[CANCER RESEARCH 55, 1617–1620, April 15, 1995]

Advances in Brief

A Novel Cancer Therapy Based on Oxygen Radicals

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Abstract

The antitumor effect of oxygen radicals produced by hypoxanthine and xanthine oxidase reaction was studied in an experimental rabbit model. VX2 carcinomas were transplanted into rabbit hind legs. Hypoxanthine was administered continuously through the ear vein, while xanthine oxidase was administered simultaneously through the femoral artery. As a result, hypoxanthine and xanthine oxidase reacted only in the hind leg, and superoxide was produced in that area. The volume of the VX2 carcinoma was measured immediately prior to treatment and 7 days later. As an index of lipid peroxidation, thiobarbituric acid-reactive substances in the tumor tissue were measured 60 min following infusion of hypoxanthine and xanthine oxidase. Tumor growth was suppressed significantly by the hypoxanthine-xanthine oxidase reaction, and thiobarbituric acid-reactive substances in the tumor tissue infused with hypoxanthine and xanthine oxidase were significantly increased. In addition, the antitumor effect of the hypoxanthine and xanthine oxidase reaction was significantly inhibited by the administration of superoxide dismutase and catalase. Pathological examination showed that oxygen radicals produced by hypoxanthine and xanthine oxidase reaction were selectively more destructive for VX2 carcinoma tissue than muscle tissue surrounding the tumor region. These results suggest that oxygen radicals produced by hypoxanthine and xanthine oxidase reaction produce an anticancer effect and that the VX2 carcinoma used in this study was more sensitive to oxygen radicals than normal muscle tissue.

Introduction

In recent years, free oxygen radicals and similar species have been implicated in various diseases (1–4), as well as carcinogenesis (5–9) and aging (10–12). Oxygen radicals are highly cytotoxic, and we have demonstrated the importance of radical-induced lipid peroxidation as a mechanism of that toxicity in various experimental animal models (13–16). Oxygen radicals give rise to lipid peroxidation, producing a variety of pathologies. As a result, free radical research has focused on disease treatment with radical scavengers. However, reversing this concept by skillfully manipulating oxygen radicals may allow their use in the treatment of cancer.

Therefore, we investigated whether oxygen radicals are cytotoxic to cancer cells. It is well known that *in vitro* hypoxanthine is catalyzed by xanthine oxidase to generate superoxide anions. We used this reaction to treat rabbits with VX2 carcinomas transplanted into their hind legs.

Materials and Methods

Experimental Animals and Tumors. Male Japanese white rabbits weighing 2.5–3.0 kg were used. The implanted tumor was rabbit VX2 carcinoma, a rabbit squamous cell carcinoma derived from virus-induced papillomas. Rabbit VX2 carcinoma that had been transplanted serially in our laboratory was excised, sliced into thin sections, filtered through a mesh, and adjusted to a tumor cell count of 1×10^8/ml with Hanks' balanced salt solution; and 1×10^7 cells were implanted into the muscle tissue of the right hind leg. Rabbits were studied 2 weeks following implantation, after confirmation with ultrasonic tomography that the tumors had grown to 1.5–2.0 cm long.

Administration of Hypoxanthine and Xanthine Oxidase. A cutdown of the left femoral artery of the rabbits was performed under i.v. sodium pentobarbiturate (20 mg/kg) anesthesia. A guidewire (SF18; Cook, Bloomington, IN) was inserted, and a 3-French-diameter polyethylene catheter (Cook) was inserted over the wire and advanced to the caudal bifurcation of the abdominal aorta for xanthine oxidase infusion. The ear vein was cannulated with polyethylene tubing (PE-10; Becton Dickinson and Co.) for hypoxanthine infusion.

HX[1] (Wako Pure Chemical Co., Osaka, Japan) and XO (Sigma Chemical Co., St. Louis, MO) were dissolved in 0.01 M PBS. HX (1 mM) was infused through the ear vein for 60 min at a rate of 240 μl/min. XO (3 units/ml) was infused through the femoral artery for 60 min at a rate of 240 μl/min. As a control, PBS was infused continuously in place of XO through the femoral artery at the same rate (Fig. 1).

