
原 著

Role of Free Radical Scavengers in Pancreatic Carcinomas of Hamsters

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Received for Publication, August 30, 1991.

Abstract

The involvement of free radicals in the carcinogenic mechanism has been suggested, however, little is known about the role of free radicals in the pancreatic cancer. In this study, the effects of active oxygen on the carcinogenesis of the tumor were examined by measuring the levels of scavengers in pancreatic cancer of Syrian golden hamsters. Pancreatic cancer was induced by di-iso-propanol nitrosamine (500 mg/kg body weight/week $\times$ 24 weeks). Activities of superoxide dismutase (SOD), catalase, glutathion peroxide (GSH-Px) and malon dialdehyde (MDA) in the tumor and border zone were compared with those in the non-tumor region and control normal tissue. Activities of SOD and catalase in the tumor and border zone were significantly lowered than those in non-tumor region and normal tissue. GSH-Px levels were significantly higher in the tumor than those in the non-tumor region and normal tissue. MDA levels also tended to be high in the tumor. These results suggest that the development of cancer in pancreatic tissue is related to a reduction of SOD and catalase. GSH-Px and MDA are suggested to be involved in the reactions of free radicals.

Introduction

Epidemiologic studies have demonstrated associations that support the view that carcinoma of the pancreas is the result of chemical carcinogenesis. Consumption of tobacco doubles the risk of development of pancreatic carcinoma^{6,11}, and nitrosamine in tobacco is well recognized as a source of carcinogens^{9,10}. Intake of animal meat (especially fat) has also been implicated as risk factors of pancreatic carcinoma^{2,8,32}. On the other hand, the involvement of free radicals in the carcinogenic mechanism has recently been suggested^{7,13,28,31}. Carcinogens are considered to promote carcinogenesis by producing free radicals such as active oxygen and lipid peroxide, but little is known about the role of free radical scavengers in the etiologic process of pancreatic carcinoma.

Key words: Pancreatic carcinoma, Free radical scavenger, Hamster, Di-iso-propanol nitrosamine, Superoxide dismutase (SOD), Catalase, Glutathion peroxide (GSH-Px), Malon dialdehyde.

索引語: 膵癌, フリーラジカル消去, ハムスター, ディイソプロパノールニトロサミン, スーパーオキシドデ
ィスムターゼ (SOD), カタラーゼ, グルタチオンペルオキシド (GSH-Px), マロンディアルデヒド

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Recently, pancreatic tumors, similar in morphologic appearance and biologic behavior to those in man, were induced in high incidence by selected nitroso compounds in Syrian golden hamsters^{20,21,22,23,24}.

We induced experimental pancreatic carcinoma in Syrian golden hamsters with di-iso-propanol nitrosamine, and studied the effects of active oxygen on the carcinogenesis and growth of the tumor by measuring the levels of scavengers such as superoxide dismutase, catalase, and glutathione peroxide as well as malon dialdehyde, a metabolite of peroxy lipid in the tumor tissue, border zone, and non-tumor tissue.

Materials and Methods

One hundred 8-week-old male Syrian golden hamsters (weighing 80–100 g) were used. Five animals were housed in each cage under controlled conditions (room temperature $23 \pm 3^\circ\text{C}$) and given pellet food (Oriental Food, Tokyo) and water.

Di-iso-propanol nitrosamine (DIPN, Nakarai Chemicals, Kyoto) was injected subcutaneously in the lumbar region once a week at a dose of 500 mg/kg body weight for over 24 weeks. Untreated animals of the same age were used as controls.

The animals were sacrificed 24–36 weeks after the administration, laparotomized, and 50–200 mg tissues were collected from the tumor, border zone and non-tumor region (Fig. 1). The tissues (10% w/v) were homogenized with physiologic saline, the supernatant was lyophilized at -40°C , and the superoxide dismutase (SOD), catalase, glutathione peroxide (GSH-Px), and malon dialdehyde (MDA) levels were determined. Histologic examination was performed simultaneously in formalin-fixed and hematoxylin-eosin-stained specimen.

