Advanced Glycation Endproducts Act as An Initiator of Skin Tumors in Mice

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Summary The role of advanced glycation endproducts (AGEs) as the initiator of tumor induction was investigated in SENCAR mice. Initiation with AGEs-modified bovine serum albumin (AGES-BSA) increased both the incidence of tumor-bearing mice and the mean number of tumors per mouse. An immunohistochemical study revealed an increase in 8-hydroxy-2'-deoxyguanosine (8-OhdG) in nuclei of epidermal cells in the AGEs-BSA treated mice. Furthermore, a Salmonella mutagenicity test revealed that AGEs-BSA was mutagenic only in Salmonella TA104, a strain more sensitive to oxidative stress than other strains. These results suggest that AGEs are mutagenic through a mechanism associated with oxidative stress.

Key Words: advanced glycation endproducts, diabetes mellitus, oxidative stress, cancer

Introduction

It has been reported that a history of diabetes is associated with an increased risk of cancers [1–3], although there is no clear explanation for this relationship. When proteins are exposed to reducing sugars, such as glucose, they undergo nonenzymatic glycation and oxidation. The ultimate result of this process is the formation of advanced glycation endproducts (AGEs) [4]. The excessive formation and accumulation of AGEs in diabetes can lead to tissue damage through a variety of mechanisms, including cellular perturbation through AGE receptors and/or the generation of reactive oxygen species [5–9].

We report that AGEs can induce skin tumors in SENCAR mice which are sensitive to multistage skin carcinogenesis [10]. This finding may explain the observation that there is an increased risk of neoplasms in individuals with diabetes.

Materials and Methods

Preparation of AGES-modified bovine serum albumin (AGES-BSA)

AGES-BSA was prepared by incubating BSA (fraction V, very low endotoxin; Miles, Kankakee, IL) in phosphate-buffered saline (PBS) (10 mM, pH 7.4) with 0.4 M glucose at 37°C for 6 weeks under sterile conditions. As a control, unmodified BSA was incubated under the same conditions.
without glucose. The unbound sugars were removed by dialysis against PBS.

**Animals and tumor induction experiments**

Specific pathogen-free female SENCAR mice were obtained from Nippon SL C Co., Ltd. (Shizuoka, Japan). The SENCAR mice were divided into 4 groups of 15 animals and their dorsal surface shaved with electric clippers. All solutions of 7,12-dimethylbenz(a)anthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA) (Sigma, St. Louis, MO) were prepared in reagent-grade acetone and were applied topically in a total volume of 0.1 ml. The control mice were initiated by a single application of DMBA at the concentration of 100 µg per mouse. AGES-BSA (100 µg and 10 µg) or unmodified BSA (100 µg) were smeared onto the mice after a single application of 0.2 ml acetone. One week after initiation, TPA (1 µg per mouse) was applied twice a week throughout the experiment. All the experimental groups were followed for 20 weeks after promotion. The incidence of papillomas (%) and number of papillomas per mouse were analyzed with Student’s t-test. All animal experiments were conducted according to the ‘Guidelines for Animal Experimentation’ at Kyoto Prefectural University of Medicine.

**Immunohistochemistry**

Immunohistochemical staining of skin specimens was performed using a monoclonal antibody specific for 8-OHdG (N45.1) (WAKO Chemicals, Osaka, Japan) according to the method described by Hattori et al. [11].

**Salmonella mutagenicity assay**

Various concentrations of AGE were tested in several Salmonella strains (TA98, TA100, TA102 and TA104; a kind gift from Dr. B.N. Ames) using a liquid preincubation procedure [12]. S9 liver homogenate from Sprague-Dawley male rats treated with phenobarbital and 5,6-benzoflavone was purchased from Oriental Yeast Co., Ltd. (Tokyo Japan). Sodium phosphate buffer (0.02 M, pH 7.4) mixed with S9 (100 µL per ml) was added to sterile 13 mm × 100 mm capped culture tubes. Next an aqueous solution of AGEs with 0.1 ml of an overnight culture of the bacterial strain was added to yield a final volume of 0.5 ml. The tubes were incubated with shaking at 37°C for 20 min, and then 2 ml of molten top agar containing histamine and biotin was added to each tube. The mixture was then plated on minimal glucose, and the number of revertants scored after 48 hr. The experiments were performed in duplicate, with 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide and Doxorubicin (Sigma, St. Louis, MO) being used as a positive control.

