Cytoprotection against neutrophil derived hypochlorous acid: a potential mechanism for the therapeutic action of 5-aminosalicylic acid in ulcerative colitis

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Abstract

The aim of the present study was to investigate the effects of 5-aminosalicylic acid (5-ASA) on the cell injury mediated by activated neutrophils. We used a system constituted of neutrophils, triggered with phorbol myristate acetate, and ^{51}Cr -labelled Daudi cells as targets. The results show that 5-ASA is capable of efficiently preventing neutrophil-mediated lysis. 5-ASA was up to 10-fold more effective than taurine, which acts as an hypochlorous acid scavenger. Moreover, 5-ASA was found to compete with taurine for the neutrophil derived hypochlorous acid. The results are consistent with the conclusion that 5-ASA is capable of limiting the neutrophil mediated cell damage by scavenging the generated hypochlorous acid. This may represent a potential mechanism for the therapeutic action of 5-ASA in ulcerative colitis.

The therapeutic benefit of 5-aminosalicylic acid (5-ASA) in ulcerative colitis is well established.^{1,2} It appears to act directly on the inflamed mucosa possibly by blocking cyclooxygenase and lipoxygenase metabolite mediated inflammation³ and/or by reducing the local generation of highly reactive oxygen species.^{4,5} This latter possibility is particularly attractive because of the neutrophil infiltration of the colonic mucosa, coupled with the well known ability of neutrophils to produce reactive oxygen species.⁶ Nevertheless, to our knowledge, the actual capacity of 5-ASA to interfere with the oxidative cell damage mediated by neutrophils has not been directly proved. In the present paper, using a cytotoxicity model generally accepted to study the neutrophil mediated cellular damage (phorbol myristate acetate – triggered neutrophils plus Daudi target cells),^{7,8} we provide evidence that 5-ASA is highly effective in limiting the neutrophil cytolytic potential.

Methods

MEDIA AND REAGENTS

Hanks' balanced saline solution with 1 mg/ml glucose and without phenol red (HBSS, Flow Lab, Ltd, Irvine, Scotland) was used as incubation medium. RPMI 1640 and fetal calf serum were purchased from Flow Lab. Taurine (TauNH_2), 5-aminosalicylic acid (5-ASA) and Triton X-100 were purchased from Sigma Chemical Co, St Louis, MO. The drug 5-ASA

(~99% pure, as assayed by Sigma; lot 15 F-0803) was dissolved at the concentration of 5 mmol/l in 0.9% NaCl (37°C) immediately before each experiment. The solution of the drug was maintained in the dark to avoid 5-ASA oxidation by light. (The assays were carried out in the dark as well.) Heparin (Liquemin) was from Roche, Milan, Italy, and Ficoll-Hypaque from Nyegaard Co, Oslo, Norway. $\text{Na}_2(^{51}\text{Cr})\text{O}_4$ was from the Radiochemical Centre, Amersham, England. Phorbol-12-myristate-13-acetate (PMA, Sigma), stored at -20°C as stock solution of 2 mg/ml in dimethylsulphoxide (C Erba, Milan, Italy) was diluted in medium and used at the final concentration of 10 ng/ml. 5-thio-2-nitrobenzoic acid (Nbs) was prepared by reducing 5-5'-dithiobis (2-nitrobenzoic) acid (Sigma), as described by Aune and Thomas.⁹ Hypochlorous acid was generated by adding sodium hypochlorite (NaOCl , BDH Ltd, Pool, UK) into solution buffered at pH 7.4.¹⁰

NEUTROPHILS

Heparinised (heparin 10 U/ml) venous blood was obtained from healthy male volunteers. Neutrophils were isolated by dextran sedimentation and subsequent centrifugation on a Ficoll-Hypaque density gradient, as previously described.¹⁰ Contaminating erythrocytes were removed by hypotonic lysis.¹⁰ Neutrophils were then washed three times with HBSS and resuspended in HBSS. Final cell suspensions contained 97% or more neutrophils and more than 98% viable cells, as evaluated by the ethidium bromide fluorescein diacetate test.¹⁰

DAUDI CELLS

The Daudi cell line (B lymphoblasts, kindly supplied by Prof G Damiani, Department of Biochemistry, University of Genova, Italy) was grown in suspension (RPMI-FCS) and subcultured every four to five days.¹¹ The cells were washed three times with HBSS and resuspended in HBSS before use. Daudi cells were labelled with 100–200 μCi sodium ^{51}Cr -chromate by incubating for one hour at 37°C .¹¹ After being washed, the cells were resuspended in HBSS.

