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ORIGINAL ARTICLE

Interleukin-1 receptor antagonist reduced apoptosis and attenuated intestinal mucositis in a 5-fluorouracil chemotherapy model in mice

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Abstract
Purpose The aim of this study was to investigate the relationship between changes in IL-1β expression and intestinal apoptosis after chemotherapy. And we further determine whether interleukin-1 receptor antagonist (IL-1Ra) reduces apoptosis in vivo after 5-fluorouracil (5-FU) chemotherapy in the small intestine.
Methods Intestinal mucositis was induced in mice by intraperitoneal injection of a single dose of 5-FU (200 mg/kg). IL-1Ra (1 mg/kg) was injected subcutaneously twice daily after 5-FU injection. 5-FU-induced intestinal apoptosis was detected by TUNEL assay. The expression of IL-1β induced by 5-FU in local intestinal tissue was examined by RT-PCR and immunohistochemistry. Assessment of 5-FU-induced mucositis (histology, diarrhea scores, bowel weight) was performed. The apoptosis-related proteins were investigated by western blotting analysis. The proliferation of intestine was examined by immunohistological staining of PCNA. Viability of IEC-6 cells was determined using the CCK-8 assay. The apoptosis of IEC-6 cells was examined by Hoechst 33342 staining.
Results The variation of IL-1β expression induced by 5-FU was in accordance with the changes in intestinal apoptosis. Administration of IL-1Ra could block the destructive effect of IL-1β and reduce apoptosis in the small intestinal crypt after chemotherapy. The protection against apoptosis was in accordance with the reduction of the up-regulation of Bax and caspase 3 and the elimination of the down-regulation of Bcl-2 and Bcl-xL. Moreover, IL-1Ra attenuated the severity of intestinal mucositis induced by 5-FU and enhanced intestinal crypt proliferation. In vitro experiments showed that IL-1Ra suppressed apoptosis and increased cell viability in enterocyte IEC-6 cells treated with 5-FU. Additionally, IL-1Ra did not affect the chemotherapeutic effect of 5-FU in tumor CT-26 xenograft mice.
Conclusions Our studies elucidate that IL-1β is quite possibly involved in and mediated the course of intestinal apoptosis after 5-FU chemotherapy. Administered with IL-1Ra protects mice against intestinal apoptosis induced by 5-FU, relieves mucosal impairment of the small intestine, and facilitates the recovery of the intestinal mucosa. IL-1Ra treatment offers a novel promising strategy for the prevention and cure of chemotherapy-induced intestinal mucositis in clinical practice.

Keywords Interleukin-1 receptor antagonist · Mucositis · Apoptosis · Chemotherapy

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Introduction

Intestinal mucositis is a common side effect during the course of clinical chemotherapy for patients with cancer. Its incidence in patients that underwent chemotherapy with the standard dosage was 40%, whereas it was nearly 100% in patients with high dose chemotherapy [1]. It is well known

that while the cytotoxic drugs used in chemotherapy have a lethal effect on tumor cells, they also generate toxic side effects on enterocytes undergoing active proliferation. These side effects are the cause of intestinal mucositis induced by chemotherapy. 5-Fluorouracil (5-FU) is a commonly used chemotherapeutic drug for the treatment of malignant tumors, which could improve the tumor-free status and survival rates of patients suffering from tumor [2]. Nevertheless, 50–80% of patients who underwent 5-FU chemotherapy showed clinical manifestations of mucositis, the symptoms of which include severe diarrhea [3]. Currently, although many studies on chemotherapy-induced intestinal mucositis have been performed, there are still no better methods available for the treatment of mucositis.

Apoptosis or programmed cell death can be physiological or induced by chemotherapy or radiotherapy [4]. The large amount of apoptotic cells generated in the intestinal crypt prior to serious mucosal destruction was observed in mice that received chemotherapy [5, 6]. A large number of apoptotic cells could be observed in the small intestinal tissue of chemotherapy model mice [5, 7] and in intestinal biopsy specimens of patients suffering from tumor undergoing chemotherapy [8]. Akira et al. demonstrated that after 5-FU chemotherapy, apoptotic cells in the small intestine of mouse started to appear at 6 h, reached a peak at 24 h, and rapidly recovered to a normal level thereafter [5]. Previous study had considered that the genesis of chemotherapy-induced intestinal mucositis was correlated with the apoptotic effect of cytotoxic drugs on small intestinal cells. For example, cytotoxic drugs caused the apoptosis of ancestral stem cells in the small intestinal crypt, inhibited regeneration of the small intestine, and therefore created a deficiency in the number of new cell supplements in small intestinal cell metabolism. This deficiency generated morphometric changes of villus atrophy and crypt hypoplasia in intestine [1, 8]. In conclusion, apoptosis plays a critical role in the mucositis induced by chemotherapy.

The mechanism of small intestinal cell apoptosis induction by chemotherapy is still uncertain but could possibly be correlated with the enhancement of proinflammatory factors such as IL-1β in patho- and physiological changes of intestinal mucosal tissue that received chemotherapy [9]. Logan et al. [9] have showed that IL-1β expression was significantly increased in intestinal mucosal tissue of rats that received chemotherapy. And high expression of IL-1β was also observed in the peripheral blood serum of animal chemotherapy model [10]. Although IL-1β was involved in the inflammatory reaction of an organism, still other studies found that IL-1β could induce the apoptosis phenomenon in multiple types of cells [11–14]. The induction of apoptosis in multiple types of cells by IL-1β was involved in the activation of a series of apoptosis-related factors such as Bax and Bcl-2 [11, 15, 16]. Chemotherapy-induced intestinal apoptosis process was accompanied by the up-regulation of proapoptotic factors (Bax, Caspase-3, Bak, and P53) and down-regulation of antiapoptotic factors (Bcl-2 and Bcl-xL) in the small intestinal crypt, which finally led to the genesis of small intestinal mucositis [5, 17–19]. Therefore, IL-1β was possibly activated and involved in cell apoptosis of the small intestinal crypt after chemotherapy.

