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Effect of hyperglycemia on hepatocellular carcinoma development in diabetes

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ABSTRACT

Compared with other cancers, diabetes mellitus is more closely associated with hepatocellular carcinoma (HCC). However, whether hyperglycemia is associated with hepatic carcinogenesis remains uncertain. In this study, we investigate the effect of hyperglycemia on HCC development. Mice pretreated with 7,12-dimethylbenz (a) anthracene were divided into three feeding groups: normal diet (Control), high-starch diet (Starch), and high-fat diet (HFD) groups. In addition, an STZ group containing mice that were fed a normal diet and injected with streptozotosin to induce hyperglycemia was included. The STZ group demonstrated severe hyperglycemia, whereas the Starch group demonstrated mild hyperglycemia and insulin resistance. The HFD group demonstrated mild hyperglycemia and severe insulin resistance. Multiple HCC were macroscopically and histologically observed only in the HFD group. Hepatic steatosis was observed in the Starch and HFD groups, but levels of inflammatory cytokines, interleukin (IL)-6, tumor necrosis factor-α, and IL-1β, were elevated only in the HFD group. The composition of gut microbiota was similar between the Control and STZ groups. A significantly higher number of Clostridium cluster XI was detected in the feces of the HFD group than that of all other groups; it was not detectable in the Starch group. These data suggested that hyperglycemia had no effect on hepatic carcinogenesis. Different incidences of HCC between the Starch and HFD groups may be attributable to degree of insulin resistance, but diet-induced changes in gut microbiota including Clostridium cluster XI may have influenced hepatic carcinogenesis. In conclusion, in addition to the normalization of blood glucose levels, diabetics may need to control insulin resistance and diet contents to prevent HCC development.

1. Introduction

Epidemiological studies have shown that the incidence of various cancers, including cancers of the bladder, breast, colon, endometrium, liver, and pancreas, are significantly elevated in patients with diabetes mellitus (DM) [1,2]. The relative risk of hepatocellular carcinoma (HCC) is the most closely associated with DM [3,4].

The incidence and mortality of HCC ranks the fifth highest in that of all cancers [5]. Important etiological risk factors of HCC include hepatitis viral inflammation, nonalcoholic steatohepatitis, obesity, and smoking. DM is also considered to be associated with a 2.5-fold increased risk for HCC [6,7]. Chronic inflammation, insulin resistance with hyperinsulinemia, and changes in gut microbiota are reported to be associated with carcinogenesis in the livers of DM patients [8–11]. Systemic and local inflammation occurs in obese DM patients [12]. Inflammatory changes in the liver

Abbreviations: DMBA, 7,12-Dimethylbenz (a) anthracene; SASP, senescence-associated secretory phenotype; HFD, high-fat diet; ND, normal diet.

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enhances production of inflammatory cytokines, which can cause cellular proliferation, angiogenesis, and apoptosis suppression, resulting in HCC development [8,13]. Hyperinsulinemia associated with insulin resistance in DM patients promotes protein synthesis, cellular proliferation, and apoptosis suppression, resulting in acceleration of tumor progression [9]. A recent study also reported that gut microbiota, particularly Clostridium cluster XI, play an important role in HCC development through senescence-associated secretory phenotype (SASP) of hepatic stellate cells in high-fat diet (HFD) fed mice [14].

Hyperglycemia is suggested to increase the risk of developing cancer [15]. Hyperglycemia in patients with type 2 DM increases the breast cancer-associated mortality risk [16]. Oxidative stress, hypoxia-inducible factor-1α (HIF-1α), and epigenetic changes induced by hyperglycemia promote tumor progression in pancreatic and breast cancer cell lines [17]. Hyperglycemia can increase the level of serum inflammatory cytokines [18]. These studies suggest that hyperglycemia can potentially promote HCC development.

However, it remains uncertain whether hyperglycemia alone, without any concomitant factors, is associated with carcinogenesis in the liver of DM patients. In this study, we investigate the effect of hyperglycemia on HCC development.

