Hydrogen peroxide generated by xanthine/xanthine oxidase system represses the proliferation of colorectal cancer cell line Caco-2

Satoru Sakuma, Muneyuki Abe, Tetsuya Kohda and Yohko Fujimoto*

Laboratory of Physiological Chemistry, Osaka University of Pharmaceutical Sciences, 4-20-1 Nasahara, Takatsuki, Osaka 569-1094, Japan

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The twin character of reactive oxygen species is substantiated by a growing body of evidence that reactive oxygen species within cells act as inducers and accelerators of the oncogenic phenotype of cancer cells, while reactive oxygen species can also induce cancer cell death and can therefore function as anti-tumorigenic species. The aim of this study was to assess a possible influence of xanthine/xanthine oxidase on the proliferation of colorectal cancer cell line Caco-2. xanthine/xanthine oxidase (2.5 μ M/0.25 mU/ml–25 μ M/2.5 mU/ml) dose-dependently inhibited the proliferation of Caco-2 cells. Experiments utilizing reactive oxygen species scavengers (superoxide dismutase, catalase and mannitol) and exogenous hydrogen peroxide revealed a major role of hydrogen peroxide in the xanthine/xanthine oxidase effect. Investigations utilizing annexin V-fluorescein/PI assay using flow cytometry, and the lactate dehydrogenase extracellular release assay indicated that hydrogen peroxide induced necrosis, but not apoptosis, in Caco-2 cells. These results suggest that hydrogen peroxide generated by xanthine/xanthine oxidase has the potential to suppress colorectal cancer cell proliferation.

Key Words: xanthine/xanthine oxidase, hydrogen peroxide, colon cancer, proliferation, reactive oxygen species

Colorectal cancer is one of the leading causes of death worldwide, being responsible for approximately 10% of total cancer-related mortality.⁽¹⁾ About 3–5% of colorectal cancers may be due to inherited genetic defects and up to 25% of patients may have some degree of familiarity for this disease, but the majority of colorectal cancers occur in a sporadic manner in the absence of a documented family history.⁽²⁾

Reactive oxygen species (ROS) are well recognized for playing a dual role as both deleterious and beneficial species. Oxidative damage primarily mediated by ROS accumulates during the life cycle, and ROS-related damage to DNA, proteins and lipids has been proposed to play a key role in the development of various cancers including colorectal cancer.⁽³⁾ In contrast, it is known that ROS can induce cellular senescence and cell death, and can therefore function as anti-tumorigenic species. Indeed, ROS are important mediators for anti-neoplastic therapy including chemotherapy, ionizing radiation, ultraviolet radiation, and possibly photodynamic therapy.^(4–6)

The enzyme xanthine oxidase (XO), which catalyzes conversion of hypoxanthine and xanthine (X) into uric acid, uses oxygen as redox partner, thus producing ROS, mainly superoxide anion radicals ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2). XO is known to circulate in many species including humans.⁽⁷⁾ There is evidence of a significant rise in plasma XO levels during pathological con-

ditions, for example in human liver disease⁽⁷⁾ and pancreatitis.⁽⁸⁾ Besides, Partridge *et al.*⁽⁹⁾ have demonstrated a process of cell death-independent, constitutive release of XO from cultured bovine pulmonary microvascular endothelial cells. An attractive role for circulating XO lies in a putative function as an antimicrobial agent, and both circumstantial and direct evidence exists for this function. Circulating XO may bind to glycosaminoglycans,⁽¹⁰⁾ which are similar to structural components found on the surface of some bacteria. Therefore, by utilizing a circulating substrate the enzyme can generate microbicidal ROS in close proximity to microbes. Indeed, the antimicrobial function of XO has been widely demonstrated in milk,⁽¹¹⁾ where it is believed that XO-generating ROS afford protection to the suckling neonate from diseases, such as gastroenteritis, in the early postpartum period. This mechanism has also been shown to be effective in Cape Buffalo where an anti-microbial role for circulating XO in the control of trypanosome infection has been established.⁽¹²⁾

In the present study, to explore further physiological or pathological role of X/XO-mediated ROS, we investigated the effect of X/XO on the proliferation of a colorectal cancer cell line, Caco-2 cells.