Interleukin-1 receptor antagonist (IL-1Ra) is a natural protein that has antagonistic biological effects on IL-1β [20], which could antagonize the inhibitory role of IL-1β on endothelial cell proliferation and promote the growth of endothelial cells by binding to IL-1R [21]. Recent studies also reported the inhibitory role of IL-1Ra on cell apoptosis in different experimental models; the mode of action was thought to be the blockage of IL-1β activity [11, 13, 22, 23]. However, there is still no related report on the role of IL-1Ra in the inhibition of cell apoptosis induced by chemotherapy in the small intestinal crypt. We hypothesized that the sharp increase in IL-1β in the organism after chemotherapy might be involved and activated in the process of small intestinal cell apoptosis. Its natural antagonist IL-1Ra was used to block the destructive effect of IL-1β and reduce apoptosis in the small intestinal crypt after chemotherapy, thereby relieving the intestinal mucositis induced by chemotherapy.

Materials and methods

Experimental animals

The pathogen-free, sex-matched, 8- to 10-week-old BALB/c mice (SLACCAS, Shanghai, China) were maintained in air-filtered units. The temperature and relative humidity were maintained at 23 ± 5°C and 50 ± 15% throughout the experiments. Mice were fed with sterile water and rodent food. Animal experiments were approved by Animal Care and Use Committee of Shanghai Jiaotong University.

Induction of intestinal mucositis by 5-FU

Intestinal mucositis was induced in BALB/c mice by intraperitoneal (i.p.) injection of 5-FU (Sigma) at a single dose of 200 mg/kg on day 0. Disease severity was assessed daily by recording the diarrhea score from day 0 to 8. Each mouse was examined twice a day. The severity of diarrhea was scored using the following scale: 0, normal (normal stool or absent); 1, slight (slightly wet and soft stool); 2, moderate (wet and unformed stool with moderate perianal staining of the coat); and 3, severe (watery stool with severe perianal staining of the coat) [24]. The average diarrhea score was used to evaluate the severity of diarrhea (n = 5 per group).

Administration of IL-1Ra

For assessment of apoptosis, 0.1 ml of IL-1Ra (Amgen; 1 mg/kg) or PBS was administered subcutaneously immediately after 5-FU injection, and 4 h later, a second administration was given, and mice were killed 6, 12 and 24 h after 5-FU treatment; For assessment of intestinal mucositis, a 3-day treatment protocol (from day 0 to 2) for IL-1Ra (1 mg/kg) or PBS was injected subcutaneously twice daily at 10 a.m. and 4 p.m. after 5-FU treatment, and mice were killed on day 3.

Establishment of mice xenografted with CT-26

The murine colonic tumor cell line CT-26 was cultured in 10% fetal bovine serum (FBS)-RPMI medium, following which 1×10^5 cells in 0.1 ml PBS were injected subcutaneously into the right flank region of each mouse. One week after tumor inoculation, the mice were divided randomly into the following groups ($n = 5$ per group): CT-26-xenografted mice treated with PBS; CT-26-xenografted mice treated with IL-1Ra; CT-26-grafted mice treated with 5-FU plus PBS; and CT-26-grafted mice treated with 5-FU plus IL-1Ra. A single dose of 5-FU (200 mg/kg) or PBS as control was injected i.p. on day 7. And treatment with either 0.2 ml of PBS or IL-1Ra (1 mg/kg) administered subcutaneously twice daily immediately after 5-FU injection from day 7 to 20. Mice were euthanized on day 20, and the tumors were excised and weighted.

Assessment of intestinal mucositis

For the assessment of intestinal mucositis induced by 5-FU, mouse intestinal tract from the pyloric sphincter to the rectum was dissected out, flushed with isotonic saline, and the wet weights of the small intestine were measured on day 3 ($n = 5$ per group). Segments of mid-jejunum were harvest and fixed in formaldehyde, embedded in paraffin, stained with hematoxylin and eosin (H&E), and then subjected to blinded histological assessment. Villus height and crypt length were measured microscopically.

Apoptosis analysis

For the assessment of 5-FU-induced intestinal apoptosis, the mice were sacrificed at the indicated time points after 5-FU administration. Segments of mid-jejunum were harvested and fixed in formaldehyde, embedded in paraffin, and 4-μm-thick cross-sections were prepared. TUNEL assay was performed using an in situ Cell Death Detection Kit (Roche) to detect apoptosis of enterocytes in the small intestine strictly according to the manufacturer's instructions. The apoptotic index was defined as the average number of TUNEL-positive cells per crypt counted from 20 to 30 crypts per mouse ($n = 5$ mice/group).

ELISA of IL-1β

Mouse peripheral blood (PB) samples were obtained by retro-orbital puncture. Each blood sample was coagulated at 4°C and centrifuged at 1,000*g* for 15 min. The serum was collected and stored at −80°C. Aliquots were used once only for the test and were not subjected to repeated freeze–thaw cycles. Murine IL-1β enzyme-linked immunosorbent assay (ELISA) kits were used to measure IL-1β serum levels according to the manufacturer's instructions (R&D Systems).