Administration of Free Radical Scavengers. The effect of CuZnSOD and catalase on the HX-XO reaction in the VX2 carcinoma was investigated. Recombinant human CuZnSOD (Nippon Kayaku Co., Tokyo, Japan) at a dose of 10,000 units/kg and catalase from bovine liver (Sigma) at a dose of 10,000 units/kg were dissolved in PBS and injected with HX through the ear vein, and XO was administered via the femoral artery. The control group was given injections of heat-inactivated (90°C for 60 min) CuZnSOD and catalase with HX.

Assessment of Antitumor Effect. The longest and shortest tumor diameters were measured by ultrasonic tomography on day 0 (immediately prior to treatment) and day 7 (7 days following treatment), and the tumor volume was calculated (17) as

$$(\text{Longest diameter}) \times (\text{shortest diameter})^2 \times \tfrac{1}{2}$$

The ratio of the tumor volume on day 7 to that on day 0 was expressed as the growth ratio.

Thiobarbituric Acid-reactive Substances. The concentration of TBARS, an index of lipid peroxidation, was measured in tumor tissue using the method of Ohkawa *et al.* (18). In brief, rabbits were killed 60 min following infusion of HX and XO by exsanguination via the abdominal aorta while under i.v. sodium pentobarbiturate anesthesia (20 mg/kg). Tumor tissues were removed and homogenized in 1.5 ml of 10 mM potassium phosphate buffer (pH 7.8) containing 30 mM KCl with a Teflon Potter-Elvehjem homogenizer. The concentration of TBARS in the tumor tissue homogenates was expressed as nmol of malondialdehyde per mg of protein using 1,1,3,3-tetramethoxypropane as the standard. Total protein in the tissue homogenates was measured by the method of Lowry *et al.* (19).

Pathological Examination. Tumor tissue slices through the center of the tumor were stained with hematoxylin and eosin 7 days after treatment.

Statistical Analysis. Results are presented as the mean ± SE from 4–8 rabbits/group. The Kruskal-Wallis analysis was used to determine variance. Statistical significance in the tumor growth ratio as shown Fig. 2 was evaluated by the two-tailed nonparametric Dunnett test. For statistical evaluation of the lipid peroxidation in tumor tissue, the nonparametric Mann-Whitney test was carried out for the concentration of TBARS.

Results

Antitumor Effect. The tumor growth ratio in the group given HX and XO was 2.6 ± 0.68. Compared to the group given HX and PBS (11.5 ± 1.67), tumor growth was significantly inhibited (Fig. 2*a*).

Received 1/24/95; accepted 3/13/95.

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[1] The abbreviations used are: HX, hypoxanthine; XO, xanthine oxidase; CuZnSOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances.

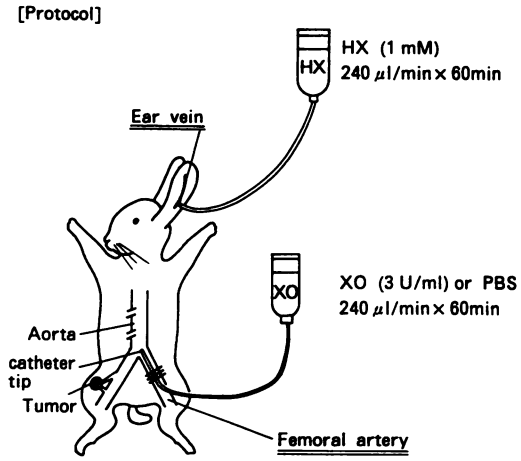

Fig. 1. HX and XO were dissolved in 0.01 M PBS. HX (1 mM) was infused through the ear vein for 60 min at a rate of 240 μl/min. XO (3 units/ml) was infused through the femoral artery for 60 min at a rate of 240 μl/min. As a control, PBS was infused continuously in place of XO through the femoral artery at the same rate.

