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Mechanism of diabetic neuropathy: Where are we now and where to go?

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ABSTRACT

Neuropathy is the most common complication of diabetes. As a consequence of longstanding hyperglycemia, a downstream metabolic cascade leads to peripheral nerve injury through an increased flux of the polyol pathway, enhanced advanced glycation end-products formation, excessive release of cytokines, activation of protein kinase C and exaggerated oxidative stress, as well as other confounding factors. Although these metabolic aberrations are deemed as the main stream for the pathogenesis of diabetic microvascular complications, organ-specific histological and biochemical characteristics constitute distinct mechanistic processes of neuropathy different from retinopathy or nephropathy. Extremely long axons originating in the small neuronal body are vulnerable on the most distal side as a result of malnutritional axonal support or environmental insults. Sparse vascular supply with impaired autoregulation is likely to cause hypoxic damage in the nerve. Such dual influences exerted by long-term hyperglycemia are critical for peripheral nerve damage, resulting in distal-predominant nerve fiber degeneration. More recently, cellular factors derived from the bone marrow also appear to have a strong impact on the development of peripheral nerve pathology. As evident from such complicated processes, inhibition of single metabolic factors might not be sufficient for the treatment of neuropathy, but a combination of several inhibitors might be a promising approach to overcome this serious disorder. (J Diabetes Invest, doi: 10.1111/j.2040-1124.2010.00070.x, 2010)

KEY WORDS: Diabetic neuropathy, Novel treatment, Pathogenesis

INTRODUCTION

Peripheral neuropathy is the most common and intractable complication of diabetes[1,2]. It involves somatic sensory and motor nerves, as well as autonomic nerves. In fact, the prevalence of diabetic neuropathy ranges from 7% within 1 year of diagnosis to 50% for those with diabetes for >25 years[3]. If patients with subclinical levels of neuropathic disturbances are included, the prevalence might exceed 90%[4]. The presence of cardiovascular autonomic neuropathy dramatically shortens the patients' longevity and increases the mortality[5,6]. Loss of feeling in the lower limbs is a high risk for limb amputation, which occurs in 1–2% of diabetic patients and necessitates extreme cost[4,7].

Despite efforts to make an early diagnosis and to halt the progression of diabetic neuropathy, currently there is no effective treatment available at a global level, except for tight control of blood glucose. This might be as a result, at least in part, of insufficient clarification of the pathogenesis of diabetic neuropathy, complicated clinical pictures that do not necessarily reflect proper progression of the disease, or inadequate design of clinical trials. There might also be a possibility that the development of a candidate drug might not be based on genuine inciting factors. To overcome this serious disorder, it is therefore essential to explore the precise role of causative factors in nerve fiber dysfunction and fiber loss. The present review summarizes the most up-to-date considerations on the pathogenesis of diabetic neuropathy and discusses the direction of its treatment.

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RISK FACTORS FOR PROGRESSION OF NEUROPATHY

The duration of diabetes and glycated hemoglobin levels have been well associated with a high incidence of neuropathy[8,9]. Classically, the Diabetes Control and Complications Trials (DCCT) confirmed the beneficial effects of meticulous control of blood glucose on the incidence of chronic complications in 1441 type 1 diabetic patients[10]. In that study, intensive insulin treatment for 6.5 years lowered HbA_{1c} levels (average 7%) by 2% compared with a conventionally treated group (average 9%) and successfully decreased the incidence of neuropathy by 60% (13 *vs* 5%)[10]. More striking are the so-called 'legacy effects' (glucose memory) of tight blood glucose control for the suppression of new development of neuropathy during a post-trial observation period for 8 years[11]. In type 2 diabetic patients, the Kumamoto study showed that intensive insulin treatment for 7 years improved nerve conduction velocity (NCV) and the vibration perception threshold (VPT) compared with those conventionally treated[12]. In contrast, the UK prospective diabetes