Materials and Methods

Materials. Xanthine, H_2O_2 , superoxide dismutase (SOD), catalase (CAT), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), propidium iodide (PI), mannitol and camptothecin were purchased from Sigma-Aldrich Corporation (St. Louis, MO). XO was obtained from Roche Diagnostics Corporation (Indianapolis, IN). Annexin V-fluorescein staining kit was obtained from Wako Pure Chem. Ind., Ltd. (Osaka, Japan). Camptothecin was dissolved in dimethyl sulfoxide (DMSO) and added to medium; the DMSO concentration (0.25%) used in the present study had no significant effect on Caco-2 proliferation.

Cell culture. Caco-2 human colon cancer cell lines were purchased from the European Collection of Cell Cultures (Salisbury, Wilts, UK) and cultured in Minimum Essential Medium (MEM; Life Technologies Corporation, Carlsbad, CA) supplemented with 10% FBS (Nishirei Biosciences Inc., Tokyo, Japan) and 1% non-essential amino acids (Life Technologies Corporation, Carlsbad, CA). The cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C.

Cell viability assay. Cell viability was measured by MTT assay, as we described previously.^(13,14) Briefly, the cells were

*To whom correspondence should be addressed.
E-mail: fujimoto@gly.oups.ac.jp

incubated with the test reagents at a density of 2.5×10^4 cells/2 ml/9.5 cm² well for 24 h. After incubation, the medium was removed and the cells were incubated with 1.1 ml of MTT solution (0.1 ml of 5 mg/ml MTT in 1 ml of medium) for 4 h. The product was eluted from cells by the addition of 20% SDS/0.01 M HCl, and absorbance at 595 nm was determined using an SH-1000Lab microplate reader (Corona Electric Co., Ltd., Ibaraki, Japan). Cell viability was calculated according to the following equation: cell viability (%) = (absorbance of experiment group/absorbance of control group) $\times$ 100.

Determination of cell apoptosis or necrosis. Apoptosis or necrosis was detected by flow cytometry using an annexin V-fluorescein staining kit. Briefly, the cells were incubated with H₂O₂ or camptothecin at a density of 1.0×10^6 cells/10 ml/55 cm² dish for 24 h, and then collected by centrifugation. The cell pellets were incubated with staining solution containing annexin V-fluorescein and PI at room temperature for 15 min. After adequate dilution according to the cell density, the samples were filtrated through a nylon mesh (35 μ m), and subjected to a FACScan™ flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ).

Cytotoxicity assay. Cytotoxicity was assessed by a Cyto Tox96® Non-Radioactive Cytotoxicity Assay (Promega Corporation, Madison, WI) based on exocytic release of lactate dehydrogenase (LDH). The cells were incubated with H₂O₂ at a density of 2.5×10^4 cells/2 ml/9.5 cm² well for 24 h. After incubation, the medium was collected, and cells were harvested and destroyed by freeze-thawing (–80°C, 30 min; 37°C, 30 min). The collected medium and cell destruction sample were centrifuged at 780 $\times$ g for 5 min, and the supernatants were then used with a Cyto Tox96® Non-Radioactive Cytotoxicity Assay kit. The product absorbance was measured with an SH-1000Lab microplate reader at a wavelength of 490 nm. Cytotoxicity was expressed as LDH release according to the following equation: LDH release (%) = (extracellular LDH activity/total LDH activity) $\times$ 100.

Statistics. Results are the means $\pm$ SEM. The significance of differences between two groups was assessed using the Student's *t* test, and differences between multiple groups were assessed by one-way analysis of variance (ANOVA), followed by Scheffe's multiple range test. *P* values less than 0.05 were considered significant.

Results

X/XO system suppressed the proliferation of Caco-2 cells.