SOD was assayed by the cytochrome reduction method¹⁷. The samples were homogenized with isotonic saline and 1% Triton X-100, and centrifuged at $7000 \times g$ for 10 minutes. The supernatant (0.1 ml) was mixed with 125 mM phosphate buffer (2 ml), 50 mM EDTA-2N (50 μl), 13.5 mg/50 ml hypoxanthine (100 μl), 1 mM KCN (23 μl), 12.4 mg/1.5 ml cytochrome C (50 μl), and 22 μl /4 ml xanthine oxidase (XOD) (100 μl), and the amount of O_2^- generated was estimated from cytochrome reduction according to the changes in the absorbance at 550 nm with a spectrophotometer (UV5260, Beckman, USA). One SOD unit (CU) was defined as the amount that inhibited O_2^- production by

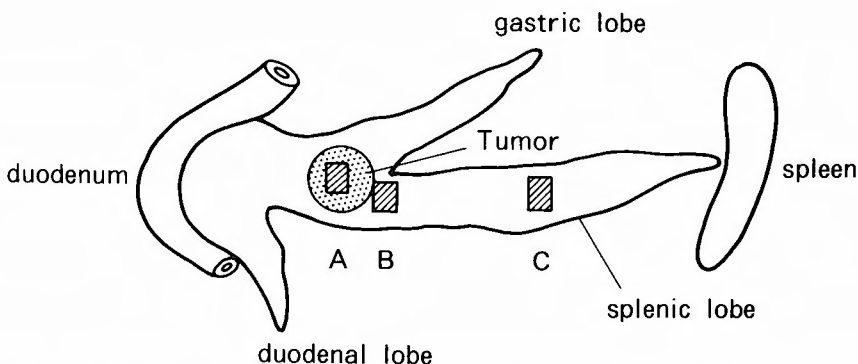


Fig. 1 Sampling of pancreatic tissue after induction of pancreatic carcinoma by DIPN injection.
A: tumor, B: border zone, C: non-tumor region.

50%. Since SOD may react directly with cytochrome C regardless of O_2^- production, the value was corrected by Asada's equation³

$$\text{SOD Unit} = \frac{a - (b - c)}{a/2}$$

a = SOD obtained by adding XO only

b = SOD obtained by adding XO and the sample

c = SOD obtained by adding the sample only

The catalase activity was determined by the method of Aebi¹ modified by Chance and Herbert. The sample was homogenized with isotonic saline and 1% Triton X-100 and centrifuged at $10,000 \times g$ for 20 minutes. The supernatant (100 μ l) was mixed with 10 mM H_2O_2 (3 ml), the absorbance of H_2O_2 was recorded continuously at 240 nm for 1 minute at 20°C from immediately after the infusion of the sample, and the catalase activity was estimated from the following formula according to the linear decrease during 18 seconds.

$$K = 2.3/\Delta T \log A1/A2$$

A1 = absorbance at the beginning of reaction (T1)

A2 = absorbance at the end of reaction (T2)

$\Delta T = T2 - T1$

1 unit = the ability of catalase to degrade 1 μ M H_2O_2

The GSH-Px activity was estimated from changes in the absorbance due to the consumption of NADPH. The sample was homogenized with isotonic saline and centrifuged at 15,000 g for 20 minutes. The supernate (100 μ l) was incubated with 0.2 M phosphate buffer (pH 7.0, 500 μ l), 40 mM EDTA (100 μ l), 2 mM NADPH (100 μ l), 40 mM GSH (100 μ l), 40 mM NaN_3 (100 μ l), and 1,000 U/4 ml glutathione reductase (4 μ l) at 37°C for 5 minutes, and the absorbance at 340 nm in the presence of 10 mM t-butyl-hydroperoxide (10 μ l) was determined.

The MDA level was determined by the TBA method^{16,18}. The values were examined by analysis of variance (ANOVA), and differences of $p < 0.05$ were considered to be significant.

Results

Forty-eight animals survived until 30 weeks after the beginning of the study. Carcinoma was observed histologically in all these animals, but macroscopic lesions were observed in 21 (43%). The tumors were grayish white nodules measuring $16 \times 11 \times 10$ mm at the maximum and $2 \times 2 \times 2$ mm at the minimum.

The tumors were found in the duodenal lobe, gastric lobe, and splenic lobe according to the nomenclature of Takahashi et al.²⁹. Although the frequency of the tumor was slightly higher in the head of the pancreas, the difference was not significant. Histologically, all lesions were adenocarcinomas, and markedly atypical epithelial cells formed irregular ducts. The basement membrane was obliterated. The tumor infiltrated into hyperplastic interstitial tissue, and papillary growth was occasionally observed. The tumor appeared to be tubular adenocarcinoma with characteristic clear nuclei of irregularity sizes, thickening of the nuclear membrane, and a single layer of cuboidal cancer cells with distinct nucleoli (Fig. 2).