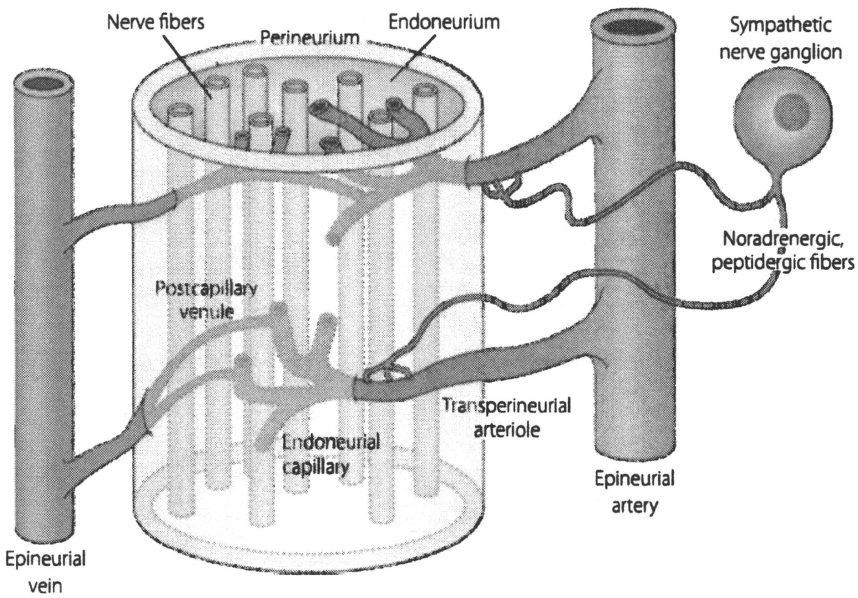

Figure 1 | Vascular supply of the peripheral nervous system is sparse and transperineurial arteriole penetrates into endoneurium. Autonomic nerve endings contact with the wall of arterioles, but vascular autoregulation is lacking in peripheral nerves as a result of sparse innervations. In diabetes, autonomic nerve endings to the arteriole are likely to be lost and therefore vasoregulation is further impaired (modified from *Pathology of Diabetes Mellitus for Clinicians* by Soroku Yagihashi, Shindan-to-Chiryo Co., Tokyo, 2004, page 110).

study (UKPDS) on 3867 type 2 diabetic patients did not find the effects of glucose control (to the extent of a 0.9% decrease in HbA_{1c}) on the prevalence of neuropathy, whereas there was a significant reduction in the risk for retinopathy and nephropathy[13]. Tesfaye *et al.* in the EURO-Diab group reported that blood glucose control, duration of diabetes, hypertension, hyperlipidemia and smoking were all significant risk factors for the development of neuropathy in type 1 diabetic patients[14]. The impact of hyperlipidemia has also been emphasized by a follow-up study of the DCCT trial[15]. However, this trend is different in cohorts of other countries, because Japanese studies could not find a significant influence of the blood concentrations of triglyceride or cholesterol on the prevalence of neuropathy[16]. It is clear after all that high blood glucose leads to peripheral nerve injury through a downstream metabolic cascade. The following section will concentrate on how hyperglycemia leads to peripheral nerve injury.

ANATOMY AND VASCULAR SUPPLY OF PERIPHERAL NERVOUS SYSTEM

Anatomical characteristics of the peripheral nervous system might explain why the pathogenesis of neuropathy is distinct from other microvascular complications[17,18]. Peripheral nerves are covered by perineurium, where only a few transperineurial arterioles penetrate into the endoneurium (Figure 1). The vascular supply in peripheral nerves is sparse and blood flow is likely to be compromised and lacks autoregulation[19]. This system makes peripheral nerves vulnerable to ischemia. Endoneurial microvessels are tightly connected with endothelial cells on their inner surface, but when destroyed they are leaky and affect the endoneurial tissue components[20]. Leaky vessels are mainly located in the ganglion with fenestrated vessels, and nerve terminals on the distal side are directly exposed to environments not covered by perineurium and are susceptible to traumatic injury.

Innervation of epineurial microvessels is involved in diabetes, resulting in impaired blood supply in diabetic nerves[21,22]. Endoneurial microvessels show thickened and multilayered basement membranes, cell debris of pericytes, as well as disrupted endothelial cells, and thus constitute salient structural changes in diabetic nerves.

Independent of vascular supply, three dimensions of neuronal architecture specific to the peripheral nervous system might account for the reason why the most distal side is susceptible in diabetes. Ganglion cells have extensively long axons covered by Schwann cells. The neuronal cell body is relatively small compared with the extremely long distance of axonal neurites, and thereby distal axons are innately too weak to support themselves for the long transport of nutrients, nerve trophic factors, as well as other signals.