**Results and Discussion**

SENCAR mice are sensitive to multistage skin carcino-

![Fig. 1. Initiating activity of AGES-BSA in SENCAR mice. (a) incidence of papillomas, (b) mean number of papillomas per mouse. Fifteen mice in each group received initiation treatment of either 100 µg of DMBA ( ), 100 µg of AGES-BSA ( ), 10 µg of AGES-BSA ( ), or 100 µg of unmodified BSA ( ). The incidence of papillomas and mean number of papillomas per mouse were increased significantly in AGES-BSA (100 µg) initiated mice compared with unmodified BSA initiated mice (P<0.01).](image-url)
genesis initiated by DMBA and promoted by TPA [13]. Skin tumors formed on mice initiated with DMBA and 100 µg of AGEs-BSA, whereas no tumors formed on mice initiated with either low dose AGEs-BSA (10 µg) or unmodified BSA. Eight weeks after initiation, skin tumors were found in 2 of 15 mice treated with 100 µg of AGEs-BSA, with the incidence of tumor-bearing mice reaching 100% 14 weeks after initiation (Fig. 1-a). The mean number of tumors per mouse in the AGEs-treated mice was 4.8 tumors at 20wk (Fig. 1-b). This result indicates that a high dose of AGEs can act as a tumor initiator in SENCAR mice. An immunohistochemical study revealed an increase in 8-OHdG, a major oxidative modified DNA base product, in the nuclei of epidermal cells in AGEs-BSA treated mice (Fig. 2) [14]. It has been shown that AGEs produce reactive oxygen species thereby enhancing cellular oxidant stress [5, 15–18]. Our results therefore suggest that AGEs may be mutagenic by inducing oxidative stress.

We next investigated whether AGEs are mutagenic using the Salmonella mutagenicity test, otherwise known as the Ames test. This test measures back-mutations in several specially constructed mutant strains of Salmonella [19]. In our study we used Salmonella strains TA98, TA100, TA102 and TA104. As shown in Fig. 3, AGEs were mutagenic only in strain TA104 and not in the other strains. TA104 was constructed from a base-pair reversion at the chromosomal histidine locus hisG428 (T:A to G:C, A:T or C:G). This reversion also enhances cell wall permeability due to insertion of an rfa mutation, and R factor on plasmid pKM101 which carries the uvrB gene for an SOS system, a type of error-prone DNA polymerase which inhibits the repair of damaged DNA [20]. The other Salmonella strains (TA98, TA100 and TA102) lack at least one of these mechanisms. It has been reported that TA104 is more sensitive to oxidative stress than another strains and is therefore used as a test strain for mutagens which are thought to damage DNA through the generation of free radicals. This result also suggests that the mutagenicity caused by AGEs is due mainly to oxidative stress.

It has been reported that oxidative stress is increased in vivo in individuals with diabetes [21, 22]. Oxygen-free radicals play an important role in damaging cells including oncogenesis [23]. Furthermore, the accumulation of AGEs on long-lived connective tissue and matrix components is accelerated in diabetes [24]. Taken together, the present results may explain why there is an increased risk of neoplasms in individuals with diabetes.

Fig. 2. Immunohistochemical examination using specific antibodies to 8-OHdG, MoAb N45.1. The epidermis from a mouse after a 24h treatment with either (a) 100 µg of AGEs-BSA or (b) 100 µg of unmodified BSA stained using MoAb N45.1. ×400 magnification.

Fig. 3. Reverse mutation test (Ames test) with AGEs-BSA. The effect of AGEs-BSA on the number of revertant colonies in the mutation tests using various Salmonella strains: TA98 (■), TA100 (□), TA102 (●) and TA104 (○). AGEs caused significant increases in the number of revertants only in strain TA104.
References