The SOD activity was 6.0 ± 0.6 unit/mg protein (mean $\pm$ SEM) in the tumor, 5.6 ± 0.7 unit/mg protein in the border zone, 11.6 ± 1.5 unit/mg protein in the non-tumor region, and 10.5 ± 0.7



Fig. 2 Histological finding of adenocarcinoma in hamster pancreas after carcinogenic induction by DIPN. Hematoxylin-eosin staining, $\times 200$.

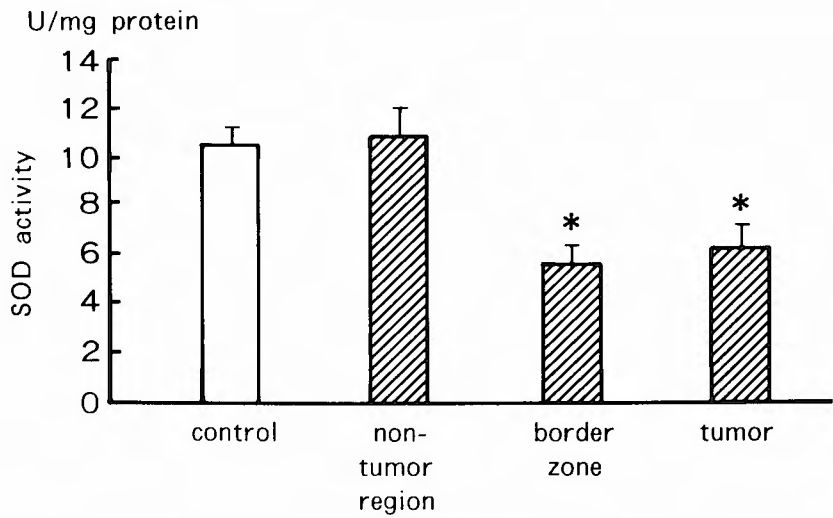


Fig. 3 SOD activity in pancreatic tissue of normal control hamsters and in tissues from three sites in hamsters with pancreatic carcinomas. $n=6$, mean $\pm$ SEM, $*p<0.05$ vs. non-tumor region and control.

unit/mg protein in the normal controls, with significant differences between the tumor of the border zone and the non-tumor region or the control (Fig. 3).

The catalase activity was 40.8 ± 6.4 unit/mg protein in the tumor, 45.1 ± 10.7 unit/mg protein

in the border zone, 97.0 ± 16.5 unit/mg protein in the non-tumor region, and 97.1 ± 20.0 unit/mg protein in the controls. It decreased from the non-tumor region to the tumor, and the differences between the tumor or border zone and non-tumor region or controls were significant (Fig. 4).

The GSH-Px activity was 0.05 ± 0.01 unit/mg protein in the tumor, 0.037 ± 0.007 unit/mg protein in the border zone, 0.025 ± 0.002 unit/mg protein in the non-border zone, and 0.017 ± 0.002 unit/mg protein in the controls. It was high in the tumor and border zone, with a significant difference between the tumor and the control (Fig. 5).

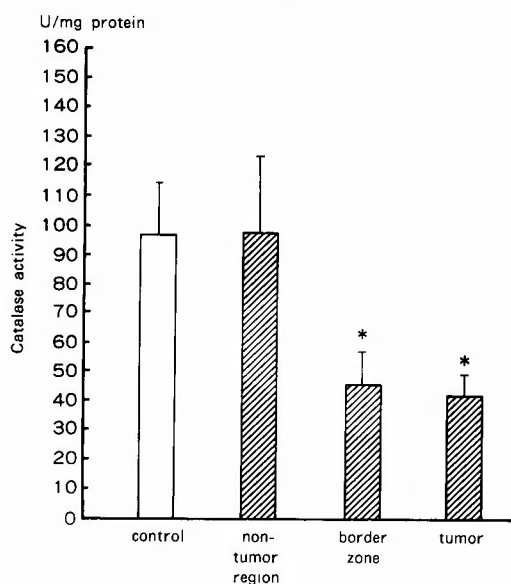


Fig. 4 Catalase activity in pancreatic tissue of normal control hamsters and in tissues of hamsters with pancreatic carcinomas. $n=6$, mean $\pm$ SEM, * $p < 0.05$ vs. non-tumor region and control.

