Abstract

We investigated the polyol pathway activity and the gene expression profiles in immortalized adult mouse Schwann cells (IMS32) under normal (5.6 mM) and high (30 mM and 56 mM) glucose conditions for 7-14 days in culture. Messenger RNA and the protein expression of aldose reductase (AR) and the intracellular sorbitol and fructose contents were up-regulated in IMS32 under the high glucose conditions compared to the normal glucose condition. By employing DNA microarray and subsequent RT-PCR/Northern blot analyses, we observed significant up-regulation of the mRNA expressions for serum amyloid A3 (SAA3), angiopoietin-like 4 (ANGPTL4) and ecotropic viral integration site 3 (Evi3), and the down-regulation of aldehyde reductase (AKR1A4) mRNA expression in the cells under the high glucose (30 mM) condition. The application of an AR inhibitor, SNK-860, to the high glucose medium ameliorated the increased sorbitol and fructose contents and the reduced AKR1A4 mRNA expression, while it had no effects on mRNA expressions for SAA3, ANGPTL4 or Evi3. Considering that the exposure to the high glucose (≥30 mM) conditions mimicking hyperglycemia *in vivo* accelerated the polyol pathway in IMS32, but not in other previously reported Schwann cells, the culture system of IMS32 under those conditions may provide novel findings about the polyol pathway-related abnormalities in diabetic neuropathy.

Keywords: Diabetic neuropathy, polyol pathway, immortalized Schwann cells, DNA microarray, aldehyde reductase

Running title: Polyol metabolism and gene expressions in IMS32

Introduction

Peripheral neuropathy is one of the most common complications of diabetes mellitus, as are retinopathy and nephropathy. Both metabolic alterations in the cellular components (mainly neurons and Schwann cells) and microvascular abnormalities are thought to play major roles in the development of diabetic neuropathy (Mizisin and Powell 2003), although the detailed pathogenesis remains unclear. Schwann cells are responsible for the action potential velocity through the insulation of axons, the maintenance of axonal caliber, effective nerve regeneration after axonal injury and other neural functions in the peripheral nervous system (Eckersley 2002). Therefore, Schwann cell abnormalities due to hyperglycemia can be a cause of nerve dysfunction, such as reduced nerve conduction velocity, axonal atrophy and impaired axonal regeneration (Song *et al* 2003; Dyck and Giannini 1996; Yasuda *et al* 2003). The role of Schwann cells in diabetic neuropathy is often discussed in relation to polyol pathway hyperactivity (Mizisin and Powell 2003; Eckersley 2002). Aldose reductase (AR: EC 1.1.1.21) is the first enzyme in the polyol pathway and converts glucose to sorbitol using NADPH as a cofactor. Since AR is localized to Schwann cells in the peripheral nerves (Kern and Engerman 1982), it has been proposed that the activation of AR in Schwann cells under hyperglycemic conditions affects nerve functions through various mechanisms:

1) Sorbitol accumulation leads to osmotic stress and the depletion of myo-inositol and taurine (Tomlinson DR 1999; Pop-Busui *et al* 2001).

2) The increase in AR activity competes with nitric oxide (NO) synthase or glutathione reductase for NADPH. The inhibition of NO synthase and the subsequent decrease in NO in the nervous tissue causes diminished nerve flow, whereas the depletion of reduced glutathione (GSH) by glutathione reductase inhibition results in the excessive production of free radicals (Low *et al* 1999).

3) Sorbitol is converted to fructose by sorbitol dehydrogenase (SDH: EC 1.1.1.14), the

second enzyme in the polyol pathway. Fructose and its metabolites, such as fructose-6-phosphate and triose-phosphate can be triggers of non-enzymatic glycation of cellular proteins and lipids (Takagi *et al* 1995).

Culture systems of Schwann cells appear to be useful for investigating the role of the polyol pathway hyperactivity in the pathogenesis of diabetic neuropathy. Thus far, an established Schwann cell line, JS1 (Mizisin *et al* 1996), and primary cultured adult rat Schwann cells (Suzuki *et al* 1999; Maekawa *et al* 2001) have been introduced to study polyol metabolism, but these cells did not display intracellular sorbitol accumulation or enhanced AR expression / enzyme activity under high glucose (25-30 mM) conditions unless hyperosmotic stress (greater than 100 mM) was applied. The reasons for this remain unknown. We have established a spontaneously immortalized Schwann cell line, IMS32, from long-term cultures of adult mouse dorsal root ganglia (DRG) and peripheral nerves (Watabe *et al* 1995). Since IMS32 possesses some biological properties of mature Schwann cells and a high proliferation activity (Watabe *et al* 1995, Sango *et al* 2004), this cell line is suitable for functional and biochemical studies of the peripheral nervous system. Kato et al. reported that the proliferation activity of IMS32 was decreased by exposure to high glucose (20-40 mM) conditions (Kato *et al* 2003, Nakamura *et al* 2003), but the polyol metabolism in the cells has not yet been characterized. If the polyol pathway is activated in IMS32 in response to the high glucose concentrations mimicking hyperglycemia *in vivo*, this cell line would be a valuable tool for the study of diabetic neuropathy. In the present study, we investigated the mRNA expression of AR and SDH, the protein expression of AR and the intracellular contents of sorbitol and fructose in IMS32 under high glucose (30 mM and 56 mM) and hyperosmotic (50 mM of sodium chloride) conditions. We also employed DNA microarray and subsequent RT-PCR/Northern blot analyses to see the high glucose (30 mM)-induced alterations in the gene expression profiles, especially in association with the polyol pathway activity.