Determination of IL-1β gene expression in intestinal tissues using semi-quantitative RT-PCR

Mice intestinal tissues were removed and homogenized for specific mRNA detection at the indicated time points after 5-FU chemotherapy. Total RNA was extracted using an RNA simple Total RNA Kit (TIANGEN). Primers are as follows: IL-1β forward primer 5′-CTGTGTCTTTCCCGT GGACC-3′ and reverse primer 5′-CAGCTCATATGGGT CCGACA-3′; GAPDH forward primer 5′-AGGGGTCTA CATGGCAACTG-3′ and reverse primer 5′-CGACC ACTTTGTCAAGCTCA-3′. Each assay was performed in triplicate. The expression levels of IL-1β were normalized to that of the housekeeping gene GAPDH.

Immunohistochemistry

Expression of IL-1β was assessed using anti- IL-1β polyclonal antibody (Santa Cruz Biotechnology) at a 1:50 dilution followed by use of the Vectastain ABC Kit (Vector Laboratories) according to the manufacturer's instructions. For proliferating cell nuclear antigen (PCNA) detection, intestinal sections were stained with mouse monoclonal anti-PCNA antibody (Santa Cruz Biotechnology) at a 1:400 dilution followed by use of the Vectastain ABC Kit (Vector Laboratories) according to the manufacturer's instructions.

Western blotting

Intestinal tissues were harvested at the indicated time points, and total protein was extracted with lysis buffer. Equivalent amounts of protein (40 μg) were loaded onto a 12% SDS–PAGE for electrophoresis and then transferred onto nitrocellulose membranes. The membranes were incubated with a 1:1,000 dilution of anti-Bax, cleaved caspase-3, Bcl-2, Bcl-xL, and β-actin (Cell signaling Technology) polyclonal antibodies and incubated with HRP-conjugated secondary antibody (Sigma). Chemiluminescent detection

was conducted using supersignal substrate (Pierce Biotechnology) according to the manufacturer's instructions.

Cell lines and cytotoxicity assays

Murine intestinal epithelial IEC-6 cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen-Gibco) complemented with 5% fetal calf serum, 0.1 unit/ml of insulin, 50 unit/ml of penicillin, and 50 μg/ml of streptomycin at 37°C with 5% CO_2-in-air. IEC-6 cells were seeded in 96-well plates at an intensity of 5,000 cells/100 μl/well, and 10 μl of various concentrations of 5-FU alone or with various concentrations of IL-1Ra was added to the culture medium and incubated for 24 h. To measure the cell viability, 10 μl of the Cell Counting Kit-8 solution (Dojindo Laboratories) was added to each well and incubated for 4 h, and then the plates were read at 450 nm in a spectrophotometer.

Hoechst 33342 staining of IEC-6 cells

Apoptosis was observed by chromatin staining with Hoechst 33342, as previously described. IEC-6 cells were treated with 10 μl of 5-FU (100 μg/ml) alone or with IL-1Ra (10 μg/ml) for 24 h. After additional 30-min incubation in the presence of 10 μM Hoechst 33342, the fluorescent and phase contrast microscopic images were presented. The results were expressed as the average number of cells with apoptotic chromatin changes in 5 continuous microscopic fields at 100× magnification.

Statistical analysis

All data were expressed as the mean ± standard deviation (SD). Differences between groups were assessed using analysis of variance (ANOVA) followed by post hoc Student–Newman–Keuls test and the non-parametric Mann–Whitney *U* test. P values less than 0.05 were considered statistically significant.

Results

IL-1β expression is induced after 5-FU chemotherapy in accordance with intestinal crypt apoptosis

As indicated in Fig. 1a and b, the number of apoptotic cells in the small intestinal crypt at the initial stage after 5-FU chemotherapy increased rapidly. A small number of apoptotic cells appeared in the small intestinal crypt at 6 h, a large number of apoptotic cells appeared at 12 h, and the peak of apoptosis occurred at 24 h. Subsequently, the apoptosis level gradually decreased and returned to the normal level before chemotherapy on the third day.

The role of IL-1β in damage from chemotherapy and its correlation with small intestinal cell apoptosis were uncertain.