Fig. 1 illustrates a dose-dependent alteration in Caco-2 cell growth by the X/XO system. When Caco-2 cells were incubated with X/XO at concentrations ranging from 2.5 μ M/0.25 mU/ml to 25 μ M/2.5 mU/ml, a dose-dependent suppression in cell growth was obtained, and the inhibition reached 52% of the control value at 12.5 μ M X/1.25 mU/ml XO. Addition of X (12.5 μ M) and denatured XO (1.25 mU/ml XO was boiled for 5 min before use) to Caco-2 cells did not show any significant effect on the cell growth, implying an inhibitory role of a certain product formed by the enzymatic reaction.

H₂O₂ formed from X/XO system induced a suppression of Caco-2 proliferation. It has been known that XO generates O₂^{•–} and H₂O₂ in the presence of oxygen as an electron acceptor. There is also a possibility that trace metal ions (Fe²⁺, Cu²⁺, etc.) in vital cells catalyze the production of hydroxyl radicals ([•]OH) from H₂O₂. SOD catalyzes the dismutation of O₂^{•–} to O₂ and H₂O₂. CAT decomposes H₂O₂ to form water. To clarify a possible involvement of O₂^{•–}, H₂O₂ or [•]OH in the inhibition of cell proliferation elicited by the X/XO system, the effects of SOD, CAT, mannitol (an effective [•]OH scavenger^(15,16)) on the X/XO effect and of exogenous H₂O₂ were examined (Fig. 2 and 3, and Table 1). As shown in Fig. 2, co-addition of SOD at concentrations of 0.02, 0.1 and 0.5 U/ml had no significant effect on the X/XO (12.5 μ M/

Fig. 1. Effect of X/XO on the proliferation of Caco-2 cells. Caco-2 cells were treated with xanthine/xanthine oxidase (X/XO) for 24 h. Proliferation was assayed by an MTT. *1.25 mU/ml XO was boiled at 100°C for 10 min. Data are expressed as the mean $\pm$ SEM (*n* = 3). ^a*p* < 0.01; significantly different from the corresponding value in the absence of X/XO. ^b*p* < 0.01; significantly different from the corresponding value in the presence of 12.5 μ M X/1.25 mU/ml XO.

1.25 mU/ml)-induced inhibition of Caco-2 proliferation. In contrast, co-addition of CAT at the same concentration range dose-dependently attenuated the suppression of Caco-2 proliferation by X/XO, and almost nullified the X/XO effect at 0.5 U/ml. This CAT action completely disappeared when the enzyme was denatured by boiling. As shown in Fig. 3, exogenously added H₂O₂ at concentrations of up to 50 μ M decreased Caco-2 proliferation in a dose-dependent manner (20 μ M H₂O₂, 38% inhibition). Also, co-addition of mannitol (10 and 100 mM) did not attenuate the suppression of Caco-2 proliferation by X/XO (12.5 μ M/1.25 mU/ml) or H₂O₂ (20 μ M) (Table 1). Results shown in Fig. 2 and 3, and Table 1 showed that the X-XO action was not due to O₂^{•–} and [•]OH, and was mainly due to H₂O₂.

H₂O₂ induced necrosis on Caco-2 cells. Fig. 4 illustrates the percentages of necrotic or apoptotic cells, measured by the annexin V-fluorescein/PI assay using flow cytometry, when Caco-2 cells were exposed to H₂O₂ (20 μ M) at 24 h. Positive staining with annexin V-fluorescein correlates with a loss of membrane polarity, and a complete loss of membrane integrity will lead to apoptosis or necrosis. In contrast, PI can only enter cells after the complete loss of membrane integrity. The fraction of the cell population in different quadrants was analyzed using quadrant statistics.⁽¹⁷⁾ Cells in the upper left quadrant [B: annexin V(–)/PI(+)] represented necrotic cells. Cells in the lower right quadrant [C: annexin V(+)/PI(–)] represented early apoptotic cells, and cells in the upper right quadrant [D: annexin V(+)/PI(+)] represented late apoptotic cells (a representative analytic datum of the control is shown in the left-hand side of Fig. 4). The experiment using camptothecin, which is a known inducer of apoptosis,⁽¹⁸⁾ verified the validity of the cell analysis; 10 μ M camptothecin significantly increased the early apoptotic cell population (C). The treatment of Caco-2 cells with 20 μ M H₂O₂ induced a significant increase in the necrotic cell population (B).