Effect of Free Radical Scavengers on the Antitumor Effect Attributed to the Infusion of HX and XO. The tumor growth ratio of the untreated control group was 15.1 ± 1.69. The control group, given HX and XO with heat-inactivated CuZnSOD and catalase, had a growth ratio of 2.69 ± 1.17, showing that the infusions of HX and XO prevented the growth of the tumor. When CuZnSOD plus catalase was given along with the HX and XO, the growth ratio was 10.3 ± 3.1. Thus, the antitumor effect of HX-XO reaction was significantly inhibited by the use of CuZnSOD plus catalase (Fig. 2*b*).

TBARS in Tumor Tissue. TBARS were measured in the tumor tissue 60 min following infusion of HX and XO. The control group, which received HX and PBS, had TBARS of 0.29 ± 0.035. The group, which received HX and XO, had TBARS of 0.44 ± 0.029. Thus, there was a significant increase of TBARS in the tumor tissue following the infusion of HX and XO (Fig. 3).

Pathological Examination. Fig. 4 shows microphotographs of tumor tissue and muscle tissue surrounding the tumor region. When HX (1 mM) was infused through the ear vein and PBS was simultaneously infused through the femoral artery at which the suppressive effect on VX2 tumor growth was not observed, the pathological observation was that macular liquefaction necrosis was observed inside the tumor, normally appearing tumor cells were widely detectable surrounding the necrotic part, and muscle tissue surrounding the tumor region appeared normal (Fig. 4*a*). When HX (1 mM) through the ear vein and XO (3 units/ml) through the femoral artery were infused simultaneously at which VX2 tumor growth was suppressed, the pathological observation substantially differed from control; the necrosis was massive, and viable tumor cells could not be detected or were only slightly detectable. On the other hand, necrosis of muscle tissue surrounding the tumor region was not observed (Fig. 4*b*).

Discussion

The potent cytotoxicity of oxygen radicals can cause various diseases but also may serve as a powerful weapon capable of destroying harmful agents (*e.g.*, cancer cells). We have investigated novel cancer therapies based on free radical destruction of cancer cells, specifically by superoxide anion. It is well known that *in vitro* hypoxanthine is catalyzed by xanthine oxidase to generate superoxide anions. We used this reaction to treat rabbits with VX2 carcinomas transplanted into their hind legs. Hypoxanthine was administered continuously through the ear vein, while xanthine oxidase was administered simultaneously through the femoral artery. As a result, hypoxanthine and xanthine oxidase reacted only in the hind leg, and superoxide was produced in that area. The volume of the VX2 carcinoma was measured immediately prior to treatment and 7 days later. Tumor growth was suppressed significantly by the hypoxanthine-xanthine oxidase reaction.