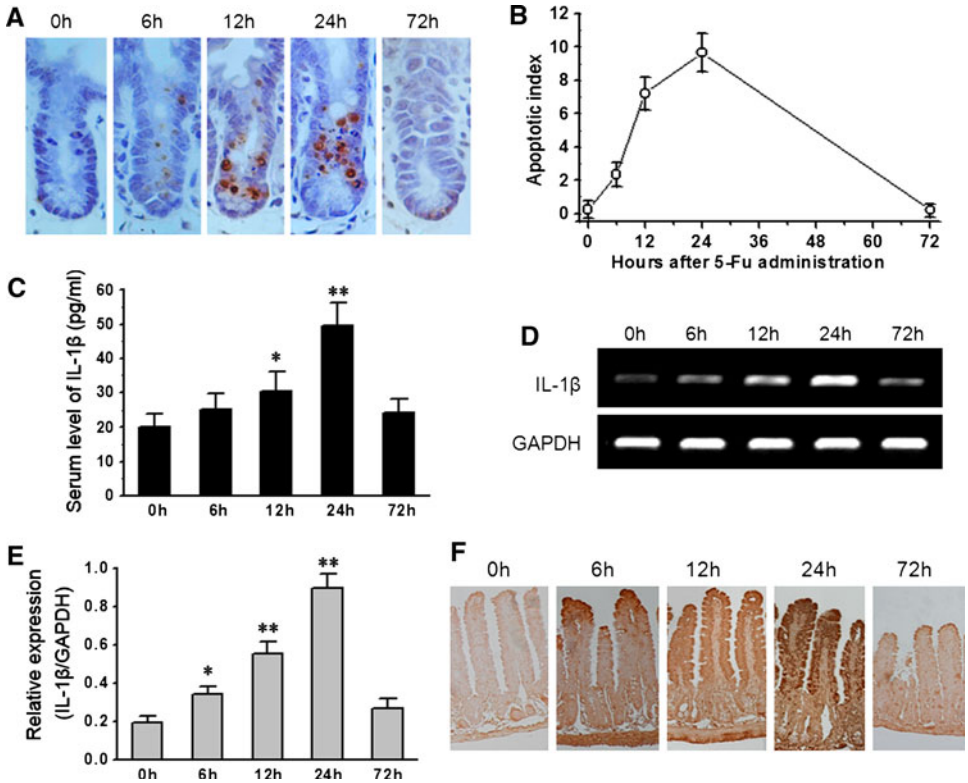

Fig. 1 Apoptosis and IL-1β expression are induced by 5-FU chemotherapy. **a** Apoptosis is induced after 5-FU treatment in a time-dependent manner. Magnification, ×400. **b** The apoptotic index was defined as the average number of TUNEL-positive cells per crypt counted from 20 to 30 crypts per mouse (n = 5 mice/group). Serum **c** and gene **d** and **e** expression of IL-1β after 5-FU treatment for each mouse were shown as the mean ± SD. * $P < 0.05$, ** $P < 0.01$ versus 0 h. **f** Immunohistochemical staining of the mid-jejunum for the expression of IL-1β was performed. Magnification, 100×

This study investigated the relationship between changes in IL-1β expression at the gene and protein levels and cell apoptosis after chemotherapy. As indicated in Fig. 1c, we observed that the level of IL-1β in blood serum was significantly increased at 12 h, reached a peak at 24 h, and returned to its normal level before chemotherapy at 72 h. Subsequently, we used RT-PCR to detect variations in IL-1β mRNA expression at different time points after chemotherapy. Our results showed that the IL-1β gene level was significantly enhanced 6 h after chemotherapy and reached a peak at 24 h, while the expression was nearly undetectable at 72 h (Fig. 1d and e); this variation was in accordance with changes in the blood serum. Immunohistochemistry results are shown Fig. 1f. Only a small amount of IL-1β was expressed in the top of the intestinal villus 6 h after chemotherapy, and slight positive expression in the whole villus was observed at 12 h. A great quantity of hyperchromatic positive expression was observed from the bottom to the top until 24 h; however, almost no positive expression was observed at 72 h. In conclusion, the variation in IL-1β expression in the local intestinal tissue was in accordance with the results seen in the peripheral blood, which indicated that IL-1β played a critical role in the genesis and development of intestinal mucositis after chemotherapy. The variation in IL-1β expression after chemotherapy was in accordance with the change in apoptosis. The peak value of expression occurred at 24 h with the most evident intestinal apoptosis but not at 72 h with the most severe impairment of the intestinal mucosa. This result suggested that IL-1β was quite possibly involved in and mediated the course of intestinal apoptosis in the initial stage of chemotherapy.

IL-1Ra reduces intestinal apoptosis induced by 5-FU chemotherapy

To better test our hypothesis that increased expression of IL-1β in the local region of the small intestine after 5-FU chemotherapy facilitated intestinal apoptosis, we used IL-1Ra, the natural antagonist of IL-1β, to try to inhibit the activation of IL-1β to reduce apoptosis of the intestinal crypt cells after chemotherapy. There were no detectable apoptotic cells in the intestinal crypt of control group. A large number of apoptotic cells appeared in the intestinal crypt of PBS group, whereas the number of apoptotic cells was significantly reduced in the IL-1Ra group 24 h after 5-FU treatment (Fig. 2a). As indicated in Fig. 2b, the number of apoptotic cells count in the small intestinal crypt of the control group was 1.23 ± 0.56 and 9.67 ± 1.12 in the PBS group 24 h after 5-FU chemotherapy. However, the apoptotic cells count in the crypt of the IL-1Ra group was 5.78 ± 0.98. These results indicated that IL-1Ra could significantly reduce the intestinal crypt cell apoptosis induced by 5-FU.

To elucidate the mechanism of IL-1Ra in the prevention of intestinal apoptosis induced by chemotherapy, we investigated the locations of apoptotic cells in the intestinal crypt after chemotherapy. As indicated in Fig. 2c, at cell locations 4, 5, 6, and 7 of the small intestinal crypt 24 h after chemotherapy, the IL-1Ra protein group showed a lower proportion of apoptotic cells compared to the PBS group. There is a significant difference between the groups, and these positions were only locations of the small intestinal stem cells [25]. Therefore, we believe that the antiapoptotic effect of IL-1Ra mainly operated through inhibiting the apoptosis of crypt stem cells.

To determine the molecular mechanism of IL-1Ra inhibition of small intestinal cell apoptosis after chemotherapy, we analyzed the variation in expression of cell apoptosis-related proteins induced by 5-FU and IL-1β. As indicated in Fig. 2d, in the PBS group after 5-FU chemotherapy, the expression of proapoptotic factors, including Bax and caspase-3, was increased in a time-dependent manner. Although expression of the two aforementioned factors in the IL-1Ra group was enhanced, the increase was still significantly lower than that in the PBS group. We also found that the expression of antiapoptotic factors including Bcl-2 and Bcl-xL was significantly reduced after 5-FU chemotherapy. The expression of Bcl-2 and Bcl-xL basically disappeared 24 h after 5-FU treatment. Nevertheless, IL-1Ra could significantly inhibit the reduction of both antiapoptotic factors after 5-FU treatment. Moreover, elevated expression of Bcl-2 was still apparent 24 h after chemotherapy, and this result indicated that IL-1Ra is able to inhibit the small intestinal cell apoptosis induced by 5-FU by affecting the expression of apoptosis-related factors.