Materials and Methods

Preparation of the Plasmid Containing Mouse cDNAs for AR and SDH

The plasmids containing mouse AR and SDH cDNA fragments were created by PCR cloning, as described previously (Sango *et al* 2004). Briefly, total RNA was isolated from IMS32 using Sepazol reagent (Nacalai Tesque, Kyoto, Japan), and was reverse transcribed with M-MLV reverse transcriptase (Invitrogen, Groningen, Netherlands) and pd(N)$_6$ random primer (Amersham Biosciences Corp., Piscataway, NJ, USA). The synthesized cDNA was used as a template for the PCR reaction. The PCR primers were designed to amplify the 896 bp of mouse AR (aldo-keto reductase 1B3 (AKR1B3)) gene (sense primer, 5'-ATGGCCAGCCATCTGGAACTC-3' and antisense primer, 5'-CACACCCTCCAGTTCCTGTT-3') (GenBank Accession No. NM_009658) and the 1074 bp of mouse SDH gene (sense primer, 5'-ATGGCAGCTCCAGCTAAGGGC-3' and antisense primer, 5'-CTAGGGGTTTTGGTCATTGGG-3') (GenBank Accession No. NM_146126). The PCR products were subcloned into pGEM-T Easy Vector (Promega Corp., Madison, WI, USA) according to the manufacturer's instructions, and the resulting plasmid DNA was sequenced (Promega / Bex Co. Ltd., Tokyo, Japan).

Cell Culture

Immortalized adult mouse Schwann cells (IMS32) were seeded on 75 cm^2 flasks (Nalge Nunc International, Naperville, IL, USA) at a density of $5x10^4/cm^2$ and cultured in Dulbecco's Modified Eagle's medium (DMEM) (Sigma, St Louis, MO, USA) supplemented with 10% fetal calf serum (FCS, Invitrogen). The medium contained 5.6 mM glucose. When the cells reached approximately 80-85% confluency, they were maintained in DMEM supplemented with 1% FCS and containing 5.6 mM glucose [Glc-5.6], 30 mM glucose [Glc-30], 56 mM glucose [Glc-56] or 5.6 mM glucose and 50 mM sodium chloride [NaCl-50]. The media containing a low concentration (1%) of serum slowed the proliferation of IMS32, and made it possible to keep the cultures in

the same culture flasks for up to 14 days without detachment and the re-seeding of cells. The cells under each experimental condition for 7 days were rinsed with phosphate-buffered saline (PBS, Sigma) and detached from the flasks by cell scrapers (Sumitomo Bakelite Co. Ltd, Tokyo, Japan). These cells were suspended in 2 ml of sterile water and collected in sterile tubes for use in Northern blotting or Western blotting.

Northern blotting

The cDNA fragments of AR and SDH (10 ng/ml) were prepared from the plasmids, and labeled with alkaline phosphatase (AlkPhos Direct (Amersham Biosciences)) according to the manufacturer's instructions. Total RNA was isolated from the cells using Sepazol reagent, and the concentration of RNA was determined with a spectrophotometer (Gene Quant Pro; Amersham Biosciences). Twenty-five micrograms of total RNA was electrophoresed in 1% agarose-formaldehyde gel and transferred to Hybond N+ membrane (Amersham Biosciences). We prepared two membranes with the same RNA samples; one was stained with methylene blue (Waldeck-Gmbh & Co KG, Münster, Germany) and the other was hybridized overnight at 50 C with the labeled probes. The CDP-Star^{TM} chemiluminescent detection reagent and Hyperfilm ECL (Amersham Biosciences) were used for visualization of the positive signals (Sango *et al* 2004). The band intensity was quantified with Edas 290 1D Image Analysis software (Eastman Kodak, New York, NY, USA) (Sango *et al* 2002), and the abundance of mRNA was expressed as a 'relative expression' (the intensity in each experimental group relative to that in [Glc-5.6]). 28S ribosomal RNA stained with methylene blue on the duplicate membranes was used for standardization.