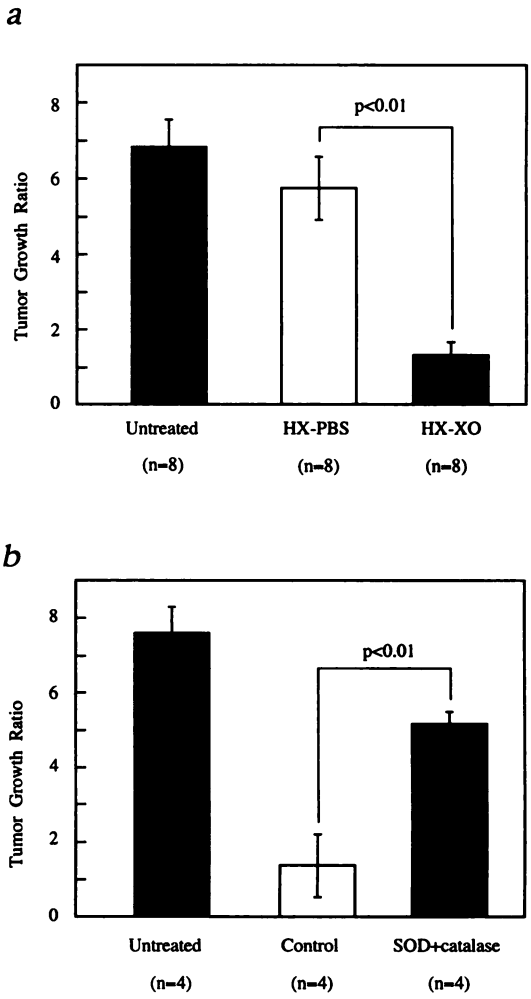

Fig. 2. *a*, antitumor effect of hypoxanthine and xanthine oxidase infusion on VX2 carcinomas in rabbits. Mean tumor volume in comparison to pretreatment control for groups of 8 rabbits are shown. Results are expressed as mean ± SE (*bars*). *b*, effect of CuZnSOD and catalase on the antitumor activity of hypoxanthine and xanthine oxidase infusion. Controls recieved injections of heat-inactivated CuZnSOD and catalase with hypoxanthine and xanthine oxidase infusion. CuZnSOD and catalase dissolved in PBS were injected simultaneously with hypoxanthine and xanthine oxidase infusion. Results are expressed as mean ± SE.

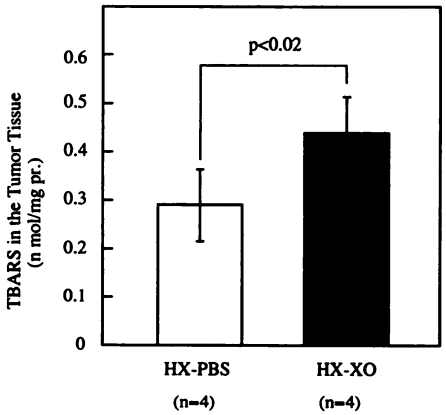

Fig. 3. Thiobarbituric acid-reactive substances in the tumor tissue after infusion of hypoxanthine and xanthine oxidase. Results are expressed as mean ± SE (*bars*).

