Anti-Cancer Drugs 1996, **7**, pp. 235–239

Prophylactic action of allopurinol against chemotherapy-induced stomatitis—inhibition of superoxide dismutase and proteases

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The activities of superoxide dismutase (SOD) and several proteases were measured in kidney of mice treated with allopurinol in order to elucidate the mechanism of prophylactic action of allopurinol against chemotherapy-induced stomatitis. The following results were obtained. Following 3 day administration of allopurinol 20 mg/day per os (Group C), the concentrations of allopurinol and oxipurinol in the renal tissue were 203.9 ± 52.1 and 1141.7 ± 194.8 μg/g, respectively. The SOD activity was significantly lower in Group C than in the untreated control group ($p < 0.01$). The enzyme activities of papain and trypsin were suppressed in Group C. However, the other proteases tested were not affected by the administration of allopurinol, indicating only weak anti-protease action of allopurinol. These results suggest that allopurinol may be effective to prevent chemotherapy-associated stomatitis via both direct and indirect actions to oral mucosa, that include inhibitory actions on xanthine oxidase as well as protease.

Key words: Allopurinol, mouthwash, stomatitis, superoxide dismutase, protease.

Introduction

Stomatitis is a common side effect of anti-cancer chemotherapy. Being a limiting factor of chemotherapy, it causes intolerable pain and sometimes becomes so severe as to interfere with any oral intake. Relieving or eliminating this side effect will be beneficial for further advancement of anti-cancer chemotherapy as well as improvement of patient's quality of life.

In 1985, Clark *et al.*[1] paid attention to allopurinol modification of the toxicity of 5-fluorouracil (5-FU)[2] and reported a lower incidence and severity of stomatitis in patients on 5-FU therapy who used allopurinol mouthwash. We also reported the high efficacy of allopurinol mouthwash in patients with anti-tumor chemotherapy.[3]

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Although the mechanism of chemotherapy-induced stomatitis is not well understood, it seems beyond doubt that superoxide plays a role in the development of stomatitis following anti-cancer chemotherapy because quinone chemotherapeutic agents exert their anti-cancer actions by producing active oxygens in their reduction–oxygenation cycles.[4] In addition, as proteases are involved in the activation of NAD(P)H oxidase,[5] they may also possibly contribute to the development of chemotherapy-induced stomatitis.

The present study determined the inhibitory effects of allopurinol on the activities of superoxide dismutase (SOD) and several proteases in mice to help clarify the prophylactic mechanism of chemotherapy-induced stomatitis with allopurinol, which shows a suppressive effect on the production of active oxygens by inhibiting xanthine oxidase.[6]

Materials and methods

Materials

Allopurinol was supplied by Tanabe Pharmaceutical Co. (Osaka, Japan). Tris-(hydroxymethyl)aminomethane, acetic acid, 2-mercaptoethanol, dimethyl sulfoxide, anhydrous disodium hydrogen phosphate, monobasic potassium phosphate, sodium cholide, potassium chloride (all the above were guaranteed reagents; Nacarai Tesque, Kyoto, Japan), SOD-525 (Bioxytech SA, France) and DC Protein Assay (Bio-Rad, USA) were used. Trypsin, chymotrypsin, elastase, papain and cathepsin B (all from Sigma, St Louis, MO) were tested. Fluorescence-labeled peptide as a substrate for each enzyme was obtained from Peptide Institute (Osaka, Japan) (Table 1). Male mice of the ddy strain weighing 20–24 g were used.

Table 1. Enzymes and MCA substrates

Enzyme	MCA substrates
Papain	Boc-Leu-Thr-Arg-MCA
Trypsin	Boc-Phe-Ser-Arg-MCA
Cathepsin B	Z-Alg-Alg-MCA
Elastase	Suc-Ala-Pro-Ala-MCA

Boc, tert-butyloxycarbonyl; Suc; succinyl; MCA, 4-metylcoumarin amide.

Methods

Procedures of administration of allopurinol. Allopurinol was administered to mice orally once daily. Four dose groups were employed: Group A mice ($n=4$) received 2 mg/animal/day for three consecutive days; Group B mice ($n=5$) received 2 mg/animal/day for two consecutive days, then, 20 mg/animal for 1 day; Group C mice ($n=4$) received 20 mg/animal/day for three consecutive days; and Control Group mice ($n=5$) did not receive allopurinol.