Western blotting

Cells were homogenized with a cell sonicator (Tomy Seiko Co. Ltd., Tokyo, Japan), and the concentration of protein was determined by using a DC Protein Assay (Bio-Rad, Hercules, CA, USA) according to the manufacturers' instructions. Twenty micrograms of protein were electrophoresed on 14-16% gradient sodium dodecyl sulfate (SDS) /

polyacrylamide gels (Daiichi Pure Chem. Co. Ltd, Tokyo, Japan) under non-reducing conditions and transferred to nitrocellulose paper (Bio-Rad). The blotted paper was then blocked with 5% skim milk and incubated for 1 h at room temperature with goat polyclonal anti-AR antibody (ALR2 (P-20), 1:2,000; Santa Cruz Biotech. Inc., Santa Cruz, CA, USA) or mouse monoclonal anti-β-actin antibody (1:1,000; Sigma), followed by incubations with biotinylated anti-goat IgG or anti-mouse IgG (1:1,000; Vector Laboratories Inc., Burlingame, CA, USA), and streptoavidin-alkaline phosphatase (1:1,000; Promega). Reactions were visualized by color development using Western blue® stabilized substrate for alkaline phosphatase (Promega).

Immunocytochemistry

IMS32 were seeded on wells of 8-well chamber slides (Nalge Nunc) or Aclar fluorocarbon coverslips (Nissin EM Co. Ltd, Tokyo, Japan; 9 mm in diameter) at a density of 1-2 x 10^4 /cm^2, and kept in [Glc-5.6], [Glc-30], [Glc-56] or [NaCl-50] for 7 days. Then, the cells were fixed with 4% paraformaldehyde for 15 min at 4 C with the following antibodies (diluted with 20 mM phosphate-buffered saline (PBS) containing 0.5% skimmed milk):

1) goat anti-AR polyclonal antibody (1:3,000);

2) rabbit anti-S100 polyclonal antibody (1:3,000, DAKO, Carpinteria, CA, USA);

3) rabbit anti-p75 low affinity NGF receptor ($p75^{NTR}$) polyclonal antibody (1:1,000, Promega).

After rinsing with PBS, the cells were incubated for 1 h at 37 C with peroxidase-conjugated anti-goat IgG (for AR) or anti-rabbit IgG (for S100 and $p75^{NTR}$) antibody (1:100, Vector Laboratories). The immunoreaction was visualized under a light microscope using 0.01% diaminobenzidine tetrahydro-chloride (DAB) (Wako Co., Tokyo, Japan) and 0.01% hydrogen peroxide in 50 mM Tris buffer (pH7.4) at room temperature for 15 min (Sango *et al* 2004).

Measurement of the intracellular contents of sorbitol and fructose

IMS32 were seeded on wells of 6-well plates (Corning Inc., Corning, NY, USA) at a density of 5 x 10^4 /cm^2, and kept in [Glc-5.6] or [Glc-30] for 14 days. A subset of [Glc-30] was treated with 1 μM of an AR inhibitor, SNK-860 (Sanwa Kagaku Kenkyusho) for 7 days (from 7 days to 14 days in culture). This culture condition was termed as [Glc-30/SNK]. Cells under each experimental condition were rinsed in ice-cold PBS, and homogenized in 2ml of cold water with a cell sonicator. Protein concentrations were determined by using a DC Protein Assay (Bio-Rad) according to the manufacturers' instructions. The polyol level in each lyophilized sample was determined by liquid chromatography with tandem mass spectrometry, according to the method of Guerrant & Moss (1984). The value of polyol was expressed as nanomols (nmol) /mg protein.

DNA microarray analysis

IMS32 cells were kept in [Glc-5.6] or [Glc-30] for 14 days in 175 cm^2 flasks (Nalge Nunc), and the total RNA was isolated from the cells using a RNeasy mini kit (QIAGEN K.K., Tokyo, Japan). The DNA microarray analysis was performed with these samples (Custom Technology Service by Kurabo Industries Ltd, Osaka, Japan). Briefly, total RNA was reverse transcribed to cDNA with T7 oligo d(T) primer (Amersham Biosciences). The cDNA synthesis product was used in an *in vitro* transcription reaction containing T7 RNA polymerase and biotinylated UTP. Then, the labeled cRNA products were fragmented, loaded onto CodeLink™ Uniset Mouse 20K Bioarray (Amersham Biosciences) and hybridized according to the manufacturer's protocol. Streptavidin-Cy5 (Amersham Biosciences) was used as the fluorescent conjugate to detect hybridized target sequences. Raw intensity data from the Codelink Bioarray were analyzed in Microsoft Excel (Microsoft, Redmond, WA, USA) and gene expression levels were expressed as relative intensities (intensity data in [Glc-30] compared to those in [Glc-5.6]). Only the fold changes of relative intensities > 2.0 and < 0.5 were considered to be significant up-regulations and down-regulations, respectively.