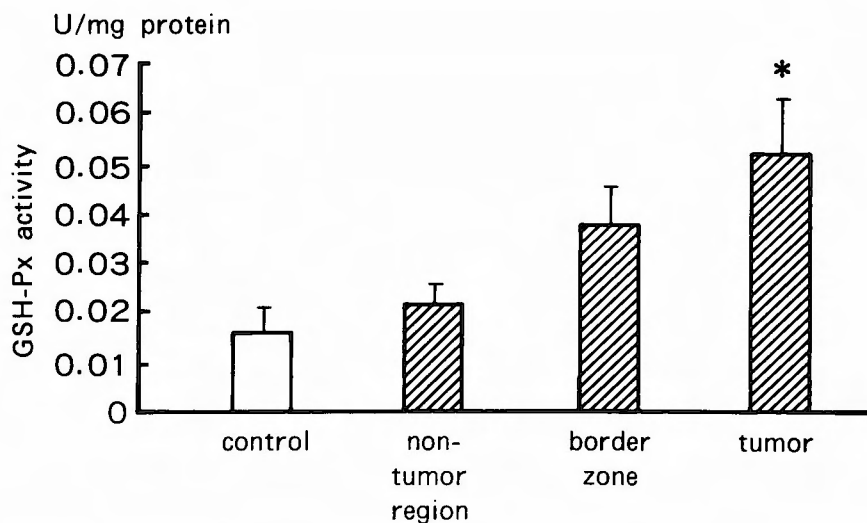


Fig. 5 Glutathione peroxidase activity in pancreatic tissue of normal control hamsters and in tissues of hamsters with pancreatic carcinomas. $n=6$, mean $\pm$ SEM, * $p < 0.05$ vs. non-tumor region and control.

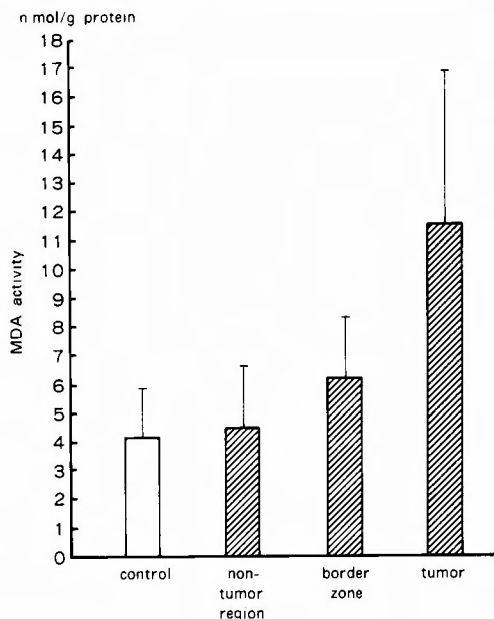


Fig. 6 Malon dialdehyde activity in pancreatic tissue of normal control hamsters and in tissues of hamsters with pancreatic carcinomas. $n=6$, mean $\pm$ SEM.

The MDA level was 11.3 ± 5.3 nmol/g protein in the tumor, 6.0 ± 2.0 nmol/g protein in the border zone, 4.3 ± 2.4 nmol/g protein in the non-tumor region, and 4.1 ± 1.7 nmol/g protein in the controls. Similarly to the GSH-Px activity, it tended to be higher in the tumor than in the control or the non-tumor region (Fig. 6).

Discussion

In 1974 Pour et al.²², in an American and German common study, observed subcutaneously injected DIPN (B-oxidized substance) of N-nitroso-(di-propyl) amine (DPN) into Syrian golden hamsters once a week, resulting in the development at a very high rate of duct cell adenocarcinoma similar to human pancreas carcinoma. Since then, there have been many similar studies using nitroso compounds²⁰⁻²⁴

There is no established theory for the mechanism of carcinogenesis. However, the even distribution of the site of carcinogenesis over the entire pancreas after ligation of the bile duct and pancreatic duct indicates that the carcinogen is more likely to be transported via the blood rather than bile²⁶.

Levitt et al.¹⁵ performed autoradiography using N-nitroso 2-6 dimethylmorphine, a complex of DIPN, and observed greater uptake by acinar cells than by duct cells. This finding suggests that acinar cells are the principal site of the action of metabolic activators.

Since nitroso compounds including DIPN are indirect carcinogens, they must be activated to become mutagenic^{5,25}. Nitrosamines are reported to be metabolized by mixed function oxidase (MFO). They are considered to be metabolized into mutagenic forms by MFO enzymes, which are distributed in abundance in acinar and duct cells. Carcinogens thus activated are considered, then,

to act on their target, namely the ductal epithelium.

Pour et al.²³ observed carcinogenesis in hamsters by chronic administration of DHPN after a minimum period of 13 weeks, but we noted small nodular carcinomas already after 12 weeks. Macroscopic lesions were observed after 20 or more weeks. Carcinogenesis was confirmed in all animals that survived until 30 weeks after the beginning of the study, and lesions had macroscopic dimensions in 41% of these animals. This percentage was higher than that reported by Kajikawa¹² (25%, 13/52).

