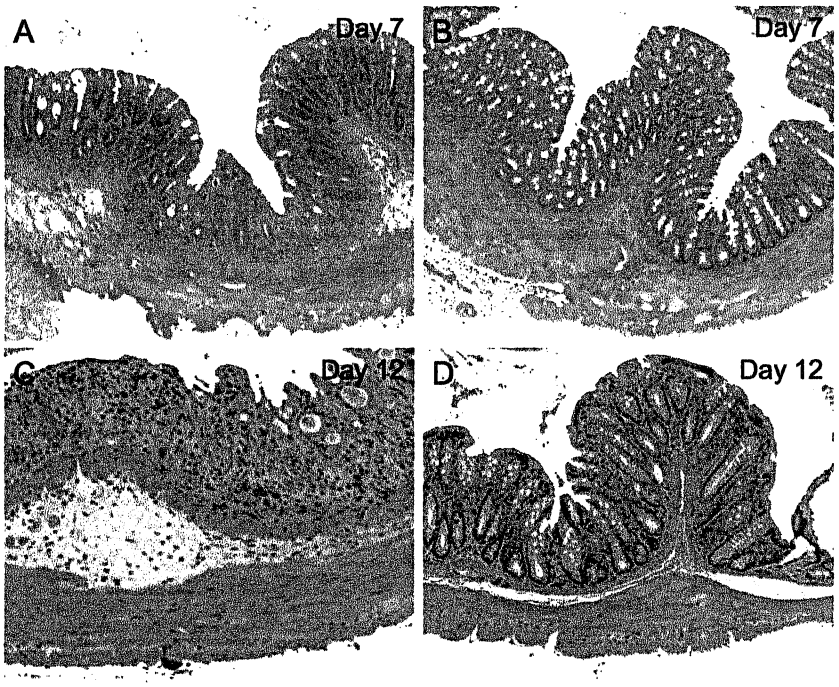

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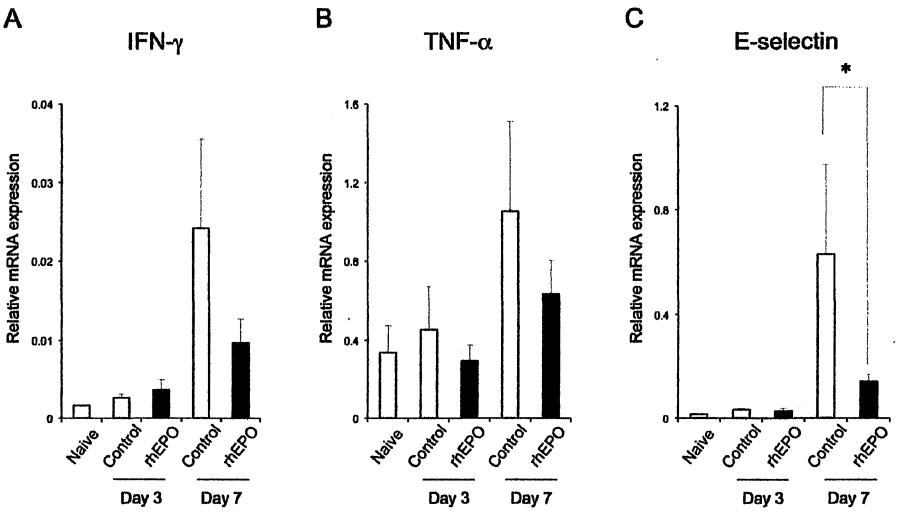

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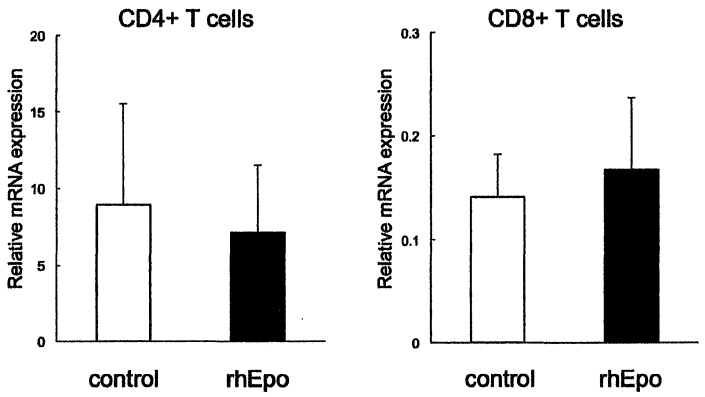