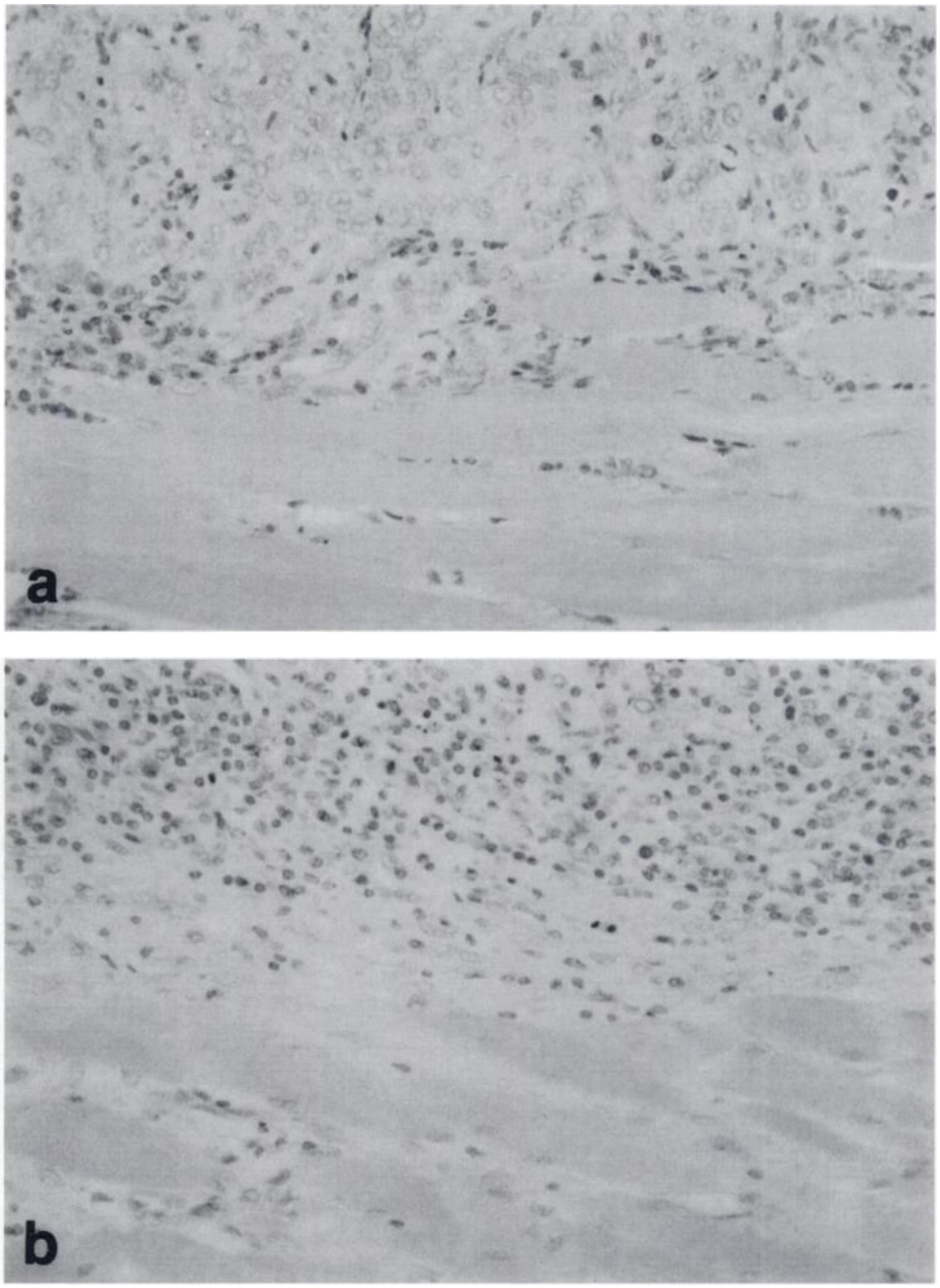

Fig. 4. Microphotographs of tumor tissue and muscle tissue surrounding the tumor region. *a*. When HX and PBS was infused at a point at which the suppressive effect on VX2 tumor growth was not observed, macular liquefaction necrosis was observed inside the tumor, normally appearing tumor cells were widely detectable surrounding the necrotic part, and muscle tissue surrounding the tumor region appeared normal. *b*. When HX and XO were infused at a point at which VX2 tumor growth was suppressed, the pathological observation substantially differed from control; the necrosis was massive, and viable tumor cells could not be detected or were only slightly detectable. On the other hand, necrosis of muscle tissue surrounding the tumor region was not observed.

In addition, when hypoxanthine, CuZnSOD, and catalase were administered simultaneously via the ear vein and xanthine oxidase was administered via the femoral artery, the reduction in tumor growth was significantly less than in a control experiment using heat-inactivated CuZnSOD and catalase. Thus, the antitumor effect is inhibited when superoxide produced by the hypoxanthine-xanthine oxidase reaction is scavenged by CuZnSOD and catalase. We hypothesized that the superoxide produced by the hypoxanthine-xanthine oxidase reaction damaged the VX2 carcinoma cells by membrane lipid peroxidation. To address this point, the concentration of TBARS, an index of lipid peroxidation, was measured in tumor tissue 60 min following the administration of hypoxanthine and xanthine oxidase. TBARS in the tumor tissues were significantly increased compared to those of the control. The increase in TBARS demonstrates that the superoxide produced by the hypoxanthine-xanthine oxidase reaction promotes lipid peroxidation in the tumor tissues, presumably causing carcinoma cell death. Although our basic goal was to generate oxygen radicals within and around the carcinoma cells, damage to the surrounding normal tissues had to be considered because of the nonspecific cytotoxicity of oxygen radicals. Fortunately, most cancer cells contain lower concentrations of radical scavengers than normal cells. In this study, because the hypoxanthine and xanthine oxidase reacted throughout the hind leg of the rabbit, superoxide likely was produced in a broad anatomical area; however, only the carcinoma tissue was injured, leaving the surrounding muscle virtually intact.

There are two conclusion from these results: (*a*) oxygen radicals produce an anticancer effect; (*b*) the VX2 carcinoma used in this study was more sensitive to oxygen radicals than normal muscle tissue. These may be the basic conditions necessary for an effect of free radicals on cancer tissue. In summary, these results support a new concept of cancer therapy based on oxygen radicals.

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Cancer Res 1995;55:1617-1620.