Galactosemic Neuropathy in Transgenic Mice for Human Aldose Reductase

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We studied the functional consequences of an enhanced polyol pathway activity, elicited with galactose feeding, on the peripheral nerve of transgenic mice expressing human aldose reductase. Nontransgenic littermate mice were used as controls. With a quantitative immunoassay, the expression level of human aldose reductase in the sciatic nerve was 791 ± 44 ng/mg protein (mean ± SE), about 25% of that in human sural nerve. When the transgenic mice were fed food containing 30% galactose, significant levels of galactitol accumulated in the sciatic nerve. Galactose feeding of nontransgenic littermate mice led to a 10-fold lower accumulation of galactitol. Galactose feeding for 16 weeks caused a significant and progressive decrease in motor nerve conduction velocity in transgenic mice to 80% of the level of galactose-fed littermate mice, which was not significantly different from that of galactose-free littermate mice. A morphometric analysis of sciatic nerve detected >10% reduction of mean myelinated fiber size but no alterations of myelinated fiber density in galactose-fed transgenic mice compared with other groups. The functional and structural changes that develop in galactose-fed transgenic mice are similar to those previously reported in diabetic animals. The results of these studies suggest that transgenic mice expressing human aldose reductase may be a useful model not only for defining the role of the polyol pathway in diabetic neuropathy but also for identifying and characterizing effective inhibitors specific for human aldose reductase. ***Diabetes*** **45:56–59, 1996**

Polyol pathway has been suggested to play an important role in the development of vascular and neurological complications in diabetic patients (1–4). Galactose intoxication is a frequently used procedure that examines the effect of exaggerated polyol pathway activity on the target tissues of diabetic complications (5–8). The major advantage of this animal model of diabetic complications is that an increased flux of substrate through the polyol pathway can be studied separately from insulinopenia (5,6). By exploiting the data obtained from diabetic and galactosemic animal models, various aldose reductase inhibitors (ARIs) have been developed for the treatment of diabetic complications. In spite of the availability of these tools, the role of the polyol pathway in the development of diabetic complications and the efficacy of ARIs in the prevention and reversal of diabetic or galactosemic tissue injuries has remained controversial (8–11).

The incorporation and overexpression of a specific gene into transgenic animals greatly facilitate studies aimed at characterizing the function of the gene products. We have succeeded in producing transgenic mice that express human aldose reductase (hAR) in a variety of tissues (12,13), and in the present study we have characterized the consequences of an exaggerated flux of substrate through the polyol pathway on peripheral nerve function and structure.

RESEARCH DESIGN AND METHODS

Animals. Mice transgenic for hAR were developed by injecting full-length hAR cDNA (14) with a mouse major histocompatibility antigen class I promoter into the eggs of B6D2F2 female mice (13). One transgenic line designated K^d-AR1 was crossed to B6D2F1 female mice (C57BL/6 × DBA2). Their litters were examined for hAR transgene expression with the polymerase chain reaction (PCR) using a set of transgene specific primers. The sequences of primers were as follows: upstream primer 5′-CTGCTAACCATGTTCATGCC-3′ and downstream primer 5′-TTCACGGCCTCAGTCACCT-3′. PCR was performed with 30 cycles through the following temperature sequence: 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min. The PCR reaction mixture consisted of 50 mmol/l KCl and 10 mmol/l Tris-HCl, pH 8.4, with 2.5 mmol/l $MgCl_2$, 0.2 mmol/l dNTPs, 2.5 U of *Taq* polymerase, 100 ng of tail DNA, and 10 pmol/l of each primer. DNA was recovered from the tail biopsy as previously described (15). Mice negative for transgenic expression were used as controls (Lm).

After birth, all animals were maintained in plastic cages in rooms with a constant temperature of 25°C and 12-h light and dark cycle. All animals were given free access to water.

Determination of hAR protein levels in sciatic nerve. hAR protein levels in the sciatic nerve were measured with an enzyme-linked immunosorbent assay (ELISA) method, as previously described (16). For this purpose, 4-week-old hAR transgenic (Tg) and littermate (Lm) mice were killed, as described above, and the sciatic nerves were dissected, weighed, and stored at −20°C before the analysis.