IL-1Ra treatment attenuated the severity of intestinal mucositis induced by 5-FU chemotherapy

We already demonstrated that IL-1Ra could inhibit the small intestinal apoptosis induced by 5-FU, and we further studied whether its inhibitory effect on apoptosis could attenuate intestinal mucositis after chemotherapy. As indicated in Fig. 3b, we found that the extent of diarrhea in the IL-1Ra group after chemotherapy was significantly lower than in the PBS group, and this result was direct evidence of the attenuation of mucositis by IL-1Ra. Subsequently, we studied morphological changes of the mucous membrane of the small intestine on the third day, when the most severe intestinal impairment was observed after 5-FU chemotherapy (Fig. 3a, *a–c*). We found that the intestinal structure in the PBS group was significantly destroyed, which was reflected by atrophy of the villi and vacuoles at the top. Nevertheless, the structure of intestinal villi in the IL-1Ra group remained quite complete. As indicated in Fig. 3c and d, the height of the intestinal villi and length of

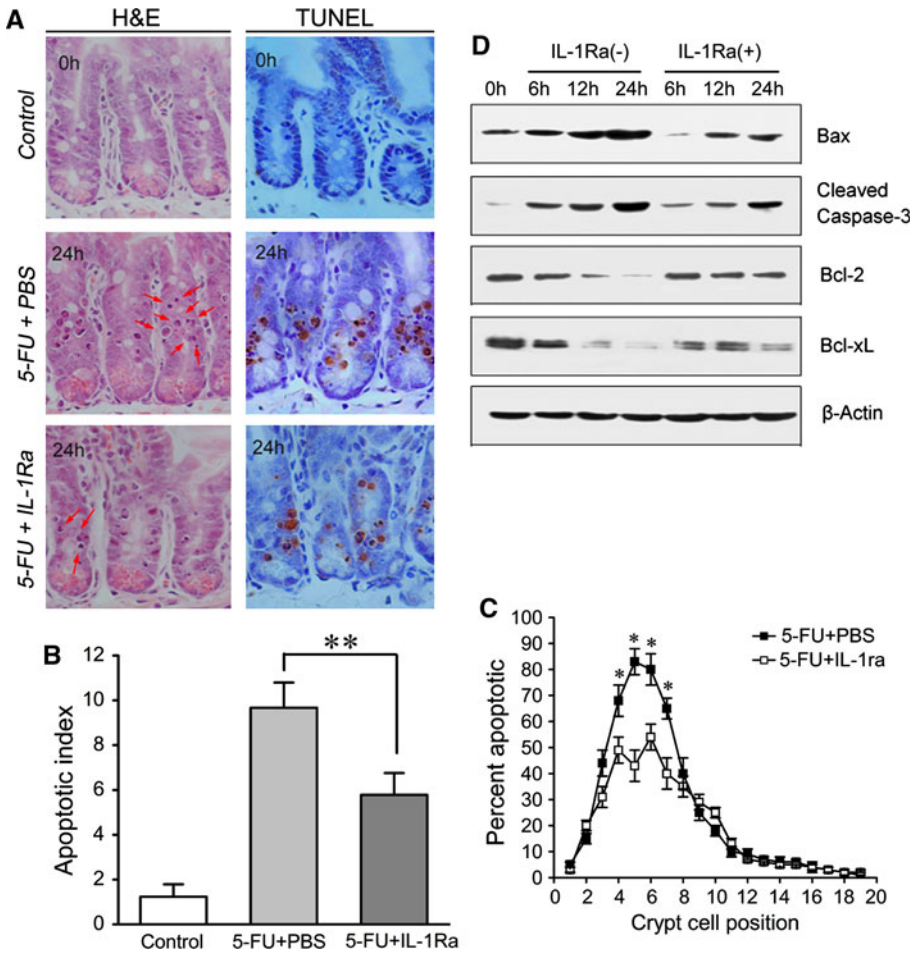

Fig. 2 IL-1Ra prevents 5-FU-induced intestinal apoptosis in mice. **a** H&E or TUNEL staining demonstrated that the incidence of apoptotic cells in the crypts was reduced in the IL-1Ra-treated mice when compared to the PBS-treated mice. Magnification, ×400. The apoptotic index **b** was defined as the average number of TUNEL-positive cells per crypt counted from 20 to 30 crypts ($n = 5$ mice/group). **c** Percentage of apoptotic cells at various crypt positions. Values are shown as the mean ± SD. * $P < 0.05$, 5-FU + PBS versus 5-FU + IL-1Ra. **d** Changes in the expression of Bax, caspase 3, Bcl-2, and Bcl-xL after 5-FU treatment were analyzed by western blotting

the crypt in the IL-1Ra group were significantly greater than that in the PBS group. This type of structural preservation was further verified by the weight of the small intestine. The weight of the small intestine in the IL-1Ra group was elevated 40.2% in comparison with the PBS group (Fig. 3f), which indicated that IL-1Ra could attenuate the intestinal mucositis induced by 5-FU chemotherapy.

To further investigate whether the protective role of IL-1Ra correlated with the enhancement of cell proliferation, we used immunohistochemistry to study proliferating cell nuclear antigen (PCNA) of the intestinal crypt on the third day after chemotherapy (Fig. 3a, *d–f*). As illustrated in Fig. 3e, the proliferation index of the crypt in the control group was (29.23 ± 1.59%), while the proliferation index in the PBS group was significantly inhibited after chemotherapy and was only 18.37 ± 5.04%. Nevertheless, plenty of PCNA-positive cells were observed in the intestinal crypt of the IL-1Ra group. The crypt proliferation index was 48.57 ± 4.13%, which indicated that IL-1Ra could enhance the proliferation activity of the intestinal crypt by inhibiting crypt cell apoptosis, thereby facilitating the recovery of the impaired mucous membrane after chemotherapy.

