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Pivotal role of oxidative stress in tumor metastasis under diabetic conditions in mice

Mai Ikemura [a], Makiya Nishikawa [b,*], Kosuke Kusamori [b], Miho Fukuoka [a], Fumiyoshi Yamashita [a], Mitsuru Hashida [a,c]

[a] Department of Drug Delivery Research, Kyoto University, Sakyo-ku, Kyoto, Japan
[b] Department of Biopharmaceutics and Drug Metabolism, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto, Japan
[c] Institute for Integrated Cell-Material Sciences, Kyoto University, Sakyo-ku, Kyoto, Japan

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ABSTRACT

Diabetic patients are reported to have a high incidence and mortality of cancer, but little is known about the linkage. In this study, we investigated whether high oxidative stress is involved in the acceleration of tumor metastasis in diabetic mice. Murine melanoma B16-BL6 cells stably labeled with firefly luciferase (B16-BL6/Luc) were inoculated into the tail vein of streptozotocin (STZ)-treated or untreated mice. A luciferase assay demonstrated that tumor cells were present largely in the lung of untreated mice, whereas large numbers of tumor cells were detected in both the lung and liver of STZ-treated mice. Repeated injections of polyethylene glycol-conjugated catalase (PEG-catalase), a long-circulating derivative, reduced the elevated fasting blood glucose levels and plasma lipoperoxide levels of STZ-treated mice, but had no significant effects on these parameters in untreated mice. In addition, the injections significantly reduced the number of tumor cells in the lung and liver in both untreated and STZ-treated mice. Culture of B16-BL6/Luc cells in medium containing over 45 mg/dl glucose hardly affected the proliferation of the cells, whereas the addition of plasma of STZ-treated mice to the medium significantly increased the number of cells. Plasma samples of STZ-treated mice receiving PEG-catalase exhibited no such effect on proliferation. These findings indicate that a hyperglycemia-induced increase in oxidative stress is involved in the acceleration of tumor metastasis, and the removal of systemic hydrogen peroxide by PEG-catalase can inhibit the progression of diabetic conditions and tumor metastasis in diabetes.

1. Introduction

Diabetes, characterized by hyperglycemia, is associated with a variety of serious complications, including microvascular and macrovascular diseases [1]. The number of diabetic patients has been increasing and this trend is predicted to continue all over the world [2]. Therefore, the need to prevent and treat diabetes and associated diseases is steadily increasing. Epidemiological studies have suggested that diabetic patients have a high risk of cancer as well as a high risk of cancer-related mortality [3–5]. These clinical data clearly indicate a close link between diabetes and cancer. These two types of diseases share many risk factors, including lifestyle, that could accelerate the incidence and progression of these diseases, but little is known about the mechanism underlying the linkage.

Reactive oxygen species (ROS) have also been proposed to be involved in many serious diseases. In diabetes, oxidative stress that is caused by excess ROS is systemically increased, because high glucose concentrations generate ROS in a variety of cell types [6–8]. In addition, ROS are reported to be involved in several steps of cancer progression, including carcinogenesis, proliferation, adhesion and invasion [9]. Therefore, high blood glucose-induced oxidative stress is a likely reason for the high incidence and rapid progression of cancer in diabetic patients.

One hypothesis suggests that the continuous removal of systemic ROS is a potential approach to prevent tumor metastasis and recurrence. In a previous study, we reported that insulin resistance, one of the characteristic features of type 2 diabetes, can be improved by repeated injections of a catalase derivative conjugated with polyethylene glycol (PEG-catalase) in insulin resistant *ob*/*ob* and high fat diet-induced obese mice [10]. These findings indicate that PEG-catalase inhibits the oxidative stress induced under diabetic conditions. Meanwhile, we have also demonstrated that targeted delivery of catalase effectively inhibits tumor metastasis in some metastatic mouse models [reviewed in 11]. Therefore, if oxidative stress is involved in the acceleration of tumor metastasis in diabetic mice, PEG-catalase is a promising candidate to inhibit it.

In this study, we investigated whether the distribution of tumor cells was affected by the pathophysiological conditions of diabetes using streptozotocin (STZ)-induced hyperglycemic mice. Then, to investigate

* Corresponding author at: Department of Biopharmaceutics and Drug Metabolism, Graduate School of Pharmaceutical Sciences, Kyoto University, 46-29 Shimoadachi-cho, Yoshida, Sakyo-ku, Kyoto 606-8501, Japan.
E-mail address: makiya@pharm.kyoto-u.ac.jp (M. Nishikawa).

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the influence of scavenging ROS generated under diabetic conditions, we also examined the effect of PEG-catalase on diabetic parameters, including blood glucose levels, and tumor metastasis.

2. Material and methods

2.1. Chemicals

Catalase (bovine liver) and streptozotocin (STZ) were obtained from Sigma Chemical (St. Louis, MO, USA). Dulbecco's modified Eagle's minimum essential medium (DMEM) and Hank's balanced salt solution (HBSS) were obtained from Nissui Pharmaceutical (Tokyo, Japan). Penicillin–streptomycin glutamine and serum- and glucose-free DMEM were obtained from Invitrogen Corp. (Grand Island, NY, USA).

2.2. Animals

C57BL/6 mice (male, 5 weeks old) were purchased from Japan SLC, Inc. (Shizuoka, Japan). Animals were maintained under conventional housing conditions and all animal experiments were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The protocols for animal experiments were approved by the Animal Experimentation Committee of the Graduate School of Pharmaceutical Sciences of Kyoto University.