Polyol levels in the sciatic nerve of galactosemic mice. Eight-week-old hAR Tg and Lm mice were fed with a 30% galactose diet (wt/wt, Nihon Clea, Tokyo) and killed after 1, 2, 3, or 4 weeks of galactose feeding. Each group consisted of four animals. Sciatic nerves were removed and stored at −20°C before analysis. Tissue galactitol levels were quantitated with gas chromatography (MD204 gas chromatogram, Shimadzu, Tokyo) as previously described (18).

Motor nerve conduction velocity (MNCV) of galactose-fed mice. At 8 weeks of age, hAR Tg and Lm mice were each separated into two groups of 20 animals. These groups were fed either normal food or food with 30% galactose for 16 weeks. MNCV was measured in these four groups of animals with previously described methods (19). For these measurements, all the mice were anesthetized with ether and the body

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AR, aldose reductase; ARI, aldose reductase inhibitor; ELISA, enzyme-linked immunosorbent assay; hAR, human aldose reductase; Lm, littermate; MNCV, motor nerve conduction velocity; PCR, polymerase chain reaction; Tg, transgenic.

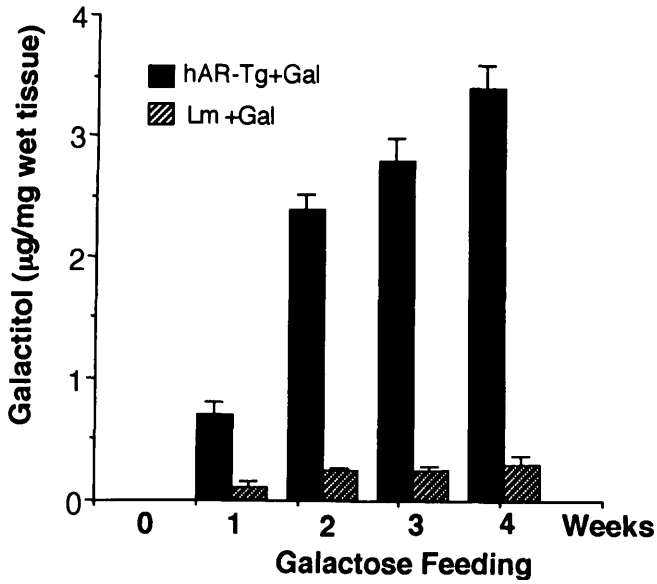

FIG. 1. Time-dependent polyol accumulation in the sciatic nerve of transgenic mice expressing human aldose reductase (hAR-Tg) with galactose feeding (hAR-Tg + Gal). Control littermate mice fed galactose (Lm + Gal) show small but significant accumulation, although to a much lesser extent than hAR Tg mice.

temperature was maintained at 37°C on the thermostatically controlled heated mat. MNCV was quantitated on the left sciatic-tibial nerve using an MS92 electromyogram device (Medelec, London, U.K.) (19).

Structural analysis of sciatic nerve. After 16 weeks of galactose feeding, all the mice (each group consisted of five animals) were killed with an overdose of pentobarbital. Sciatic nerves from the right side were removed and immediately fixed in 2.5% glutaraldehyde. Nerve samples were postfixed in 1% osmium tetroxide, dehydrated through an ascending series of ethanol, and immersed in Epon. After polymerization of the Epon-embedded nerve samples, semithin sections, 0.5 μm in thickness, were stained with toluidine blue and were examined with light microscopy. Micrographs of transverse sections were enlarged at a magnification of 1,600×, and myelinated fibers were digitized using an image analyzer connected with a data-storing computerized system (NIH image, Agfa-Arcus scanning system, Macintosh). Myelinated fiber size, axon-to-fiber size ratio, and fiber density were quantitated from the digitized images. Myelinated fiber size and axon size were defined by the area delineated by the outer and inner borders of myelin sheath, respectively. Axon-to-fiber size ratio was accordingly calculated in each myelinated fiber. More than 250 fibers randomly chosen from 5 frames of a single sciatic nerve fascicle were measured in each mouse. Percentage population of fibers with areas greater or less than 50 μm^2 was also calculated in each mouse. Fiber density was obtained by calculating total fiber number of myelinated fibers divided by the area of a whole sciatic nerve fascicle. Group values were represented by the mean of five animals.