Confirmation of high glucose-responsive gene expression by RT-PCR and Northern blot analyses

Total RNA was isolated from the cells kept in [Glc-5.6], [Glc-30] or [Glc-30/SNK] for 14 days in 75 cm^2 flasks、by using Sepazol reagent. Semi-quantitative RT-PCR analysis was carried out, as described previously (Sango *et al* 2002). Briefly, first-strand cDNA was synthesized from 4 μg of total RNA and PCR amplification was performed in a reaction volume of 25 μl containing 2 μl of diluted cDNA, 0.625 U of AmpliTaqGold™ DNA polymerase (Applied Biosystems, Foster City, CA, USA), each dNTP at 0.2 mM, 50 mM KCl, 10 mM Tris-HCl (pH8.3), 1.5 mM $MgCl_2$ and sense and antisense primers each at 0.5 μM. Oligonucleotide sense and antisense primers for the PCR were as follows:

Serum amyloid A3 (SAA3) (GenBank Accession No. NM_011315), 5'-ATGAAGCCTTCCATTGCCA-3' and 5'-TATCTTTTAGGCAGGCAGGCCAGCA-3' (a 365-bp product);

Angiopoietin-like 4 (ANGPTL4) (GenBank Accession No. NM_020581), 5'-AGGGGCCCAAGGGAAAAGAT-3' and 5'-TAGCCTCCATGGGCTGGAT-3' (a 978-bp product);

Ecotropic viral integration site 3 (Evi3) (GenBank Accession No. NM_145492), 5'-TGGGGAGGCAGTAGACTG-3' and 5'-CTCCATCCTGGAGCCAGA-3' (a 640-bp product);

β-Actin (the internal standard) (GenBank Accession No. NM_007393), 5'-AGAAGCTGTGCTATGTTGCC-3' and 5'-ATCCACACAGAGTACTTGCG-3' (a 382-bp product).

All PCR reactions were carried out under conditions in which the amplification was linear by using the appropriate number of cycles. The PCR products were resolved by electrophoresis in a 2 % agarose gel at 100 V for 30 min. The intensity of the PCR fragments visualized by ethidium bromide staining were quantified with Edas 290, and

the abundance of the respective mRNAs was expressed as the intensity of each cDNA fragment/intensity of the β-actin cDNA fragment.

For Northern blot analysis, the plasmid containing mouse aldehyde reductase (AKR1A4; GenBank Accession No. NM_021473) cDNA fragment was created by PCR cloning with the following primers,
5'-TCCAGTGTCCTCCTGCACA-3' and 5'-TCAGTATGGGTCATTAAAGGG-3' (a 969-bp product).

mRNA expression of SAA3, ANGPTL4, Evi3 and AKR1A4 in the peripheral nerves of adult mice

Three-month-old female ICR mice were anesthetized by ether and sacrificed, in accordance with the Guideline for the Care and Use of Animals (Tokyo Metropolitan Institute for Neuroscience). Thirty dorsal root ganglia (from the thoracic to sacral levels) with associated spinal nerve bundles were dissected from each mouse, and total RNA was isolated from the dissected tissue as previously described (Sango *et al* 2002). The cDNA synthesis and subsequent RT-PCR analysis was carried out as described above, using the PCR primers for SAA3, ANGPTL4, Evi3 and AKR1A4.

Statistical analysis

For statistical comparison, post hoc tests were performed using Bonferroni/Dunn post hoc analyses. P values of <0.05 were considered significant.

Results

IMS32 exhibited Schwann cell phenotypes under the normal and high glucose conditions

IMS32 cells showed distinct Schwann cell phenotypes such as the spindle-shaped morphology (Fig.1A-C) and the expression of Schwann cell markers such as S100 (Fig.1D-F) and $p75^{NTR}$ (not shown) under normal ([Glc-5.6] (Fig.1A,D)) and high glucose ([Glc-30] (Fig.1B,E) and [Glc-56] (Fig.1C,F)) conditions.. We observed no

significant difference in the morphological appearances of living cells or immunoreactivity to S100/p75^{NTR} between [Glc-5.6] and [Glc-30] or [Glc-56]. The application of SNK-860 to the high glucose conditions failed to alter these phenotypes of IMS32 (not shown).

Up-regulated AR and SDH mRNA expressions in IMS32 under the high glucose and hyperosmotic conditions

By Northern blot analysis with alkaline-phosphatase-labeled cDNA probes, mRNA for AR and SDH were detected as single bands corresponding to the molecular weight of around 1.4 kilobase (kb) and 2.4 kb molecular size, respectively (Fig.2A, top). These results were consistent with those in the previous studies (Gui *et al* 1995; Lee *et al* 1995). The blot showed more intense signals for AR mRNA in [Glc-30], [Glc-56] and [NaCl-50] than those in [Glc-5.6], and for SDH mRNA in [Glc-56] and [NaCl-50] than those in [Glc-5.6]. Methylene blue (MB)-stained images of the duplicate membrane (Fig.2A, bottom) showed that a relatively equal amount of RNA was loaded. The average values of the relative expression of AR mRNA were 1 in [Glc-5.6], 1.88 in [Glc-30], 2.41 in [Glc-56] and 2.78 in [NaCl-50], respectively (Fig.2B); AR mRNA expression was up-regulated under the high glucose ([Glc-30] and [Glc-56]) and hyperosmotic ([NaCl-50]) conditions, although the difference in the values between [Glc-30] and [Glc-5.6] was not statistically significant. The average values of relative expression of SDH mRNA were 1 in [Glc-5.6], 1.19 in [Glc-30], 2.03 in [Glc-56] and 2.99 in [NaCl-50]; SDH mRNA expression was significantly up-regulated under the high glucose ([Glc-56]) and hyperosmotic ([NaCl-50]) conditions.