PATHOLOGICAL BACKGROUND OF NEUROPATHY

Most characteristic findings of the peripheral nervous system in diabetic patients are distal and sensory predominant nerve fiber degeneration, axonal loss and endoneurial microangiopathy[23,24]. Both large and small caliber sizes of nerve fibers are affected. Based on this anatomical condition, Dyck *et al.* proposed that microvascular injury is the most probable factor for focal fiber loss and its summation appears to be the cause of diffuse fiber

loss of distal predominant axonal neuropathy in diabetes[25,26]. However, this explanation is too simplistic and does not explain why hyperglycemia and duration of diabetes are crucial for its occurrence. There also emerges a controversy as to whether there is any predominance for the involvement of small fibers in early diabetic neuropathy. Questions on this issue were further raised by the report that the focality of nerve fiber loss was not universally demonstrated, indicating that microangiopathy does not always account for the fiber loss[27]. Nevertheless, vascular influence on the development of neuropathy was further supported by subsequent studies on humans. Malik *et al.* showed that patients who did not have clinically evident neuropathy at the time of nerve biopsy, but who showed high-grade microangiopathic changes of endoneurial microvessels later, developed overt neuropathy, whereas the patients without microvessel changes did not develop neuropathy[28]. The extent of microangiopathic changes correlated well with subsequent nerve fiber loss in diabetic nerves[29]. We ourselves found a correlation between the thickness of the basement membrane of endoneurial microvessels and reduced myelinated fiber density[30].

The most distal axons of small fibers distribute in the epidermis of the skin, sensing pain or pricking. Currently, punched skin biopsy immunostained with protein gene product (PGP)-9.5 is widely used for the evaluation of peripheral neuropathy[31]. The method is simple and minimally invasive, but requires the equipment of confocal laser scan microscopy and skills for the staining and measurement. Usually, skin over the calf muscle is used, but other sites might also be added. In diabetes, the nerve fibers in the epidermis of the skin are significantly affected, resulting in distortion, twisting, focal swelling or beading, and finally, disappearance of nerve fibers[32–34] (Figure 2). The reduction was found even in subjects of impaired glucose tolerance (IGT) and the extent of fiber loss was marked in established diabetic patients[35,36]. The nerve fiber loss in the skin was associated with fiber loss in the nerve trunk of the sural nerve, thus in keeping with the presence of clinically evident neuropathy[32]. In relation to the alteration of epidermal innervation, a non-invasive method using corneal confocal microscopy has now been developed for the evaluation of neuropathy[37,38]. With this method, small nerve fibers distributed in the cornea can be observed without tissue sampling in live conditions[38,39]. Diabetic patients showed significant loss of nerve fibers, twisting and increased branching on the cornea[38,39]. Taking advantage of non-invasiveness, it is easy to follow by repeated observations and to evaluate the treatment effects on neuropathy by this method. In fact, the recovery of nerve fibers by regeneration was detected in long-standing type 1 diabetic patients 6 months after pancreas transplantation[40]. To understand the cause and the development of neuropathy, spatial and temporal changes of nerve pathology and their clinical significance should be explored in more detail.

To compensate for the paucity of information on human materials, animal models have served the basis of functional and biochemical changes that might be translated into human diabetic neuropathy. Unfortunately, diabetic animal models did not show the pathological features in the peripheral nerves trunk observed in human diabetic patients. However, recent studies