All of the histological evaluations were performed with materials labeled to mask the observers to the treatment groups of the source of the tissues.

Statistical analysis. All the values in each group were represented by mean ± SE. Comparison of the mean values was performed with one-way analysis of variance with a post hoc Bonferroni test. P values <0.05 were considered statistically significant.

RESULTS

hAR protein level in sciatic nerve. With ELISA, the mean level of hAR protein in the sciatic nerve of hAR Tg mice at 4 weeks of age was 791 ± 44 ng/mg protein (n = 5). Only background levels of hAR protein, i.e., 3 ± 1 ng/mg protein (n = 4), were observed in Lm mice.

Polyol levels in the sciatic nerve of galactosemic mice. Feeding hAR Tg mice a 30% galactose diet for 4 weeks resulted in a rapid and time-dependent accumulation of galactitol in the sciatic nerve (Fig. 1). After a 4-week exposure to the galactose diet, the level of galactitol in sciatic nerve of Lm mice was ~10-fold less than that in hAR Tg mice (Fig. 1). Galactitol was not detected in sciatic nerves of either hAR Tg or Lm mice fed normal food.

MNCV in galactose-fed mice. During the 16-week experimental period, all of the animals gained 5–8 g of body weight. There were no significant differences in body weight gain between animals fed normal food or food with 30% galactose.

The MNCV was similar for all of the animal groups before galactose feeding (Fig. 2). During the 16-week observation, MNCV slightly increased in Lm mice fed normal food. Sixteen weeks of galactosemia had no significant effects on MNCV in Lm mice, although a slight decrease was found compared with the value before galactose feeding. In contrast, a progressive slowing of MNCV was observed in galactose-fed hAR TG mice. As early as 8 weeks after the introduction of the galactose diet, MNCV in hAR Tg mice was reduced compared with the value observed in the animals before galactose feeding. After 16 weeks of galactosemia, MNCV in hAR Tg mice decreased to ~80% of that in galactose-fed Lm mice ($P < 0.02$).

Structural analysis of galactose-fed mice. There was no significant difference in myelinated fiber density in the sciatic nerve of transgenic mice with or without galactose feeding compared with either galactose-fed or galactose-free Lm mice. Morphometric analysis of the transverse sections of sciatic nerves disclosed a significantly smaller value of the mean of mean myelinated fiber size in galactose-fed hAR Tg mice (29.6 ± 1.1 μm^2) than in galactose-free Lm mice (33.6 ± 0.7, $P < 0.02$), galactose-fed Lm mice (33.7 ± 1.2, $P < 0.02$), and galactose-free hAR Tg mice (34.1 ± 0.9, $P < 0.01$) (Fig. 3). The mean of mean axon-to-fiber size ratio was slightly lower in galactose-fed Lm mice (0.457 ± 0.019) than those in galactose-free Lm mice (0.478 ± 0.017) and hAR Tg mice (0.488 ± 0.015), but the differences were not significant. By contrast, it was significantly low in galactose-fed hAR Tg mice (0.425 ± 0.024) compared with hAR Tg and Lm mice fed normal food ($P < 0.05$ for both). Percentage population of myelinated fibers >50 μm^2 was significantly reduced in galactose-fed hAR Tg mice (15.5 ± 0.8%) as compared with galactose-fed Lm mice (23.2 ± 1.8, $P < 0.02$) and galactose-