Up-regulated AR protein expression under the high glucose and hyperosmotic conditions

By Western blot analysis with a polyclonal anti-AR antibody, the expression band of AR was identified as the level of around 36 kDa in molecular size (Fig.3A, top). The blot showed more intense signals for AR protein in [Glc-30], [Glc-56] and [NaCl-50]

than those in [Glc-5.6]. There was no significant difference in the signal intensity to β-actin between the normal and high glucose / hyperosmotic conditions (Fig.3A, bottom). The average values of relative expression were 1 in [Glc-5.6], 1.79 in [Glc-30], 2.15 in [Glc-56] and 1.95 in [NaCl-50], respectively; AR protein expression was up-regulated under the high glucose ([Glc-30] and [Glc-56]) and hyperosmotic ([NaCl-50]) conditions, although the difference in the values between [Glc-30] and [Glc-5.6] was not statistically significant. Immunocytochemical analysis revealed the localization of AR protein in the cytoplasm of IMS32. The immunoreactivity for AR was markedly enhanced in [Glc-30] (Fig.4B) compared to [Glc-5.6] (Fig.4A), whereas we saw no significant difference in the immunoreactivity for S100 between the two culture conditions (Fig.1).

Intracellular polyol levels under normal and high glucose conditions

The intracellular contents of sorbitol and fructose were significantly higher in [Glc-30] (17.9±1.7 nmol/mg and 77.7±6.7 nmol/mg) than those in [Glc-5.6] (1±0.2 nmol/mg and 13.6±3.4 nmol/mg) (Fig.5A). The polyol levels in the cells under [Glc-56] were extremely high and beyond the range of measurement. The application of an AR inhibitor, SNK-860, to the high glucose ([Glc-30]) condition for 7 days significantly reduced the polyol contents to levels close to those under the normal glucose ([Glc-5.6]) condition (*i.e.*, sorbitol: 17.9±1.7 nmol/mg in [Glc-30] *vs.* 3.4±0.4 nmol/mg in [Glc-30/SNK] and fructose: 77.7±6.7 nmol/mg in [Glc-30] *vs.* 29.2±4.8 nmol/mg in [Glc-30/SNK]). The western blot analysis performed in parallel with the measurement of the polyols revealed that the level of AR protein in [Glc-30] was not reduced by treatment with SNK-860 (not shown).

Gene expression profiles in IMS32 under the normal and high glucose conditions

Among nearly 20,000 mouse genes, we identified 28 genes with altered expression in the high glucose ([Glc-30]) condition; 10 genes were expressed at a 2.0-fold or greater level, while 18 genes were down-regulated by 2.0-fold or more (Table 1). The

relative expression of AR mRNA was 1.2 (< 2-fold), and therefore AR was not included in the up-regulated genes in the table. To confirm the microarray results, we measured the relative expression of these genes in the cells under [Glc-5.6], [Glc-30] and [Glc-30/SNK] by RT-PCR or Northern blot analysis. Thus far, RT-PCR analysis resulted in the significant up-regulation of 3 genes in [Glc-30] compared with [Glc-5.6]: serum amyloid A3 (SAA3), angiopoietin-like 4 (ANGPTL4) and ecotropic viral integration site 3 (Evi3) (Fig.6). Northern blot analysis revealed the down-regulation of mRNA expression for aldehyde reductase (EC 1.1.1.2, *aka* aldo-keto reductase 1A4 (AKR1A4)) (Fig.7). Treatment with SNK-860 had no effects on the mRNA expressions for SAA3, ANGPTL4 or Evi3, but restored the diminished mRNA expression for AKR1A4 to a level equivalent to [Glc-5.6] (Figs.6&7). These 4 genes were expressed in DRG and spinal nerves of adult normal mice, as well as in IMS32 cells (Fig.8).