IL-1Ra prevents 5-FU-induced apoptosis in intestinal epithelial IEC-6 cells

Our in vivo experiments in mice verified the antiapoptotic effect of IL-1Ra. We next tried to demonstrate that IL-1Ra could also protect against the impairment of intestinal cells by 5-FU in vitro. As shown in Fig. 4b, the cell viability of IEC-6 cells was inhibited by 5-FU in a dose-dependent manner. At a 5-FU concentration of 100 μg/ml, 61.29% of cell viability was inhibited, which indicated that IEC-6 cells were quite sensitive to the effects of 5-FU. Different concentrations of IL-1Ra and 5-FU were cultured together for 24 h; we found that 1 and 10 μg/ml of IL-1Ra could significantly increase cell viability from 38.71 to 48.21% and 59.87%, respectively. However, a higher dose of IL-1Ra (100 μg/ml) was unable to enhance cell viability. To study whether its protective role correlated with the inhibition of apoptosis, we used the Hoechst 33342 staining method to detect cell apoptosis (Fig. 4). When IL-1Ra (10 μg/ml) was cultured together with 5-FU for 24 h, the apoptotic numbers were significantly reduced. As indicated in Fig. 4c, cell apoptosis was reduced 31% in the IL-1Ra group when compared to the

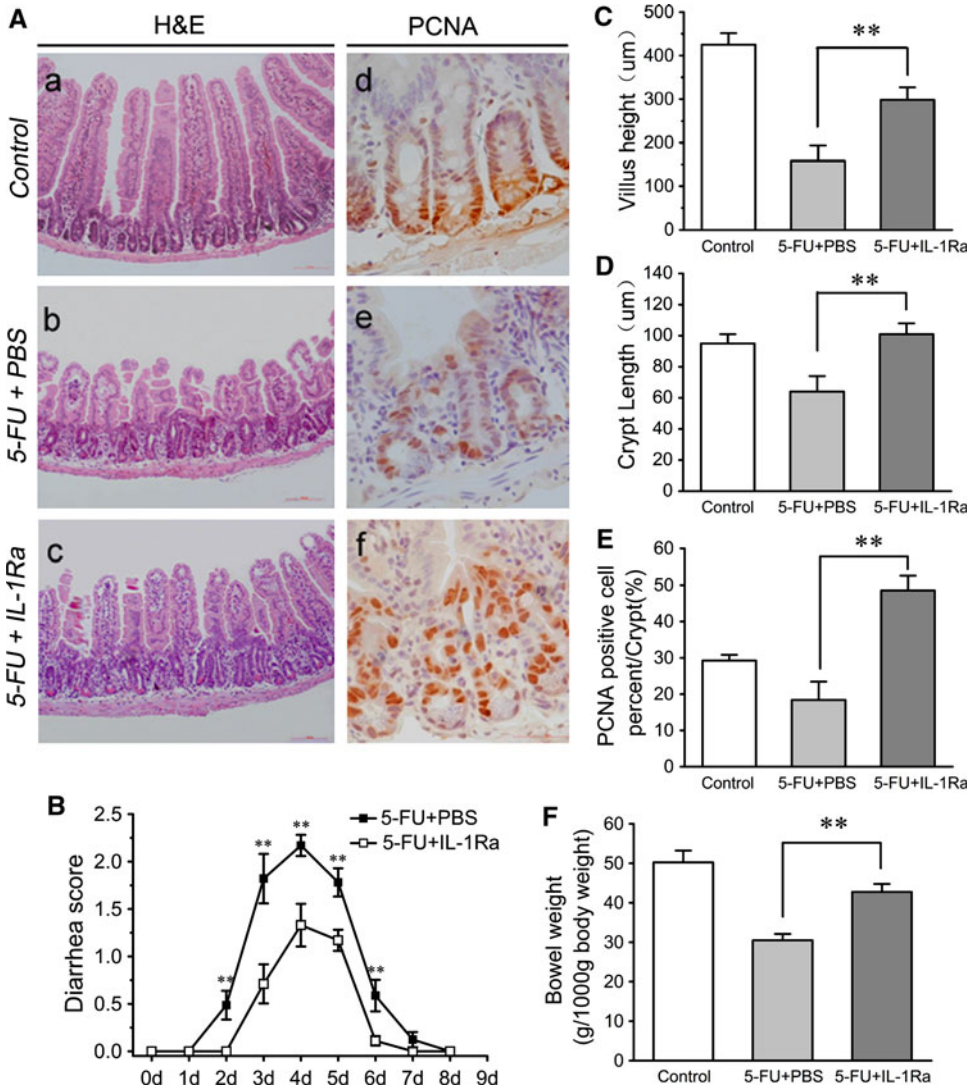

Fig. 3 IL-1Ra attenuated the severity of 5-FU-induced intestinal mucositis in mice. **a** H&E staining (*a–c*, 100×) and PCNA Immunostaining (*d–f*, ×400) were performed on Day 3. **b** Mice were monitored daily with regard to diarrhea score, 5 mice per group continued the 8-day diarrhea evaluation experiment. Some mice ($n = 5$ mice/group) were sacrificed on Day 3 to examine the effects of IL-1Ra on villus height **c** and crypt length **d** in the mid-jejunum. Longitudinal tissue sections of 20 villi and crypts were counted per mouse. **e** The proliferation index was derived from the percentage of PCNA-positive cells per crypt in 25 crypts. The weights of the small intestine were shown in graph **f**. Values are shown as the mean ± SD; ** $P < 0.01$ represents significant differences between the indicated pairs

5-FU single use group (24.45 ± 2.91 vs. 35.34 ± 3.32), which indicated that IL-1Ra could also protect intestinal cells by inhibiting the cell apoptosis induced by 5-FU in vitro.