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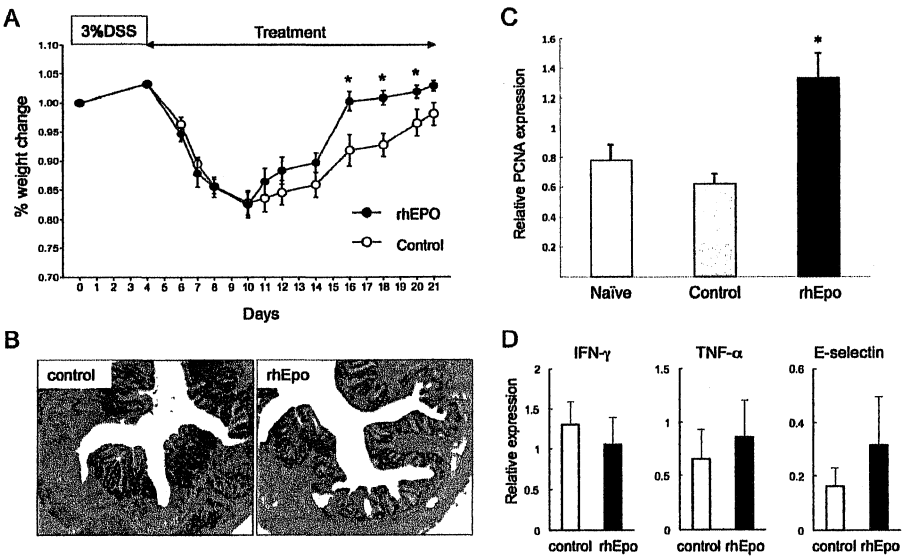

Figure 5. Regenerative effect of erythropoietin. Mice were given dextran sulfate sodium (DSS) for 4 days in the drinking water to induce colitis. And the treatment was started on 4th day after DSS administration. (A) Body weight change. EPO treatment significantly improved the recovery of body weight loss compared to control (n = 12 of each group; *p < 0.05). (B) Representative histological appearances of distal colon at day 21 by hematoxylin and eosin staining from control and EPO-treated mice (original magnification, x100). (C) PCNA expression in the inflamed colon. PCNA expression was up-regulated in the DSS-induced inflamed colon treated with EPO compared to naïve and control colon (n = 10 of each group; *p < 0.05). (D) Local expressions of cytokine and adhesion molecule. There were no differences in the local expressions of IFN-γ, TNF-α, and E-selectin between control and EPO treatment (n = 10 of each group).
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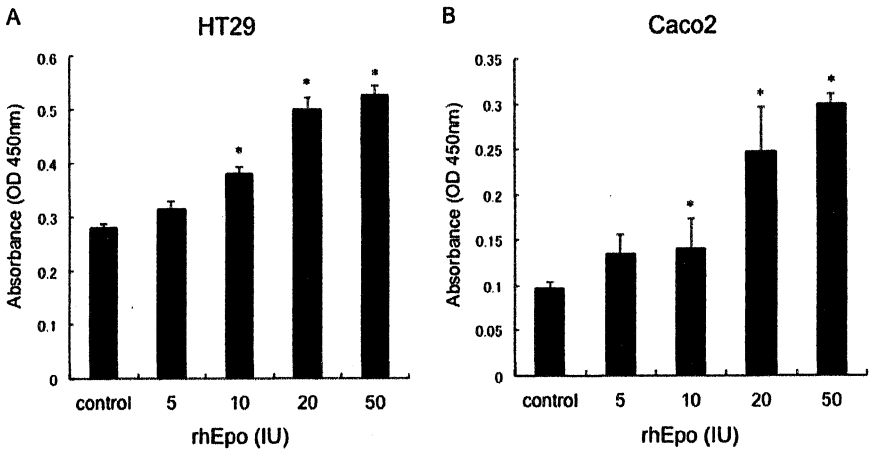

Figure 6. Cell proliferation analysis. EPO treatment inhibited proliferation of human colon epithelial cells in a dose-dependent manner (*p < 0.05).
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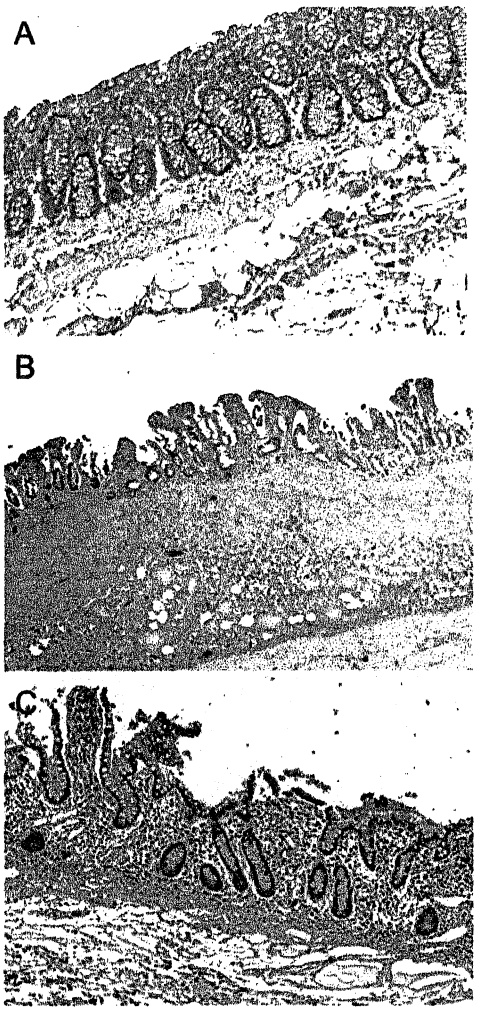

Figure 7. EPO-R expression in human intestinal tissues. EPO-R expression was found in human intestinal tissues form patients with normal control (A), ulcerative colitis (B), and Crohn's disease (C)(original magnification, x100).
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