Discussion

Phenotypic and biochemical features of the Schwann cell line IMS32

IMS32 cells showed distinct Schwann cell phenotypes, such as spindle shaped morphology and intense immunoreactivity for S100 (Fig. 1), $p75^{NTR}$, laminin and several Schwann cell-specific transcription factors and neurotrophic factors (Watabe *et al* 1995, 2003). Similar to primary and long-term cultured Schwann cells, IMS32 cells exhibit mitogenic responses to several growth factors (*e.g.*, platelet-derived growth factor (PDGF)-BB, acidic and basic fibroblast growth factor (aFGF, bFGF), transforming growth factor (TGF)-β1, 2). On the other hand, IMS32 cells are different from primary and long-term cultured Schwann cells in that the former were not contact-inhibited and formed ball-shaped subcolonies when cultures reached confluence (Watabe *et al* 1995). We failed to show that the cell line could myelinate a mouse axon (Watabe *et al*, unpublished observation), in the same way as endogenous Schwann cells in the peripheral nerves and primary cultured Schwann cells (Suzuki *et al* 1999). In

spite of those differences from normal Schwann cells, IMS32 cells have such advantages for the study of diabetic neuropathy, as the activation of the polyol pathway (shown in this study) and the decreased proliferative activity (Kato *et al* 2003; Nakamura *et al* 2003) under high glucose conditions mimicking hyperglycemia *in vivo*.

Activation of the polyol pathway in IMS32 under high glucose (≥30 mM) environments

In the present study, we observed the significant up-regulation of the mRNA expression for AR and SDH and protein expression for AR under [Glc-56] (Figs.2 and 3), and marked increases in the intracellular sorbitol and fructose contents under [Glc-30] (Fig.4) and [Glc-56] (beyond the range of measurement), compared to those under [Glc-5.6]. Although not statistically significant, AR expressions at both mRNA and protein levels under [Glc-30] were approximately 1.8-fold higher than those under [Glc-5.6]. These findings were in line with the enhanced AR immunoreactivity under [Glc-30] according to immunocytochemistry (Fig.4). We did not measure the enzyme activity in the cells, but the excessive production of sorbitol and fructose under [Glc-30] and [Glc-56] suggests the high glucose-induced activation of AR and SDH. Furthermore, the application of an AR inhibitor, SNK-860, to [Glc-30] for 7 days diminished the intracellular polyol contents to a level close to [Glc-5.6]. Taking these findings together, the exposure of IMS32 to the high glucose (≥30 mM) environments is likely to enhance the expression and activity of AR, thereby leading to exaggerated flux through the polyol pathway. Significant up-regulation of mRNA and protein expressions for AR under [Glc-56] and [NaCl-50] suggests that AR is induced by not only high glucose but hyperosmotic conditions. The increased expression of AR under hyperosmotic environments has been reported in a variety of cells, and indicates the osmoregulatory role of AR (Yabe-Nishimura 1998). Compared with AR, much less information is available concerning the expression of SDH. The mRNA expression of SDH was not induced by hyperosmolality in rat Schwann cells (Maekawa *et al* 2001) or renal collecting duct cells (Grunewald *et al* 1998). Those findings are contrary to the results

in this study, *i.e.* the significant up-regulation of SDH mRNA expression under [Glc-56] and [NaCl-50]. Although the reasons for such variable results among the cells are unknown, we clearly demonstrated that the exposure to high glucose or hyperosmotic environments could induce both AR and SDH mRNA in IMS32.

It remains to be elucidated why an increase in the glucose concentration to 20-30 mM, corresponding to the plasma level in poorly controlled diabetic patients, accelerated the polyol pathway in IMS32, but not in other cultured Schwann cells (Mizisin *et al* 1996; Suzuki *et al* 1999; Maekawa *et al* 2001). In adult rat Schwann cells, neither AR expression / activity nor intracellular sorbitol levels were enhanced under the 30 mM glucose condition. On the other hand, the extracellular sorbitol level in that condition was increased significantly compared with that in 5.6 mM glucose condition (Suzuki *et al* 1999). These findings suggested that sorbitol was released from the cells into the media by an unidentified transport mechanism. In JS1 Schwannoma cells, sorbitol did not accumulate under 25 mM glucose or 100 mM NaCl conditions unless a SDH inhibitor was applied (Mizisin *et al* 1997). In contrast with those cells, conspicuous increases of intracellular sorbitol (18-fold) and fructose (6-fold) levels were observed in IMS32 under [Glc-30]. It seems possible that IMS32 cells possess a much higher capacity than other Schwann cells to store sorbitol and other glucose-derived metabolites. To verify this possibility, we are now investigating if the levels of dicarbonyl intermediates, such as methylglyoxal and 3-deoxyglucosone (Kikuchi *et al* 1999) in IMS32, are elevated under [Glc-30]. It is also noteworthy that immortalized Schwann cells were established from not only normal adult mice, but also from mouse models of metabolic diseases such as Niemann-Pick disease type C (NPC; *spm/spm*, npc^{nih}/npc^{nih}) (Watabe *et al* 2001, 2003), globoid cell leukodystrophy (twitcher) (Shen *et al* 2002) and G_{M2} gangliosidosis (Ohsawa *et al* 2005). The cells originated from those mouse models were able to survive and proliferate in culture, despite the progressive accumulation of undegraded substrates in the cytoplasm.