Furthermore, an extracellular release of LDH, which is known to be a necrosis indicator,⁽¹⁹⁾ was measured during incubation with H₂O₂; 20 μ M H₂O₂ showed about two-fold increase in the LDH extracellular release [control, 8.6 ± 1.1 ; H₂O₂, 17.8 ± 0.4 (*n* = 3; *p* < 0.01)] (% of total LDH activity).

These findings revealed that H₂O₂ induced necrosis, but not apoptosis, in Caco-2 cells.

Fig. 2. Effect of X/XO in the presence of SOD or CAT on the proliferation of Caco-2 cells. Caco-2 cells were treated with 12.5 μ M xanthine (X)/1.25 mU/ml xanthine oxidase (XO) in the presence of superoxide dismutase (SOD) or catalase (CAT) for 24 h. Proliferation was assayed by an MTT. *0.5 U/ml CAT was boiled at 100°C for 10 min. Data are expressed as the mean $\pm$ SEM ($n = 3-4$). ^a $p < 0.01$, ^b $p < 0.05$; significantly different from the corresponding value in the absence of X/XO, SOD and CAT. ^c $p < 0.05$, ^d $p < 0.01$; significantly different from the corresponding value in the presence of X/XO. ^e $p < 0.01$; significantly different from the corresponding value in the presence of X/XO and 0.5 U/ml CAT.

Fig. 3. Effect of H₂O₂ on the proliferation of Caco-2 cells. Caco-2 cells were treated with hydrogen peroxide (H₂O₂) for 24 h. Proliferation was assayed by an MTT. Data are expressed as the mean $\pm$ SEM ($n = 3-4$). ^a $p < 0.05$, ^b $p < 0.01$; significantly different from the corresponding value in the absence of H₂O₂.

Table 1. Effects of X/XO and H₂O₂ in the presence of mannitol on the proliferation of Caco-2 cells

Treatment			Cell viability (% of control)
Control			100.0 ± 5.2
X/XO			33.8 ± 0.5 ^a
H ₂ O ₂	+ mannitol	10 mM	31.0 ± 1.4
		100 mM	30.0 ± 1.6
			46.0 ± 0.8 ^a
		+ mannitol	10 mM
		100 mM	33.0 ± 1.7

Caco-2 cells were incubated with or without 12.5 μ M xanthine (X)/1.25 mU/ml xanthine oxidase (XO) or 20 μ M H₂O₂ in the presence or absence of indicated concentrations of mannitol for 24 h. Proliferation was assayed by an MTT. Data are expressed as the means $\pm$ SEM ($n = 4-5$). ^a $p < 0.01$; significantly different from control.

Discussion

In the present study, using Caco-2 cell lines, we investigated a possible role of X/XO in colorectal cancer cell proliferation. We found that X/XO at a concentration of 12.5 μ M/1.25 mU/ml or higher potentially decreased the cell viability of Caco-2 cells. Experiments utilizing ROS scavengers (SOD, CAT and mannitol) and exogenous H₂O₂ revealed a major role of H₂O₂ in the inhibition of Caco-2 proliferation elicited by X/XO. Investigations by annexin V-fluorescein/PI assay using flow cytometry and an LDH extracellular release assay indicated that H₂O₂ induced necrosis, but not apoptosis, in Caco-2 cells.

Wu *et al.*⁽²⁰⁾ noted that cyclooxygenase-2 (COX-2) expression is dysregulated in many types of cancer including colorectal cancer, and COX-2-derived eicosanoids elicit multiple oncogenic signals to promote carcinogenesis. We therefore examined the effect of H₂O₂ on the mRNA and protein expression of COX-2 in Caco-2 cells using real-time RT-PCR and western blotting at 6-h, 12-h or 24-h incubation. 20 μ M H₂O₂ did not have any significant effect on either COX-2 mRNA nor protein expression levels when compared to controls (data not shown). Thus, it seems likely that H₂O₂ inhibits Caco-2 proliferation independently of COX-2. Further studies are needed to clarify the mechanism of inhibition of Caco-2 proliferation induced by H₂O₂ or X/XO.