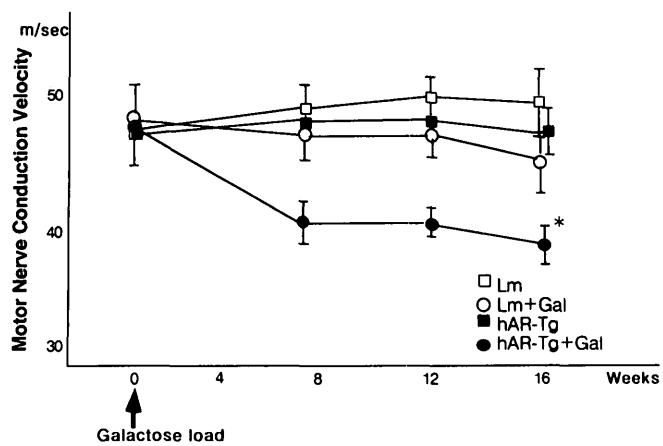

FIG. 2. Serial changes of MNCV in the transgenic mice expressing human aldose reductase (hAR-Tg) and control littermates (Lm) with or without galactose (Gal) feeding. As early as 8 weeks after galactose feeding, the transgenic mice (hAR-Tg + Gal) revealed conspicuous delay of MNCV, which further deteriorated with continuous feeding of galactose over 16 weeks (*$P < 0.01$ vs. Lm mice and hAR Tg mice, $P < 0.02$ vs. Lm mice + Gal). Bars indicate SE.

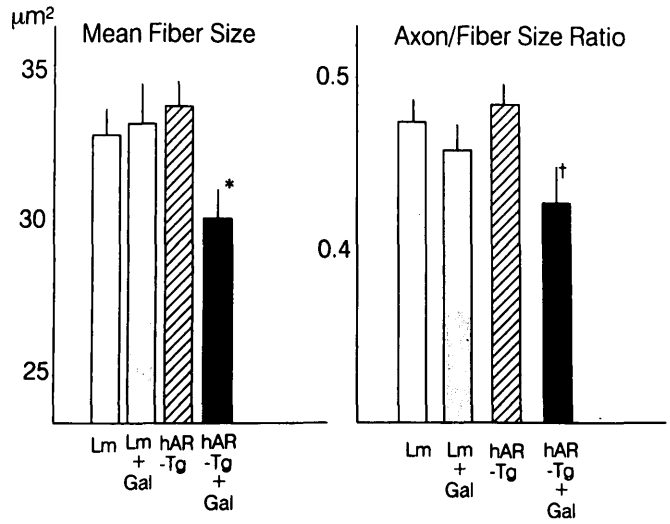

FIG. 3. Morphometric analysis of the transverse section of myelinated fibers in the sciatic nerve. Reduction of the mean of mean myelinated fiber size was found in galactose-fed transgenic mice (hAR-Tg + Gal) as compared with galactose-free littermate controls (Lm), transgenic mice (hAR-Tg), and galactose-fed Lm mice (Lm + Gal) (*$P < 0.01$ vs. Lm and Lm + Gal and $P < 0.02$ vs. hAR Tg + Gal). The mean of mean axon-to-fiber size ratio in hAR TG mice + Gal was also significantly decreased as compared with hAR Tg (†$P < 0.05$). Slight reduction in the mean of mean axon-to-fiber size ratio was also found in Lm + Gal compared with Lm and hAR Tg mice, but this was not significant.

free Lm mice (23.2 ± 2.1, $P < 0.02$) but not significantly compared with galactose-free hAR Tg mice (21.0 ± 3.4, $P = 0.06$). Percentage of smaller myelinated fibers <50 μm^2 was reciprocally increased in galactose-fed hAR Tg mice as compared with other groups.

DISCUSSION

In the present study, hAR Tg mice were shown to contain large amounts of hAR protein in the peripheral nerve. The expression level of hAR in the sciatic nerve was about 25% of the level observed in human sural nerve and was similar to that observed in human kidney cortex (21). Enhancement of hAR-mediated polyol pathway activity, elicited by galactose feeding, induced functional and structural abnormalities of the peripheral nerve in hAR Tg mice, similar to those reported in other diabetic animal models (5,19,22). An early and significant reduction of MNCV was detected only in galactose-fed hAR Tg mice, in which myelinated nerve fibers showed reduction of mean fiber size. These results suggested that the expression of hAR in transgenic animals was essential for the development of the significant changes that occurred in the peripheral nerves of this animal model of diabetic neuropathy.