In the present study, the tumors were grayish white and nodular with a maximum dimensions of $16 \times 11 \times 10$ mm. All the macroscopic tumorous lesions were defined histologically as tubular adenocarcinoma or papillary adenocarcinoma, and none were acinar cell adenocarcinoma, demonstrating the appropriateness of the present animals as a model of human pancreatic cancer.

Concerning scavengers of O_2^- , McCord and Fridovich¹⁷ first discovered SOD, which specifically consumes O_2^- in an unequalized reaction. Subsequent fundamental studies on O_2^- that followed have indicated relationships between free radicals and various processes such as inflammation, carcinogenesis, and aging.

Active oxygen produced by the administration of carcinogens is mostly inactivated by lipids of the cell membrane and enzymes of the cytoplasm such as GSH-Px, catalase, and SOD before it reaches DNA in the nucleus. Bauer et al.⁴ observed high SOD, GSH-Px, and MDA levels but a low catalase level in colon cancer tissues and speculated that the reduced catalase activity localized in peroxisomes leads to an increase in H_2O_2 , which, in turn, increases hyperoxidation of lipids in tumor tissues but that SOD and GSH-Px exert antioxidant effects against this lipid hyperoxidation.

In this study, we measured the SOD, H_2O_2 scavengers, catalase, GSH-Px and MDA. In the tumor and the border zone, SOD and catalase activities were lower, and GSH-Px and MDA levels were higher, than the control values. A reduction in the SOD activity in cancer tissues has long been known, and Leuthauer and Oberley¹⁴ ascribed it particularly to a decrease in mitochondrial Mn-SOD. Peskin¹⁹ measured Mn-SOD (mitochondrial), Cu, and Zn-SOD (cytoplasmic) in various cancer cells and reported reductions in both SOD levels in cancer cells. Whether these reductions in SOD and catalase in cancer tissue are simply the results of malignant transformation remains to be clarified.

Shinkai et al.²⁷ induced hypoxanthin-xanthinoxidase reaction productive of superoxide radicals in methothelial cell monolayer cultures of rat ascites hepatoma cells with and without simultaneous addition of SOD and catalase and observed inhibition of penetration into the methothelial cell monolayer in the presence of SOD plus catalase. This finding suggests that the reduced SOD and catalase activities in the tumor and the border zone promote the growth of cancer tissue and its infiltration into the surrounding tissues.

The GSH-Px activity is considered to be variable except for the organ-specific high levels particularly in the liver and kidney³⁰. The high activity in our measurement indicates an increase in GSH-Px, which disposes H_2O_2 and peroxy lipids via GSH, as a result of the body's defense reactions to these active oxygens. Also, the increase in MDA, a metabolite of lipid peroxide, suggests an involvement of chronic reactions of free radicals in the pancreas.

Thus, in the pancreatic cancer produced in hamsters by DIPN administration, the O_2^- scavenger SOD and H_2O_2 scavenger catalase were reduced in the neoplastic and borderline regions, and GSH-Px, which disposes lipid peroxide via GSH, was increased in the neoplastic region. These findings suggest an involvement of free radicals in the growth and infiltration of pancreatic cancer

produced with a nitroso compound.

Acknowledgment

This study was supported by grants, Scientific Research B-03454319 and C-01570754, from the Ministry of Education, Science and Culture of Japan. The experiment was done in the Institute of Laboratory Animals, Faculty of Medicine, Kyoto University.

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和文抄録

ハムスター膵癌におけるフリーラジカルスカベンジャーの役割

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癌の発生, 進展機序にフリーラジカルが関与することはよく知られているが, 膵癌とフリーラジカルの関連性については明らかではない. 本研究では, 膵癌におけるフリーラジカルの関わりを明らかにするために, ハムスターを用い, Di-iso-propanol nitrosamine 500 mg/kg 体重/週×24週投与により, 膵管癌を発生せしめ, 膵腫瘍部, 境界部, 非腫瘍部, 正常対照膵部の組織を摘出し, 組織中のスーパーオキシドディスムターゼ (SOD), カタラーゼ, グルタチオンペルオ

キシド, (GSH-Px), マロンディアルデハイド (MDA) 活性を測定し, 比較検討した. その結果, 腫瘍部および境界部における SOD およびカタラーゼ活性は非腫瘍部, 正常膵と比し有意に低下し, GSH-Px および MDH 活性は上昇することが明らかとなった. これらの事実は膵癌の進展機序にフリーラジカル消去系の機能低下が関与することを示唆した. GSH-Px, MDA は反応性に増加するものと考えられた.