



Short communication

Binding affinity of anti-xylitol antibodies to canine hepatic vessels

Akihiro Imai^a, Toshiho Nishita^b, Nobutsune Ichihara^c, Kinji Shirota^d, Kensuke Orito^{a,*}^a Department of Physiology II, School of Veterinary Medicine, Azabu University, Sagamihara, Kanagawa 252-5201, Japan^b Department of Physiology I, School of Veterinary Medicine, Azabu University, Kanagawa, Japan^c Department of Anatomy I, School of Veterinary Medicine, Azabu University, Kanagawa, Japan^d Department of Pathology, School of Veterinary Medicine, Azabu University, Kanagawa, Japan

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ABSTRACT

Xylitol is used as a sugar substitute in food products. Dogs have been reported to experience lethal liver injury after accidental ingestion of xylitol. Because liver injury may be a serious consequence of canine immune-mediated reactions, antibodies produced against xylitol may attack the liver. Therefore, in the present study, we evaluated whether binding sites for xylitol antibodies are located at the liver or not. Anti-xylitol antibodies were generated by immunization of rabbits with a xylose–bovine serum albumin conjugate. Immunohistological examination showed that binding sites for the anti-xylitol antibodies were located in the hepatic arteries and the portal veins. Western blotting analyses by using a canine liver homogenate showed 4 protein bands with different molecular weights which reacted with anti-xylitol antibodies. Therefore, binding of anti-xylitol antibodies to the vessels may be the first step in an immune-mediated pathogenic response in xylitol toxicity. Further studies are necessary to determine the effects of anti-xylitol antibodies on the liver in the pathogenesis of xylitol toxicity.

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1. Introduction

Xylitol, a 5 carbon sugar alcohol, has been widely used as a sugar alcohol sweetener in human diets since its approval by the US Food and Drug Administration (FDA) in 1960 (Jaffe, 1978; Makinen, 2000). On the other hand, xylitol has been reported to act as a contact allergen and induces oral erosions and skin erythema in humans (Jaffe, 1978; Makinen et al., 1995; Mattila et al., 1995; Makinen, 2000; Venkatesh, 2003). It is also a known toxin in dogs and induces excessive production of insulin in the pancreas with resultant severe hypoglycemia (Dunayer, 2004). In addition, there is a clinical report on 8 adult dogs that were treated for lethargy and vomiting after ingestion of xylitol, and some of these dogs had widespread petechial, ecchymotic, or gastrointestinal tract hemorrhages.

Clinicopathological findings include moderately to severely elevated serum activities of liver enzymes, hyperbilirubinemia, hypoglycemia, hyperphosphatemia, prolonged clotting times, and thrombocytopenia. In other words, xylitol not only caused hypoglycemia but also caused acute life-threatening hepatopathy and coagulopathy in these dogs (Dunayer and Gwaltney-Brant, 2006). Hypoglycemia was evoked within an hour of xylitol ingestion; however, hepatic damage was induced several hours after ingestion (Campbell and Bates, 2008, 2010). Thus, it is highly likely that the hepatic damage induced by xylitol is not related to hypoglycemia. The pathogenic mechanisms underlying hepatic toxicity of xylitol in dogs remain to be elucidated.

Dengue virus commonly causes hepatic involvement characterized by raised transaminases and sometimes induces acute hepatic failure (Giri et al., 2008). When dengue virus nonstructural protein 1 (NS1), which was detected in the serum of patients with dengue virus infection, was injected into mice, antibodies against NS-1

* Corresponding author. Tel.: +81 42 850 2474; fax: +81 42 850 2474.
E-mail address: oritok@azabu-u.ac.jp (K. Orito).

were generated and cross-reacted with host components, including endothelial cells (Falconar, 1997). The antibodies against NS1 induced hepatic injury by disrupting vascular endothelium in the liver (Hollyfield et al., 2008). These findings indicate the molecular mechanism for the induction of an autoimmune pathogenesis in dengue virus infection (Jaeschke et al., 2002; Lin et al., 2006; Martina et al., 2009). When a conjugate of xylose and bovine serum albumin (BSA) was injected subcutaneously in rabbits, specific antibodies against xylitol were generated (Sreenath and Venkatesh, 2007). Thus, xylitol may act as a haptenic antigen, and the induced anti-xylitol antibodies may be pathogenic in dogs. If this is true, anti-xylitol antibodies are hypothesized to bind to the structures in the liver as the first step in an immune-mediated hepatopathy. In the present study, specific antibodies against xylitol were first generated, and hepatic binding sites of the anti-xylitol antibodies were delineated in dogs.