2.3. Synthesis of PEG-catalase

PEG-catalase was synthesized by conjugating catalase with 2,4-bis(O-methoxypolyethylene glycol)-6-chloro-s-triazin (Seikagaku Corporation, Japan) as previously described [12]. The enzymatic activity was measured by monitoring the decomposition of hydrogen peroxide spectrophotometrically at 240 nm.

2.4. Production of a mouse model of diabetes and treatment with PEG-catalase

C57BL/6 mice received an intraperitoneal injection of STZ dissolved in sodium citrate buffer (pH 4.5) at a dose of 100 mg/kg body weight. A group of mice received intraperitoneal injections of PEG-catalase in saline three times a week at a dose of 1000 catalase units/shot starting on the day of the induction of hyperglycemia. Another group of mice was treated similarly except for PEG-catalase.

2.5. Tumor metastasis model

Murine melanoma B16-BL6 tumor cells (obtained from the Cancer Chemotherapy Center of the Japanese Foundation for Cancer Research, Tokyo, Japan) expressing firefly luciferase (B16-BL6/Luc) were established as reported previously [13]. They were grown in DMEM supplemented with 10% heat-inactivated FBS, 0.15% $NaHCO_3$, 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C in humidified air containing 5% CO_2. At 4 weeks after STZ injection, each mouse received a tail vein injection of 1×10^5 B16-BL6/Luc cells in 0.1 ml HBSS. Mice were killed on predetermined days after inoculation, and the lungs and liver were excised, washed with saline and homogenized. The number of B16-BL6/Luc in organs was determined by measuring luciferase activity as reported previously [13].

2.6. Evaluation of diabetic conditions

Blood samples were obtained after a 12-h fast by cardiac puncture from non-tumor-bearing mice. Blood glucose levels were determined with an ACCU-CHEK Active meter (Sanko Junyaku Co., Ltd, Japan). The level of malondialdehyde was determined by an assay of the thiobarbituric acid reactive substances (TBARS) using commercially available test reagents (Cayman Chemical Company, MI, USA).

2.7. Proliferation of tumor cells under high glucose conditions

B16-BL6/Luc cells were seeded on 6-well plates at a density of 1×10^4 cells per well. At 6 days after incubation in medium containing 0, 45, 90 or 360 mg/dl glucose, the number of tumor cells was determined using the methylthiazole tetrazolium (MTT) assay. Separately, B16-BL6/Luc cells were seeded on 96-well plates at a density of 1×10^3 cells per well in serum- and glucose-free DMEM. Blood was collected from 12 h-fasted non-tumor-bearing-mice at 4 weeks after STZ injection. Plasma obtained from the blood by centrifugation was added to cultured B16-BL6/Luc cells at a final concentration of 10% plasma. At 3 days after treatment, the number of tumor cells was determined as described above.

2.8. Statistical analysis

Differences were statistically evaluated by one-way ANOVA followed by the Student–Newman–Keuls multiple comparison test. The significance of differences was set at $P < 0.05$.

3. Results

3.1. Distribution and proliferation of tumor cells in STZ-treated mice

Fig. 1 summarizes the characteristics of STZ-treated mice used in this study. At 4 weeks after STZ injection, the body weight of the mice was significantly reduced (Fig. 1A), and fasting blood glucose levels were significantly increased more than two-fold (Fig. 1B). Plasma malondialdehyde levels were significantly higher in the STZ-treated mice (Fig. 1C) than in the control mice. Thus, the STZ-treated mice were referred as hyperglycemic mice.

Then, the distribution and proliferation of tumor cells in control and STZ-treated mice were examined at 3 weeks after intravenously inoculating the mice with B16-BL6/Luc cells. Tumor cells were detected in the lungs of all control mice (Fig. 2A) and in the liver of one out of five control mice (Fig. 2B). Fig. 2C and D shows typical examples of the lung and liver of the control mice, respectively. There are several black colonies of B16-BL6/Luc cells on the surface of the lung. A significantly greater number of tumor cells were detected in the lung of the STZ-treated mice than in the control mice (Fig. 2A). In addition, tumor cells were detected in the liver of all the STZ-treated mice, and the average number (6×10^6 cells/liver) was far greater than the number in the control mouse (Fig. 2B). Reflecting these quantitative results, there were greater and more numerous metastatic colonies in the lung (Fig. 2E) and liver (Fig. 2F) of the STZ-treated mice.

3.2. Effect of PEG-catalase on the physiological parameters in STZ-treated mice

Fig. 3 shows the effect of repeated injections of PEG-catalase on the physiological parameters of control and STZ-treated mice. The molecular weight of PEG was 10,000, and about 24% of amino groups of catalase were estimated to be modified with PEG in PEG-catalase. The remaining activity of PEG-catalase was 96% of unmodified catalase [12]. The body weight of the STZ-treated mice was significantly lower than that of the control mice (Fig. 3A). PEG-catalase had no significant effects on the body weight of the two groups. A significantly high fasting blood glucose level was detected in the STZ-treated mice, and this was significantly reduced by repeated injections of PEG-catalase (Fig. 3B). The plasma malondialdehyde level was also higher in the STZ-treated mice than in the control mice (Fig. 3C). Again, repeated injections of PEG-catalase significantly reduced the