2. Materials and methods

2.1. Animal model

C57Bl/6J mice (Chubu Kagaku Shizai Co. Ltd., Japan), were housed in a temperature-controlled room under conditions of the 12 h light/dark cycle, with free access to food and water. All procedures were performed according to a protocol approved by the Nagoya University Institutional Animal Care and Use Committee. Carcinogenesis was initiated in male mice using 7,12-dimethylbenz (a) anthracene (DMBA; Sigma). Treatment comprised a single application of 50 μL of 0.5% DMBA solution in acetone to the dorsal surface on postnatal day 4. Next, the mother mice and the pups were fed with a normal diet. At 4 weeks old, pups were weaned and subsequently fed with a normal diet, high-starch diet (Starch) or HFD, until euthanization at 40 weeks after DMBA administration. Control groups were fed on a normal diet (ND; CLEA Japan, Osaka, Japan) containing 4.2% fats and 54.6% carbohydrates. A streptozotosin (STZ; Sigma–Aldrich) group was fed with the ND and intraperitoneally (i.p.) injected with STZ. STZ was dissolved in saline and injected at 50 mg/kg body weight daily for 5 days starting at the ninth week of age. This injection helped induce the pancreatic damage causing DM. Starch groups were fed with Starch (CLEA Japan, Osaka, Japan) containing 12.8% fats and 74.1% carbohydrates. HFD group was fed with HFD (HFD32; Chubu Kagaku Shizai Co. Ltd., Japan) containing 32% fats and 29.4% carbohydrates.

2.2. Random blood glucose levels and body weight

Random blood glucose levels and body weight were measured day at 09:00. Blood glucose levels were measured with Antsense III (Horiba, Kyoto, Japan).

2.3. Intraperitoneal glucose tolerance test (IPGTT)

IPGTT was conducted at 32 weeks. After 16 h of fasting, D-glucose, 2 g/kg body weight, was injected i.p. into the treated mice. Plasma glucose levels were then measured at 0, 15, 30, 60, and 120 min after the injection. Plasma insulin levels were measured using a mouse insulin enzyme-linked immunosorbent assay kit (Morinaga, Tokyo, Japan) at the same time points.

2.4. Insulin tolerance test (ITT)

ITT was conducted at 32 weeks. After 6 h of fasting, regular insulin (Humulin U-100; Lilly, Indianapolis, IN), 0.6 g/kg body weight, was injected i.p. into the treated mice. Plasma glucose levels were then measured at 0, 15, 30, 60, and 120 min after the injections.

2.5. Area under the curve (AUC)

AUC for glucose (mg min/dL) and insulin (ng min/mL) was calculated, as described previously [19].

2.6. Triglyceride measurement

The liver tissue was isolated at 40 weeks after DMBA administration and then homogenized with isopropyl alcohol (Wako, Tokyo, Japan). The triglyceride content in the liver tissue was measured using the Triglyceride E-test kit (Wako, Tokyo, Japan).

2.7. Histological analyses

The liver was fixed in 4% paraformaldehyde, sequentially washed thoroughly in PBS, and embedded in paraffin wax. The specimens were sectioned at a thickness of 4 μm and stained with hematoxylin–eosin staining. Steatosis in the liver was assessed by the steatosis score, as described by Kleiner et al. [20], with separate scores for steatosis (0–3). Liver fibrosis was assessed using Sirius red staining, and the areas with positive Sirius red staining were measured using a BZ-9000 fluorescent microscope system (Keyence, Osaka, Japan) in five microscopic fields at a 200-fold magnification. All liver specimens were assessed by two hepatologists blinded to the identities of the study groups.

2.8. RNA expression of cytokine

Total RNA was extracted from each liver and HCC using the RNeasy® Plus Mini kit from Qiagen (Valencia, CA, USA). RNA expression of interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and IL-1β relative to GAPDH was quantified using Power SYBR Green RNA-to-CTTM 1-Step kit in a 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The following primer sets were used: For IL-6, forward primer: 5′-CCGGAGAGGAGACTTC ACAG-3′ and reverse primer: 5′-TCCACGATTTCCCAGAGAAC-3′. For TNF-α, forward primer: 5′-TATGGCTCAGGGTCCAACTC-3′ and reverse primer: 5′-CTCCCTTTGCAGAACTCAGG-3′. For IL-1β, forward primer: 5′-GATCCACACTCTCCAGCTGCA-3′ and reverse primer: 5′-CAACCAACAAGTGATATTCT-3′. For GAPDH, forward primer: 5′-CCAATGTGTCCGTCGTGGAT-3′ and reverse primer: 5′-TGCTGTTGAAGTCGCAGGAG-3′.

2.9. Bacterial 16S rRNA amplification sequencing and analysis

Fecal samples were collected from mice at 40 weeks after DMBA administration. DNA was extracted from the feces and the large intestinal microbiota was analyzed using terminal restriction fragment length polymorphisms (T-RFLP), as described previously [21–23].

2.10. Determination of the copy number of fecal bacteria

The copy number of Clostridium cluster XI was calculated from the standard curve of known bacterial copy number by quantitative real-time PCR of 16S rRNA gene using 5′-TGACGGTACYYNRKGAGG AAGCC-3′ and 5′-ACTACGGTTRAGCCGTAGCCTTT-3′ primers as described previously [24].