2. Materials and methods

2.1. Purification of rabbit anti-xylitol antibodies

Specific antibodies against xylitol were obtained as reported previously (Hegde and Venkatesh, 2007; Sreenath and Venkatesh, 2007). In brief, two 4-month-old female Japanese white rabbits (J1a: JW, Japan Laboratory Animals, Inc., Tokyo, Japan), housed in the animal house facility of this institute, were immunized with a conjugate of xylose and BSA by subcutaneous injection. The rabbits were bled by marginal ear vein puncture, and the formation of anti-xylitol antibodies was demonstrated by conventional immunodiffusion tests with xylose–BSA conjugate and BSA. Ten days after the confirmation of antibody formation, the rabbits were anesthetized with urethane (1 g/kg, i.v.), and blood was collected from the jugular vein. The rabbit affinity IgGs (antibodies) for xylitol were purified with affinity chromatography. We confirmed that the obtained anti-xylitol antibodies had high specificity to xylitol and low cross-reactivity for sugar alcohols including xylose, mannose, threitol, sorbitol, arabinitol, and ribitol by using noncompetitive ELISA (data not shown). The antibodies were stored at -80°C until use.

2.2. Immunohistochemistry

Canine liver was obtained from a 1-year-old female beagle (Japan Laboratory Animals, Inc.) and frozen in liquid nitrogen. Cryostat sections (thickness, $10\ \mu\text{m}$) were mounted on glass slides. They were washed briefly in PBS and pretreated with 0.3% H_2O_2 in methanol to block endogenous peroxidase activity. The purified anti-xylitol antibodies, diluted 1:500 with buffer A (50 mM Tris–HCl [pH 7.5] containing 0.3% BSA, 0.9% NaCl, 0.01% thimerosal, and 10 mM EDTA) was used for the detection of antigenic complexes cross-reacting with xylitol in the canine liver. Following incubation in the antibodies at 4°C overnight, immuno-positive reactions were visualized using a Histofine streptavidin-biotin-peroxidase kit (SAB-PO[®] kit; Nichirei, Inc., Tokyo, Japan). The staining procedure was performed according to the manufacturer's

protocol. All preparations were incubated with 0.02% H_2O_2 and 0.1% diaminobenzidine tetrahydrochloride in 0.05 M Tris–HCl (pH 7.6) for 2 min and 20 s, respectively. The sections were counterstained with hematoxylin to stain the nucleus (Kaseda et al., 2006).

All animals were handled in accordance with “Azabu University Animal Experiment Guidelines; April 2000”.

2.3. Western blotting

Western blotting was performed as previously described (Nishita and Matsushita, 1988). Antigen bound to the anti-xylitol antibodies obtained in the present study was diluted 1:2000 and subjected to western blotting.

3. Results and discussion

Binding sites of anti-xylitol antibodies were confirmed using immunohistological methods. When control IgGs purified with affinity chromatography from normal rabbit blood were applied to the specimen, no binding to hepatic vessels or bile ducts was observed (Fig. 1). In contrast, anti-xylitol antibodies were found to bind intensely to the hepatic arteries and moderately to the portal veins (Fig. 1) but did not bind to the bile ducts at all. Western blotting analyses by using a canine liver homogenate showed 4 protein bands with different molecular weights; 1 specific signal had a higher molecular weight and the remaining 3 specific signals had lower molecular weights as compared to the xylitol–BSA conjugate (Fig. 2).

In the present study, we first generated specific antibodies against xylitol according to previously reported methods (Hegde and Venkatesh, 2007; Sreenath and Venkatesh, 2007). Immunochemical assay showed that the anti-xylitol antibodies bound intensely to the hepatic arteries and moderately to the portal veins of the liver of the beagle dog. The antibodies likely bound to the endothelial cells of these vessels. However, the possibility that the antibodies bound to the other structures of the vessel walls cannot be excluded. Further studies using other methods such as immunogold electron microscopy, binding experiments with isolated canine hepatic endothelial cells, or confocal microscopy are necessary to examine whether the anti-xylitol antibodies really bind to the endothelial cells or not. Western blotting analyses with a canine liver homogenate showed 4 proteins that reacted with the anti-xylitol antibodies present in the canine liver. These findings strongly suggest that anti-xylitol antibodies bind to antigens located within the hepatic vessels.