Preparation of supernatant fractions of kidney extracts. On the fourth day after the start of allopurinol administration, a kidney from each animal was extracted and washed with an ice-cold buffer (1.15% KCl–0.01 M phosphate, pH 7.4). Then, the kidney was homogenized with 3 volumes of the above buffer, and the homogenate was centrifuged at 10 000 r.p.m. for 20 min at 4°C.[7] The resulting supernatant fluid was used as supernatant fraction.

Quantitative determination of allopurinol and oxipurinol in mouse kidney. Tissue concentration of these compounds in mouse kidney was measured by HPLC.[8,9]

Measurement of SOD activity. SOD activity in the kidney was measured by use of SOD-525. As shown in Figure 1, to 40 μl of the supernatant fraction of kidney, 30 μl of reagent R2 (mercaptan scavenger) and 900 μl of buffer (0.11 mM diethylenetriaminepentaacetic acid, pH 8.8) were added, mixed well and incubated at 37°C for 1 min. After incubation, 30 μl of chromogenic reagent R1 was added and mixed. Immediately after mixing, absorbance at 525 nm was measured with a spectrophotometer (Model 150-20, Hitachi). Protein concentration was measured by the DC Protein Assay.[10]

Measurement of inhibition of enzymatic activity.[11] Required concentrations of allopurinol and enzymes were prepared using an incubation buffer

40 μl of sample (supernatant fractions of kidney)
↓
add 30 μl of solution of reagent R2
↓
add 900 μl of buffer (pH 8.8, containing 0.11 mM diethylenetriaminepentaacetic acid)
↓
mix thoroughly
↓
incubate at 37°C for 1 min
↓
add 30 μl of solution of reagent R1
↓
mix rapidly
↓
measure immediately the absorbance at 525 nm

Figure 1. Measurement of SOD activity. SOD activity in kidney of mice was measured by use of the SOD-525 kit (Bioxytech SA, France). Preparation of supernatant fractions of kidney was shown in Methods.

that consisted of 50 mM Tris–HCl, 50 mM NaCl and 60 mM 2-mercaptoethanol (pH 7.9), and substrates were dissolved in dimethyl sulfoxide. As shown in Figure 2, to 5μl of a supernatant fraction of kidney, 5μl of an enzyme solution of various concentrations was added. After pre-incubation at 4°C for 15 min, 3 μl of 2 mM substrate, 10 μl of allopurinol of different concentration and 47 μl of the incubation buffer were added, and the mixture was incubated at 37°C for 15 min. Then, the reaction was stopped with the addition of 90 μl of 10% acetic acid. The reaction mixture was diluted with 1850 μl of purified water, and fluorescence of 7-amino-4-methylcoumarin in the mixture was measured with a fluorescence spectrophotometer (Model 650-10s, Hitachi) at an excitation wavelength of 380 nm and an emission wavelength of 460 nm. For the reaction, concentrations of the enzyme and the substrate were set at 5×10^{-5} μg/ml and 2.0 mM, respectively. By setting the intensity of fluorescence of the reaction mixture in the control group at 100 in the spectrophotometer, fluorescence in the test groups was examined. When the reading was 0, inhibitory activity was judged to be positive.

Results

Tissue concentration of allopurinol and oxipurinol

As is shown in Table 2, allopurinol and oxipurinol concentrations in the kidney were below the detection limit (0.5 μg/g) in Group A, the 2 mg admin-