Cells are able to detoxify excessive oxidative stressors before they react with vulnerable cellular targets. Enzymes such as SOD and CAT play key roles in the cellular defense against free radical damage.⁽²¹⁾ In fact, when 12.5 μ M/1.25 mU/ml X/XO or 20 μ M H₂O₂ was incubated with primary rat hepatocyte cultures prepared according to our previous report⁽²²⁾ for 24 h, there was no significant alteration in the cell viability [MTT assay (% of control): control, 100.0 $\pm$ 8.2; X/XO, 96.4 $\pm$ 9.3; H₂O₂, 101.2 $\pm$ 8.9 ($n = 3$)]. This may mean that normal cells have a cellular defense system against the oxidative stress induced by X/XO and H₂O₂. However, a wide body of data indicates that animal tumor cells including colon cancer cells lack the complex enzyme systems that normally exert protection by scavenging toxic free radicals such as O₂⁻, H₂O₂ and lipid hydroperoxides.⁽²³⁾ Dreher and Junod⁽²⁴⁾ reported that low levels of oxidative stress stimulate cell proliferation, whereas high levels induce cytotoxicity and cell death in later stages of carcinogenesis. Thus, there is a possibility that Caco-2 cells are sensitive to H₂O₂ by their inherent characteristics as malignant tumors, and are thus led to necrosis.

Fig. 4. Effect of H₂O₂ on apoptosis or necrosis in Caco-2 cells. Caco-2 cells were treated with 20 μ M hydrogen peroxide (H₂O₂) or 10 μ M camptothecin for 24 h. Apoptosis or necrosis was detected by flow cytometry using an annexin V-fluorescein staining kit. A representative analytic datum of the control is shown on the left-hand side. Data are expressed as the mean $\pm$ SEM ($n = 4$). ^a $p < 0.05$, ^b $p < 0.01$; significantly different from control.

In a large population-based cohort of breast cancer, Linder *et al.*⁽²⁵⁾ showed that XO was down-regulated in more than half of the breast tumors studied, and that absence of XO was an independent predictor of unfavorable outcome. They also demonstrated that the down-regulation of XO is common in patients with gastric cancer and associated with unfavorable disease-specific survival.⁽²⁶⁾ Furthermore, their group assessed XO expression in the foci of 478 patients with colorectal cancer. They found that XO was decreased in 62% and undetectable in 22% of the tumors as compared to normal tissues, and thus concluded that XO expression was associated with the histological grade of differentiation and extent of disease in colorectal cancer.⁽²⁷⁾ The circulating XO has been shown to bind to, and to be endocytosed by the vascular endothelium.⁽²⁸⁾ Normal serum XO concentrations determined by ELISA have been estimated to be up to 1 mU/ml.⁽⁷⁾ In systemic inflammation, XO has also been reported to be present in human plasma at concentrations of up to 1.5 mU/ml.⁽²⁹⁾ In the present study, we observed that H₂O₂ generated by XO at a concentration of 1.25 mU/ml or higher reduces the proliferation of colorectal cancer cells Caco-2 partially by an induction of necrosis. These findings might indicate an

important role of circulating XO and the formed H₂O₂ as an endogenous suppressor of colon cancer proliferation, and consequently also progression and metastasis.

Abbreviations

CAT	catalase
COX	cyclooxygenase
DMSO	dimethyl sulfoxide
LDH	lactate dehydrogenase
MEM	Minimum Essential Medium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
PI	propidium iodide
ROS	reactive oxygen species
SOD	superoxide dismutase
XO	xanthine oxidase

Conflict of Interest

No potential conflicts of interest were disclosed.

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