High glucose-induced alterations of gene expression profiles in IMS32

In recent studies, gene expression patterns in DRG (Burnand *et al* 2004), superior mesenteric and celiac ganglia (SMG-CG) and superior cervical ganglia (SCG) (Carroll *et al* 2004) were compared between STZ-diabetic and control rats via microarray profiling. Those approaches appear to be useful to elucidate the molecular mechanism of the development of diabetic neuropathy. However, the peripheral ganglia contain neurons, Schwann cells and other cells, and the microarray studies on them are not sufficient to specify the cells with altered gene expressions. By employing DNA microarray and subsequent RT-PCR or Northern blot analyses, we investigated the gene expression profiles in IMS32 exposed to normal ([Glc-5.6]) and high ([Glc-30]) glucose conditions. Since 30 mM glucose in culture medium ([Glc-30]) is closer to the blood glucose concentration in diabetic individuals than 56 mM ([Glc-56]), we thought that the findings from the comparison between [Glc-30] and [Glc-5.6] could be more relevant to the *in vivo* studies between diabetic and normal conditions. Although the expression of AR mRNA/protein was not significantly up-regulated by exposure to [Glc-30] (Fig.2, Fig.3 and Table 1), intracellular contents of sorbitol and fructose under [Glc-30] were markedly higher than those under [Glc-5.6]. It seems possible that the degree of increases in the enzyme activity of AR does not correlate with that in the mRNA/protein expression in IMS32 under [Glc-30].

The microarray analysis revealed 10 up-regulated genes and 28 down-regulated genes under the high glucose condition (Table 1). Among the up-regulated molecules, glutathione S-transferase alpha 2 (GSTα2) is an isozyme of GST and may be involved in the detoxification of lipid peroxidation products generated by oxidative stress under hyperglycemic conditions (Srivastava *et al* 1995; Obrosova 2002). Among the down-regulated genes, phosphofructokinase (PFK), one of the key glycolytic enzymes, is essential for the maintenance of axonal integrity: endogenous aldehydes originating from the lipid-peroxidation process are potent inhibitors of this enzyme and may cause

significant nerve damage (Novotny *et al* 1994). Peroxisome proliferator-activated receptor α (PPARα) is an isotype of PPAR, transcription factors involved in the regulation of. lipid and glucose metabolism (Staels and Fruchart, 2005). Shibata *et al* (2000) observed that treatment with JTT501, a PPARα and γ agonist, prevented the development of diabetic neuropathy in the Zucker diabetic fatty rats. However, further RT-PCR/Northern blot analyses failed to show the high glucose-induced changes of expression of these genes. In contrast, these analyses confirmed the microarray results, such as significant up-regulation of mRNA expressions for SAA3, ANGPTL4 and Evi3, and the down-regulation of aldehyde reductase (AKR1A4) mRNA expression.

i) SAA3: Serum amyloid A (SAA), a family of apolipoproteins associated with high density lipoprotein (HDL), is known as an acute phase reactant (Meek *et al* 1992). There is an increasing body of evidence that correlates the diabetic conditions with the chronic elevation of SAA (Hamano *et al* 2004) and the up-regulation of SAA3 (Lin *et al* 2001), but their significance remains to be elucidated. SAA, as well as advanced glycation end products (AGE), is one of the ligands for the receptor for AGE (RAGE) and the RAGE-ligand interaction is likely to induce cellular dysfunction, thereby being implicated in the development of diabetic complications (Schmidt *et al* 2000). In addition, Chung *et al* (2000) observed intense immunoreactivity for SAA in the brains of patients with neurodegenerative diseases such as Alzheimer's disease and multiple sclerosis: the major site of SAA staining in both diseases was the myelin sheaths and axonal membrane. Taking this finding together with the evidence that SAA can inhibit lipid biosynthesis (Schreiber *et al* 1999), up-regulation of SAA in nervous tissue under hyperglycemic conditions might affect myelin lipid synthesis.

ii) ANGPTL4: ANGPTL4 gene is predominantly expressed in the adipose tissue and liver, but its induction in ischemic tissues (Le Jan *et al* 2003) implies a role of this molecule in the compensatory angiogenesis for ischemia and hypoxia (Deindl *et al* 2001). ANGPTL4 is recognized as a downstream target gene of PPARα and γ (Kersten

et al 2000), and induces hyperlipidemia by inhibiting lipoprotein lipase (LPL) activity (Yoshida *et al* 2002). LPL is expressed in cultured Schwann cells and may play a role in myelin phospholipid biosynthesis in the peripheral nervous system (Huey *et al* 1998). Ferreira *et al* (2002) reported that the activity of LPL in the sciatic nerves was reduced in STZ-diabetic rats and restored by treatment with insulin. Taking these findings into consideration, it seems possible that up-regulated ANGPTL4 in Schwann cells under hyperglycemic conditions inhibits LPL activity, thereby being one of the causes for the impaired fatty acid metabolism and deficient phospholipid synthesis in nervous tissue (Cameron *et al* 1998; Martin *et al* 2003). SAA may also play a role in the lipid synthesis as described above, but the interaction between the two molecules remains unknown.