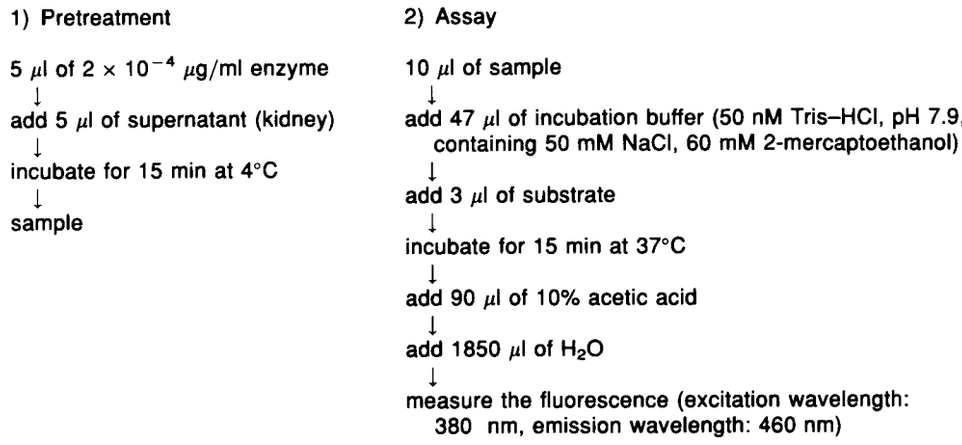

Figure 2. Measurement of inhibition of enzymatic activity. Proteases and substrates were shown in Table 1.

Table 2. Concentrations of allopurinol and oxipurinol in kidney of mice

	Allopurinol concentration (μg/g)	Oxipurinol concentration (μg/g)
Group A ($n=4$)	ND	ND
Group B ($n=5$)	41.5 ± 3.6	314.7 ± 62.7
Group C ($n=4$)	203.9 ± 52.1	1141.7 ± 194.8

Group A received 2 mg/day for three consecutive days. Group B received 2 mg/day for two consecutive days, then 20 mg for 1 day. Group C received 20 mg/day for three consecutive days. Data are presented as mean ± SD. ND, not detected (< 0.5 μg/g).

Table 3. Inhibition of enzymatic activity in kidney of mice treated with allopurinol

	Incidence of inhibition (%)			
	Papain	Trypsin	Cathepsin	Elastase
Group A ($n=4$)	25 (1[a]/4)	50 (2[a]/4)	0 (0/4)	0 (0/4)
Group B ($n=5$)	1 (1/5)	80 (4[a]/5)	0 (0/5)	0 (0/5)
Group C ($n=4$)	100 (4[a]/4)	100 (4[a]/4)	0 (0/4)	0 (0/4)

[a]Positive case. When enzymatic activity was 0, inhibiting activity was judged to be positive. Group A received 2 mg/day for three consecutive days. Group B received 2 mg/day for two consecutive days, then 20 mg for 1 day. Group C received 20 mg/day for three consecutive days.

istration group. In contrast, in Group C, the 20 mg administration group, allopurinol concentration in the kidney was 203.9 ± 52.1 μg/g. Oxipurinol concentration in this group was 1141.7 ± 194.8 μg/g in the kidney. Oxipurinol concentration was higher than allopurinol in the kidney.

SOD activity

SOD activity decreased in the order of Groups B, A and C. Especially, the activity in Group C was 1.49–1.85 (mean = 1.70 ± 0.16) × 10^3 units/mg protein and this was significantly lower than the activity in the control group (1.98–2.12, mean = 2.05 ± 0.03, × 10^3 units/mg protein) ($p < 0.01$) (Figure 3).

Inhibition of enzyme activity

As to the effect on papain, inhibition in the kidney was observed in one case of four (25%) in Group A, one of five (20%) in Group B and four of four (100%) in Group C. Against trypsin, inhibition was observed in two of four (50%) in the Group A, four of five (80%) in Group B and four of four (100%) in Group C (Table 3). No inhibitory activity against cathepsin or elastase was observed in Groups A, B or C.

Discussion

Clark and Slevin used a mouthwash of 3% methylcellulose solution containing allopurinol 1.0 mg/ml in patients treated with 5-FU and reported a lower incidence and severity of stomatitis.[1] This report drew attention to the beneficial effect of allopurinol in preventing chemotherapy-induced stomatitis and was followed by several other studies.[3,12–14] We also prepared allopurinol mouthwash solution using carboxymethylcellulose, and had it used by patients