CYTOLYTIC ASSAY

Daudi cell lysis by neutrophils in the presence of phorbol myristate acetate was measured using a ^{51}Cr release method.¹⁰ The experiments were carried out in duplicate using 2×10^6 neutrophils,

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Figure 1: Inhibition of the neutrophil cytolytic activity (ordinate) by 5-ASA. Results are expressed as mean (1) SD of at least three experiments. The % ^{51}Cr release from labelled Daudi cells, incubated with phorbol myristate acetate triggered neutrophils in the absence of 5-ASA, was 27.6 (4.3) ($\times(1)$ SD, $n=4$). The compound 5-ASA had no effect on the ^{51}Cr release from Daudi cells incubated in absence of neutrophils.

5×10^5 Daudi cells, and 10 ng/ml phorbol myristate acetate in a final volume of 1 ml. Tests were done in Falcon Plastic tubes (17 $\times$ 100 mm, Falcon Plastic, Oxnard, Calif) and in a shaking water bath (100 rpm) at 37°C. After incubation for two hours, the ^{51}Cr release from labelled target cells was determined in the cell free supernatants. The percentage of cytolysis (% ^{51}Cr release) was calculated according to the formula $(E - S)/(T - S) \times 100$, where E is the cpm released in the presence of effectors, T is the cpm released after lysing target cells with 5% Triton X-100, S is the cpm spontaneously released by target cells in the absence of effectors (in each case $\leq 10\%$).

HYPOCHLOROUS ACID ASSAY

The generation of hypochlorous acid by neutrophils in the presence of phorbol myristate acetate was measured by the taurine (TauNH₂) trapping technique,¹² as previously described.¹⁰ Briefly, 1×10^6 neutrophils and phorbol myristate acetate 10 ng/ml were incubated (37°C, one hour) in a final volume of 1 ml in the presence of 20 mmol/l TauNH₂ (17 $\times$ 100 mm Falcon Plastic tubes, shaking water bath at 100 rpm). After incubation, the amount of TauNHCl, generated by the reaction between hypochlorous acid and

TauNH₂, (TauNH₂ + hypochlorous acid $\rightarrow$ TauNHCl + H₂O) was determined in the cell free supernatants by measuring spectrophotometrically (OD=412 nm, $\epsilon = 1.36 \times 10^4$ mol/l/cm) the TauNHCl mediated oxidation of 5-thio-2-nitrobenzoic acid.¹² A series of experiments was also planned to detect the ability of 5-ASA to limit the formation of Tau NHCl from reagent hypochlorous acid and TauNH₂. These experiments were carried out by adding reagent hypochlorous acid (40 $\mu\text{mol/l}$) to a mixture of TauNH₂ (20 mmol/l) and 5-ASA in doses ranging from 0.05 to 1.0 mmol/l (final volume of the reaction = 1 ml). After incubation for 10 minutes at 37°C, the amount of the generated TauNHCl was measured as described above.

OXYGEN CONSUMPTION ASSAY

The oxygen consumption by neutrophils was measured polarographically using a Clark electrode (Oxygen Monitor, Yellow Springs Instrument Co, Yellow Spring, Ohio) and using the technique described by Metcalf and coworkers.¹³ Cell suspensions of 8×10^6 neutrophils in 2 ml HBSS were agitated continuously at 37°C. Oxygen consumption was measured after stimulation with phorbol myristate acetate (10 ng/ml) and the results expressed as nanomoles of oxygen consumed by 4×10^6 neutrophils per minute.

MYELOPEROXIDASE ASSAY

The myeloperoxidase activity released by neutrophils (10^6 cells/ml), incubated (one hour, 37°C) with 10 ng/ml phorbol myristate acetate, was determined in cell free supernatants as previously described.¹⁰ Briefly, the myeloperoxidase assay was done by using 0.167 mg/ml 0-dianisidine (Sigma) and 0.1 mmol/l H₂O₂ in 50 mmol/l phosphate buffer (pH=6). One unit of enzyme activity was defined as that oxidising 1 μmol 0-dianisidine/min/25°C (od=550, $\epsilon = 11.3$ mmol/l/cm).

Results

As shown in Figure 1, 5-ASA inhibited the neutrophil lytic activity in a dose dependent manner. Moreover, such a neutrophil function was almost completely inhibited by 20 mmol/l taurine (TauNH₂) which traps the neutrophil derived hypochlorous acid yielding TauNHCl¹² (% ^{51}Cr release from labelled Daudi cells incubated with phorbol myristate acetate triggered neutrophils: 3.5 (2.7) and 28.8 (3.2) in the presence and absence of 20 mmol/l TauNH₂ respectively, $\times(1\text{SD})$, $n=3$).