IL-1Ra did not affect 5-FU-induced tumor regression in mice with CT-26 xenografts

We established a tumor-bearing mouse model to study the effect of IL-1Ra on tumor growth in mice after 5-FU chemotherapy. As shown in Fig. 5, the use of IL-1Ra was unable to affect tumor growth, and 5-FU with or without IL-1Ra caused the tumor weight to decrease to 39.12 ± 8.2% and 44.85 ± 9.4% of the control group, respectively. Moreover, we did not detect any significant difference in tumor apoptosis and proliferation between control and IL-1Ra-treated groups (date not shown).

Discussion

Our study first used RT-PCR and immunohistochemical methods simultaneously to verify the significantly increased expression of IL-1β in small intestinal tissue at both the gene and protein levels during the initial stage of 5-FU chemotherapy. This tendency for change was in accordance with expression in the peripheral blood. It was surprising that the rapid rise in the IL-1β gene and protein levels was generated during the initial stage after chemotherapy and did not occur at the same time as the most severe impairment of the mucous membrane of the small intestine. This phenomenon was in accordance with the genesis and development of intestinal cell apoptosis. It has been reported that the expression of proinflammatory cytokines such as IL-1β, IL-6 and TNF-α in the small intestine and colon of rats after chemotherapy was significantly enhanced [9]. Logan et al.

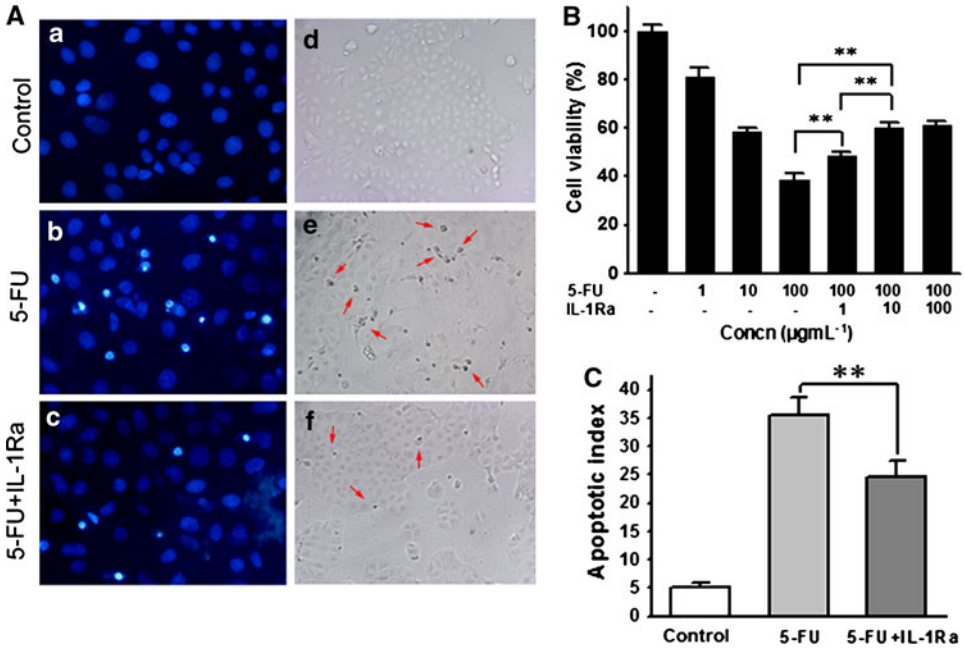

Fig. 4 IL-1Ra suppresses 5-FU-induced apoptosis in vitro. **a** The fluorescent (*a–c*, 400×) and phase contrast (*d–f*, 100×) microscopic images were presented. Red arrows indicate the apoptotic cells. **b** Cell viability was assessed with Cell Counting Kit-8 solution. Data were expressed as relative viability. The apoptotic index **c** was defined as the average number of cells with apoptotic chromatin changes per field in 5 continuous microscopic fields at 100× magnification. Values are shown as the mean ± SD of three independent experiments; ** $P < 0.01$ represents significant differences between the indicated pairs

found that the enhanced IL-1β expression in intestinal tissue after 5-FU chemotherapy appeared before the genesis of intestinal mucositis, but they did not explain the cause of this phenomenon. Additionally, previous studies reported that IL-1β could induce apoptosis in multiple cell lines [11–14]. We considered that IL-1β played a critical role in the genesis and development of intestinal mucositis after chemotherapy and that this type of effect was caused by inducing intestinal crypt cell apoptosis. We hypothesized that the application of IL-1Ra, a natural antagonist of IL-1β, could inhibit the intestinal apoptosis induced by 5-FU, thereby relieving intestinal mucositis after chemotherapy. The inhibitory effect of IL-1Ra on cell apoptosis has been verified by previous investigators. In experimental models of spinal injury [13], myocardial damage [22], and renal injury [26], IL-1Ra could inhibit the genesis of cell apoptosis by inhibiting the effect of IL-1β, thereby relieving the diseases.