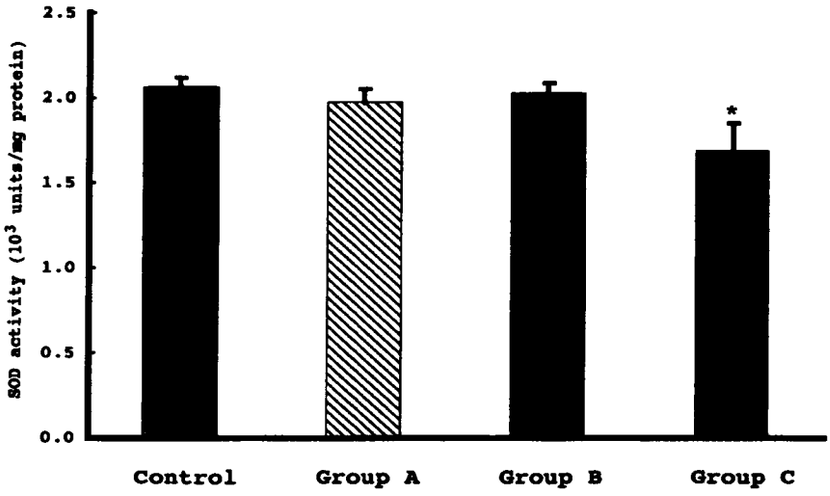

Figure 3. Inhibitory effect of allopurinol on the level of SOD in kidney of mice. Control ($n=5$) did not receive allopurinol. Group A ($n=4$) received 2 mg/day for three consecutive days. Group B ($n=5$) received 2 mg/day for three consecutive days, then 20 mg for 1 day. Group C ($n=4$) received 20 mg/day for three consecutive days. Vertical column and bar represent mean value and standard deviation, respectively. *Significantly different from control ($p<0.01$).

with uterine cancers receiving chemotherapy of 5-FU+cisplatin or vincristine ± actinomycin D+cyclophosphamide therapy. As a result, the incidence of stomatitis was markedly reduced in these patients.[3] However, there are some studies denying this anti-stomatitis effect of allopurinol.[14] Doses of chemotherapeutic agents, the concentration of allopurinol and other factors should be studied further in detail.

The onset mechanism of stomatitis occurring following anti-tumor chemotherapy has not been well clarified. The following hypothesis has been suggested by previous studies. The administered anti-cancer drugs are transferred to the oral mucosa where active oxygens are produced in the reduction–oxygenation cycles of the drugs.[4] These active oxygens accelerate the metabolism of hypoxanthine to xanthine and enhance further the production of active oxygens.[15] At the site of inflammation, on the other hand, serine protease activates NAD(P)H oxidase on the cell membrane, which then oxygenates NAD(P)H with the production of active oxygen in the process of oxygen reduction. Furthermore, accelerated vascular permeability and accumulation of leukocytes and macrophages at the site of inflammation[16] indicate the possibility that the active oxygen production by these cells may also contribute to the progress of stomatitis.

It seems that allopurinol may prevent stomatitis from occurring in association with chemotherapy by inhibiting xanthine oxidase and consequently reducing the production of active oxygen. To validate this hypothesis, we determined the inhibitory effects of allopurinol on the activities of SOD and several proteases.

The level of active oxygen can be determined directly,[17,18] indirectly, otherwise, by measuring the activity of SOD which is an active oxygen scavenger enzyme.[19] In this study, we used Bioxytech's kit and measured the SOD activity in the kidney after oral administration of allopurinol to mice. As a result, compared with the untreated control group, the SOD activity was found to be significantly lower ($p<0.01$) in the mice treated with allopurinol 20 mg/day for 3 days (Group C), in which the concentrations of allopurinol and oxypurinol in the renal tissue were as high as 203.9 ± 52.1 and 1141.7 ± 194.8 μg/g, respectively. This lowered SOD activity indicates that the production of active oxygen was inhibited by the administration of allopurinol. In the renal tissue taken from mice treated with oral allopurinol, on the other hand, the activities of trypsin and papain, which are cysteine proteases working in the inflammatory process, were also found suppressed, but other proteases were not, suggesting only weak protease inhibition of allopurinol. Although not included in this article, we performed a previous study to evaluate the inhibitory effects of allopurinol on various proteases and found that allopurinol inhibited papain. This finding is comparable to the result of the present study.

Sonis *et al.* succeeded in preparing an animal model of human chemotherapy-induced stomatitis in the cheek pouch of hamsters by combining 5-FU 60 mg/kg i.p. and mechanical mucosal irritation in Golden Syrian hamsters,[20] though they made little discussion about the onset mechanism of this stomatitis. The mechanism of allopurinol in preventing the development of stomatitis following anti-cancer chemotherapy is hoped to be clarified in the future using such animal models.

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(Received 10 October 1995; accepted 25 October 1995)