iii) Evi3: Evi3 is a common retroviral integration site in murine B-cell lymphoma and encodes a novel zinc finger protein (Warming *et al* 2003). Since very few papers described this molecule (most of them were focused on tumorigenesis in hematopoietic cells), we currently have no information or idea of how this molecule is involved in the pathobiology of diabetic neuropathy. Treatment with SNK-860 had no effects on the mRNA expressions for SAA3, ANGPTL4 or Evi3 under [Glc-30] in this study. This finding suggests that the up-regulation of these genes in IMS32 under the high glucose condition is not related to the polyol pathway hyperactivity.

iv) AKR1A4: Aldehyde reductase is a member of the aldo-keto reductase (AKR) superfamily (the AKR1A subfamily represents the aldehyde reductases and the AKR1B subfamily represents the aldose reductases) (Allan and Lohnes 2000; Hyndman *et al* 2003). Approximately 50% of the amino acid sequences are conserved between aldehyde reductase and AR (Bohren *et al* 1989), and both enzymes catalyze the reduction of reactive biogenic aldehydes (*e.g.*, glyceraldehyde, methylglyoxal (MG), hydroxynonenal (HNE) and glucosones) by using NADPH as a cofactor (Flynn 1986; Vander Jagt *et al* 1992). Unlike AR, aldehyde reductase is virtually inactive for glucose and other aldo-sugars (Kawasaki *et al* 1989). Consequently, much less attention has

been paid to aldehyde reductase than AR in the research field of diabetes and its complications (Danesh *et al* 2003; Takahashi *et al* 1995). In the present study, reduced mRNA expression of aldehyde reductase in IMS32 under the high glucose condition was completely ameliorated by treatment with an AR inhibitor, SNK-860. The activated AR enhances the flux through the polyol pathway by converting glucose to sorbitol, but it may also act against reactive aldehydes and related substances produced by lipid peroxidation and oxidative stress under hyperglycemic conditions (Obrosova 2002; Rittner *et al* 1999). Like AR, aldehyde reductase appears to be able to neutralize lipid peroxidation products (Suzuki *et al* 1998), but the results in this study suggest that the production of this enzyme is suppressed by augmented expression and activity of AR in Schwann cells during hyperglycemic conditions. Conversely, AR inhibition may up-regulate aldehyde reductase to be more active against the toxic substances induced by high glucose. This idea is partly supported by findings from previous studies which suggested the functional redundancy of the two enzymes in rat sympathetic ganglia (Kawamura *et al* 1999, 2002). Moreover, a lack of apparent phenotypes except slightly defective urine-concentrating ability in AR-knockout mice (Ho *et al* 2000) led us to speculate that the detoxification function may be taken over by aldehyde reductase in the absence of AR. However, there has been no direct evidence to indicate such redundancy of these enzymes under hyperglycemic conditions. It is also important to note that aldehyde dehydrogenase (ALDH) and alcohol dehydrogenase (ADH), as well as AR and aldehyde reductase, can participate in the detoxification of reactive aldehydes in nervous tissue (Picklo *et al* 2001). A recent report by Suzuki *et al* (2004) implied the association of ALDH2/ADH2 polymorphism with the development of human diabetic neuropathy. We expect that further studies focusing on the expression and activity of aldehyde reductase in the peripheral nervous system under hyperglycemic conditions, especially in relation to those of AR, ALDH and ADH, will elucidate the role of this enzyme in the pathogenesis of diabetic neuropathy.

The mRNA expression of SAA3, ANGPTL4, Evi3 and AKR1A4 in DRG and spinal nerves in adult normal ICR mice (Fig.8) suggests their functional roles in the peripheral nervous system. We are about to determine if the expression of these genes is altered in the nerves of STZ-diabetic mice. The antibodies to these proteins are not available from the commercial source, and we plan to collaborate with the laboratories studying these molecules. The future studies with the antibodies will help elucidate the immunochemical localization and function of these proteins in the peripheral nervous system and their involvement in the pathogenesis of diabetic neuropathy.

Conclusion

The findings in this study indicate the activation of the polyol pathway and the altered gene expression profiles (*i.e.*, up-regulation of SAA3, ANGPTL4 and Evi3 mRNA and down-regulation of AKR1A4 mRNA) in IMS32 under exposure to high glucose (≥ 30 mM) environments. Considering that an increase in the glucose concentration to 20-30 mM accelerated the polyol pathway in IMS32, but not in other previously reported Schwann cells, the culture system of IMS32 under the high glucose conditions may provide useful information about the pathogenesis of diabetic neuropathy, especially polyol pathway-related abnormalities.

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