We used the mucositis model in mice induced by a single dose of 5-FU chemotherapy to study the protective effect of IL-1Ra during chemotherapy. The results indicated that the exogenous application of IL-1Ra could significantly reduce intestinal crypt cell apoptosis 24 h after 5-FU chemotherapy, thereby relieving the severity of intestinal mucositis after chemotherapy. Moreover, the antiapoptotic effect of IL-1Ra on intestinal crypt IEC-6 cells has been verified in vitro. We also demonstrated that the antiapoptotic effect of IL-1Ra mainly occurred through a reduction in the apoptosis of stem cells at positions 4–7 of the small intestinal crypt. The reduction in stem cells apoptosis means that more rapidly proliferating cells can be preserved. This result elucidated why in the third day of the

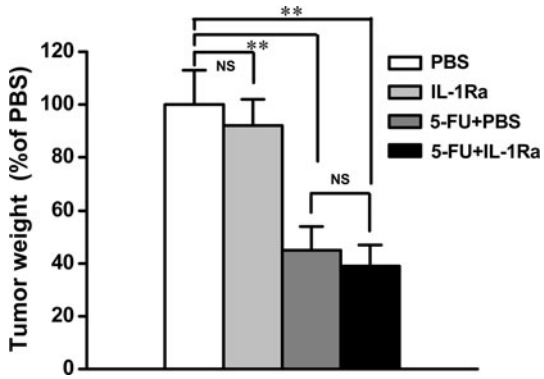

Fig. 5 Effect of IL-1Ra on tumor size in tumor-bearing mice treated with 5-FU. On Day 7 after grafting CT-26 colon tumor cells, mice were randomly divided into four groups ($n = 5$ mice/group). A single dose of 5-FU or PBS as a control was then injected. The mice were treated with either 0.2 ml of PBS or IL-1Ra administered subcutaneously twice daily from Day 7 to 20. Mice were euthanized on Day 20, and the tumors were excised and weighed. Values are shown as the mean ± SD. ** $P < 0.01$, versus PBS group; NS shows no significance

most severe impairment of intestinal mucosa, the histology results and diarrhea scores in IL-1Ra group were better than PBS group. PCNA immunohistochemistry also indicated that on the third day after chemotherapy, the intestinal crypt proliferation rate in the IL-1Ra group was significantly higher than in the PBS group. Therefore, we considered that the protection by IL-1Ra mainly occurred by inhibiting the chemotherapy-induced apoptosis in stem cells of the intestinal crypt. This inhibition simultaneously preserved more regenerative cells of the small intestine, relieved mucosal impairment of the small intestine, and facilitated the recovery of the intestinal mucosa.

To determine the mechanism of IL-1Ra, we hypothesized that the administration of excessive IL-1Ra in the body could inhibit the intestinal cell apoptosis induced by IL-1β after 5-FU chemotherapy by competitively binding to IL-1R and relieving the impairment of the intestinal mucosa. Apoptosis is the critical factor for intestinal mucositis in 5-FU chemotherapy; the genesis of intestinal cell apoptosis was accompanied by the enhancement of Bax expression in the intestinal crypt [5]. Mahr et al. found that IL-1β could facilitate cell apoptosis by altering the expression of Bax and Bcl-2 [16]. Cell apoptosis induced by IL-1β was also involved in the activation of multiple apoptosis-related factors such as Bak and Bcl-x [15]. However, the role of IL-1β in cell apoptosis of the mucous membrane of the small intestine was uncertain. Our experiment verified that exogenous injection of IL-1Ra not only significantly reduced the number of apoptotic cells in the small intestinal crypt after chemotherapy, but that this type of protection was also accompanied by the reduction of the up-regulation of Bax and caspase 3 and down-regulation of Bcl-2 and Bcl-xL. However, the balance between apoptotic and antiapoptotic markers 24 h after IL1Ra seems to be very similar to what observed 12 h after 5-FU. One possible reason is that IL1Ra delays the induction of apoptotic markers after chemotherapy. Indeed, IL1Ra had reduced the expression of apoptotic markers and increased the expression of antiapoptotic markers 24 h after 5-FU when the peak of apoptosis was observed. According to the results presented above, IL-1β quite possibly facilitated apoptosis during the course of the small intestinal apoptosis induced by 5-FU chemotherapy. Moreover, the promotion of this apoptotic effect correlated with the enhanced expression of proapoptotic factors such as Bax and caspase 3 and the reduced expression of antiapoptotic factors such as Bcl-2 and Bcl-xL. IL-1Ra could inhibit the genesis of apoptosis by inhibiting the role played by IL-1β. The detailed mechanism is still in need of further investigation.

The intestinal mucositis induced by chemotherapy in a clinical setting was mainly generated in the patients suffering from tumors. To mimic the clinical status, we used a tumor-bearing mouse model to investigate whether IL-1Ra played a role in tumor growth and genesis. A recent study reported that IL-1β generated by autocrine tumor tissue played a role in facilitating tumor invasion [27] and that IL-1Ra could not only inhibit melanoma and the growth of liver metastasis [27, 28] but also inhibited the growth of tumor cells in the hematological system [29]. Therefore, we tried to demonstrate that IL-1Ra could facilitate the chemotherapeutic effect on tumor-bearing mice by inhibiting tumor cell growth. The results indicated that the application of IL-1Ra was unable to significantly enhance the antitumor effect of 5-FU. Although our experiment did not yield the anticipated results, it did at least indicate that IL-1Ra did not affect the whole effect of 5-FU chemotherapy.

In conclusion, the exogenous application of IL-1Ra can reduce the intestinal cell apoptosis induced by 5-FU chemotherapy, and the in vitro and in vivo experiments have all demonstrated this ability. The antiapoptotic effect of IL-1Ra during the initial stage of chemotherapy significantly relieved diarrhea in the mice and impairment of the intestinal mucosa after chemotherapy. Furthermore, IL-1Ra did not affect the antitumor effect of 5-FU. Therefore, we believe that IL-1Ra could be used as a potential novel clinical therapeutic strategy for the prevention and cure of chemotherapy-induced intestinal mucositis and that the quality of life of the patients could be improved by reducing chemotherapeutic side effects such as diarrhea.

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