As depicted in Figure 2, 5-ASA lowered the recovery of TauNHCl from phorbol myristate acetate-triggered neutrophils incubated in the presence of TauNH₂ (20 mmol/l). This suggests that 5-ASA is likely to compete with TauNH₂ for the hypochlorous acid generated by neutrophils. Consistent with such a possibility, 5-ASA limited the formation of TauNHCl from reagent hypochlorous acid and TauNH₂ in a dose dependent manner (Fig 3). Thus, it appears that 5-ASA inhibits the neutrophil cytolysis

Figure 2: Inhibition of the hypochlorous acid recovery (measured as TauNHCl) from phorbol myristate acetate triggered neutrophils by 5-ASA. Results are expressed as mean (1) SD of at least three experiments. The TauNHCl recovery from neutrophils in the absence of 5-ASA was: 56.88 (4.6) nmol/10⁶ neutrophils/1 h ($\times(1)$ SD, $n=4$).

Figure 3: Effect of various doses of 5-ASA on the TauNHCl recovery from a system constituted of reagent hypochlorous acid (40 $\mu\text{mol/l}$) and TauNH₂ (20 mmol/l). Tests were performed in a final volume of 1 ml.

primarily by trapping the generated hypochlorous acid.

The compound 5-ASA gave only slight inhibition of the neutrophil oxygen uptake and myeloperoxidase positive primary granule exocytosis. When used at the concentration of 1 mmol/l, 5-ASA inhibited the neutrophil oxygen consumption by 15–8%. Concentrations of 5-ASA lower than 1 mmol/l were completely ineffective (oxygen consumption by phorbol myristate acetate triggered neutrophils in the absence of the drug: 8.74 (0.70) nmol oxygen per 4×10^6 cells per minute, $\times(1SD)$, $n=3$). Finally, a slight inhibition (18–6%) of the myeloperoxidase release from neutrophils was only observed using relatively high doses (1 mmol/l) of 5-ASA (myeloperoxidase release from phorbol myristate acetate triggered neutrophils in the absence of the drug: 2.86 (0.32) mU/ 10^6 cells/1h, $\times(1SD)$ $n=3$).

Discussion

Our results suggest that 5-ASA is capable of inhibiting the neutrophil cytolytic efficiency by scavenging the generated hypochlorous acid (HOCl). In agreement with reports from other authors,^{7,14} the generation of hypochlorous acid by the neutrophil myeloperoxidase pathway ($\text{H}_2\text{O}_2 + \text{Cl}^- \xrightarrow{\text{MPO}} \text{HOCl} + \text{H}_2\text{O}$) is crucial in promoting the neutrophil-dependent cell damage. In fact, TauNH₂, capable of efficiently trapping the generated hypochlorous acid to yield TauNHCl,¹² inhibited the lysis. When compared on a molar basis, 5-ASA was much as 10-fold more effective than TauNH₂ in reducing the neutrophil lytic activity. Also, 5-ASA was found to efficiently compete with TauNH₂ for reagent or neutrophil derived hypochlorous acid, lowering the production of TauNHCl. Thus, it appears that the reaction between 5-ASA and hypochlorous acid is efficient enough to protect the target cells from the neutrophil delivered hypochlorous acid dependent attack. Consistent with such a conclusion, 5-ASA was found to reduce the lysis of fibroblasts by a neutrophil free myeloperoxidase- H_2O_2 -Cl-system,¹⁵ presumably able to produce hypochlorous acid.¹⁴

The ability of 5-ASA reported here to limit the neutrophillytic efficiency observed at 5-ASA

doses well within those detectable in the distal part of the intestinal lumen,¹⁶ raises the possibility that this effect of the drug may contribute to its therapeutic activity in ulcerative colitis. This does not contradict the view that 5-ASA may also act by suppressing the production of prostaglandins or leucotrienes or both,^{5,16} although the interference with the arachidonic acid metabolism has been reported to occur at doses of 5-ASA clearly higher than those used in the present set.

Moreover, owing to the wide range of biologic activities exerted by hypochlorous acid,¹⁴ the cytoprotection against neutrophil attack is likely to be only one of the consequence of the hypochlorous acid-5-ASA interaction. In fact, the hypochlorous acid capacity of activating the neutrophil latent collagenase and inactivating proteinase inhibitors¹⁴ suggests that the ability of 5-ASA to scavenge neutrophil derived hypochlorous acid may limit the degradation of connective tissue components other than the tissue cell damage. Consistent with this view, 5-ASA has been recently shown to limit the inactivation of α_1 -antiproteinase by reagent hypochlorous acid.¹⁷

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