Dengue virus is associated with hepatic injury caused by disruption of endothelial cells and endothelial cell-derived inflammatory events (Jaeschke et al., 2002; Higuchi and Gores, 2003). Anti-dengue virus NS1 antibodies that cross-reacted with endothelial cells were found in dengue patients and caused cell apoptosis and inflammatory activation (Lin et al., 2002, 2003). Immune-mediated hepatic injuries are induced by a wide array of medications, herbal supplements, and dietary supplements. Drugs such as halothane, tienilic acid, dihydralazine, and anticonvulsants may trigger immune reactions. Immuncyte activation may then generate autoantibodies and cell-mediated

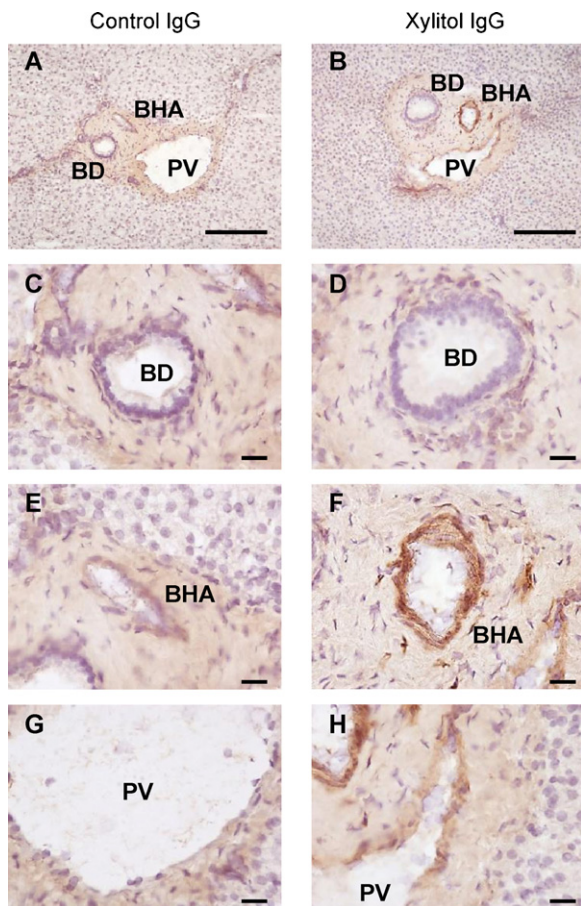


Fig. 1. Immunostaining in the hepatic triad with control and anti-xylitol IgGs. No binding to the branch of the hepatic arteries (BHA), the portal veins (PV) or bile ducts (BD) was observed in control IgGs. In contrast, anti-xylitol antibodies were found to bind intensely to BHA and moderately to PV. Bars indicate 200 μm (A and B) and 20 μm (C–H).

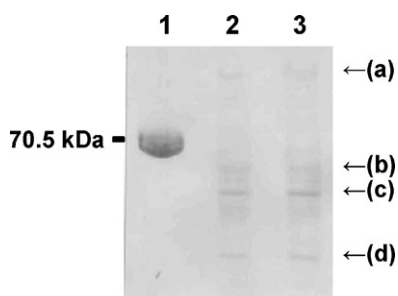


Fig. 2. Western blotting of homogenized extract from canine liver samples after 12.5% SDS-PAGE. Lane 1, xylitol–BSA conjugate; lane 2, hepatic extract (dilution, $\times 32$). Lane 3, hepatic extract (dilution, $\times 16$). Bands (a)–(d) indicate the position of western blot signals (Band a, 123 kDa; Band b, 52.5 kDa; Band c, 41.5 kDa; Band d, 12 kDa).

immune responses, which in turn damage hepatocytes (Liu and Kaplowitz, 2002). This evidence indicates the association of autoimmune responses with antibodies and hepatitis.

When xylitol was administered to dogs, plasma alanine aminotransferase and aspartate aminotransferase increased dose-dependently (Xia et al., 2009). However, accidental ingestion of only a small quantity of xylitol has been reported to induce acute hepatitis in veterinary medicine (Dunayer, 2006; Dunayer and Gwaltney-Brant, 2006). Thus, anti-xylitol antibodies may bind to hepatic vessels and trigger or promote hepatic inflammatory events, which may lead to lethal hepatitis. However, the present study merely proved that anti-xylitol antibodies bind intensely to hepatic vessels. Therefore further studies are necessary to prove the pathophysiological association between anti-xylitol antibodies and acute hepatic disease in dogs.

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