Experimental galactosemia in rats and dogs, which have higher constitutive expression levels of aldose reductase (AR) protein than mice (5), has been used to examine the effects of an enhanced polyol pathway on the target organs of diabetic complications (8,23,24). Atrophy and demyelination of myelinated fibers are the salient pathological features in rats exposed to long-term galactosemia (25,26), keeping with the findings reported in the current study with the transgenic mice. The peripheral nerve abnormalities were dependent on the concentration of loaded galactose in previous studies (7,27). Tissue hydration and increased endoneurial pressure are considered to be responsible for the peripheral nerve abnormalities in long-standing galactosemic animals (27,28). In contrast to experimental diabetes, Na^+-K^+-ATPase activity is increased in rats with acute galactose intoxication, suggesting a different aspect operating in functional and structural changes in the peripheral nerve from diabetic condition (7,29).

In the present study, we could not directly compare the extent of the galactitol concentration with functional and structural changes in the peripheral nerve in hAR Tg mice, since the polyol accumulation was examined only up to 4 weeks. In our subsequent studies, the greater accumulation of galactitol in galactose-fed hAR Tg mice over Lm mice was sustained up to 10 months (30). It is therefore likely that the higher concentration of tissue polyol may be related to the more severe changes of peripheral nerve function and structure in galactose-fed hAR Tg mice than those found in Lm mice. Excessive flux of polyol pathway related to *myo*-inositol depletion as well as other mechanisms like secondary taurine or nitric oxide depletion should be also taken into account for the functional and structural changes (31,32).

In mice, which are resistant to cataract formation under galactosemia (33,34), tissue AR contents were suggested to be extremely low (3,33). In the peripheral nerve, measurable levels of polyols were not detected in diabetic C57Bl (*db/db*) mice up to 20 weeks of age (35,36). However, it has been shown that nerve polyols accumulate in mice with longer duration of diabetes (37) or under galactosemia (38,39), which accompanied the delay of MNCV. These findings suggest that mice contain their own AR, which can be activated by galactose load or long-term diabetic condition. The present study confirmed that galactosemia elicited galactitol accumulation in the peripheral nerve in control mice, although to a much lesser extent than the levels detected in galactose-fed transgenic mice. In contrast to the data of SAS/4 mice fed with 20% galactose for 4 weeks (39), galactose-fed controls (B6D2F2 mice) in the present study did not show a significant delay of MNCV. Lower polyol concentrations in the nerve may be the reason for the nonsignificant trend toward MNCV decline in B6D2F2 mice (about 0.3 μg/mg wet weight) compared with SAS/4 mice (about 2.3 μg/mg dry weight). This variability of the polyol accumulation in response to galactose load may reflect a strain difference in the degree of polyol pathway activity. Alternatively, other AR-related metabolic factors such as galactokinase or AR activity may also affect the concentration of polyol levels. Clearly, further studies are necessary to elucidate the characteristics of constitutive AR in various strains of mice and their changes under galactosemic and diabetic conditions.

Numerous ARIs have been developed as possible therapeutic agents for the treatment of diabetic complications. The design of these ARIs has been based on their inhibitory activities with animal AR biochemically purified from heterologous tissues. Recent study suggests that there are significant differences in IC_{50} values between hAR and the enzymes purified from other animals (40). These latter results suggest that the effective use of ARIs in diabetic patients may require the design of inhibitors specific for hAR. Mice transgenic for hAR provide a convenient model for characterizing the in vivo activity of these agents.

In summary, transgenic expression of hAR has caused neuropathic changes under conditions where galactose feeding has caused an exaggerated flux of substrate through the polyol pathway. The tissue alterations found in these mice correlated well with the functional expression of hAR. In-

duction of diabetes in these mice will further clarify whether the hyperglycemia can induce diabetes-related tissue alterations.

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