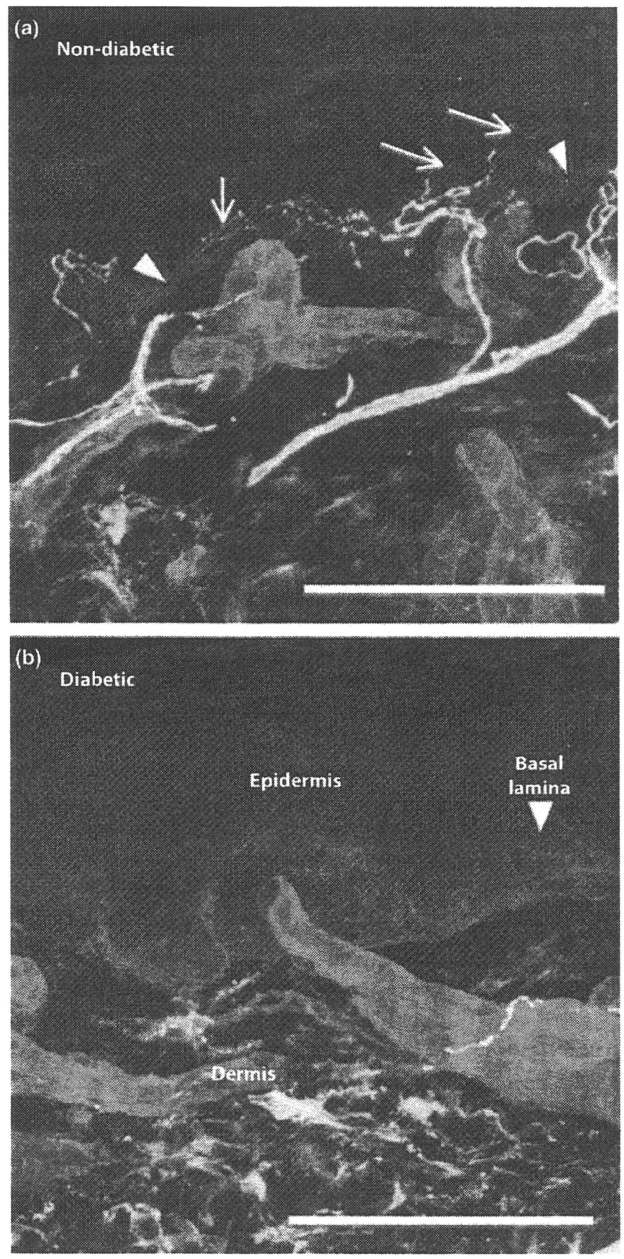

Figure 2 | Epidermal innervation in diabetic patients as shown by immunostaining with PGP9.5. (a) In a normal subject (a 32-year-old man), small branching fibers (arrows) penetrating to basal lamina (arrowhead) derived from dermis distribute diffusely and end in the surface of the epidermis of the skin. (b) In contrast, in a type 2 diabetic subject with symptomatic neuropathy (a 52-year-old woman with 15 years duration of diabetes), fibers in the epidermis are completely lost. Only a few fibers are sparsely left in the dermis. Vascular systems also develop in the upper dermis (red color of tortuous structure). Bar, 100 mm.

have overcome this discrepancy by showing significant nerve fiber loss in the skin of diabetic animal models[41,42]. It is therefore now possible to search in more detail for the contribution of possible factors to the loss of nerve fibers of the skin by studying animal models. More importantly, it provides us a great tool for the exploration of effective compounds to inhibit nerve fiber loss and promote nerve fiber regeneration[43,44].

Unlike human diabetic subjects, distinct pathological changes of endoneurial microvessels are not consistently shown in animal models, although reduced nerve blood flow is reproducibly shown[45,46]. In streptozotocin (STZ)-induced diabetic rats, there was only a modest dilatation of vascular lumina, but no reduction of microvessel density or thickening of basement membranes in the peripheral nerve[47–49]. Although some studies reported reduced microvessel density in diabetic animals that reverted to normal by intervention with vascular endothelial growth factor (VEGF) or other angiotrophic factors, the recovery of nerve blood flow by these agents might be explained by functional improvement of endoneurial vessels rather than robust angiogenesis in the endoneurium.

HOW DOES HYPERGLYCEMIA LEAD TO PERIPHERAL NERVE INJURY?

Polyol pathway

Increased polyol flux regulated by aldose reductase (AR) activation has been studied most extensively and there is no doubt that this metabolic cascade contributes to the development of neuropathy. With this premise, numerous AR inhibitors (ARI) have been developed, but clinical trials have mostly been unsuccessful, in part due to the adverse effects or insignificant improvement at the clinical end-point. Currently, epalrestat (ONO2235) is the only one licensed in Japan. It was approved after a 3-month double-blinded trial[50], which showed improvement of symptoms and nerve function. Further extended 3-year double-blinded randomized trials confirmed that ARI treatment significantly suppressed the progressive delay of nerve conduction[51]. The ARI effects were more marked in patients with early neuropathy and modestly elevated levels of glycated hemoglobin[52]. Another challenge of a new ARI will be expected to succeed in future trials, because other mechanisms do not amply replace the polyol pathway hypothesis[53,54].

Despite a long history of preclinical studies, the detailed mechanism of how the polyol pathway is involved in neuropathy remains elusive. Earlier studies proposed the osmotic theory in which increased polyol flux caused intracellular hyperosmolarity by an accumulation of impermeable sorbitol in the cytoplasm, resulting in the expansion of cells and cell lysis[55,56]. Although this theory might be applied to the genesis of diabetic cataracts[55,56], there is no consistent evidence of nerve edema or swollen cells in diabetic nerve tissues[57]. Following the osmotic hypothesis, Greene raised the poor energy utilization theory as the surrogate of osmotic theory[58,59]. With an accumulation of sorbitol, other osmolytes of *myo*-inositol, taurine and adenosine were depleted in the cytoplasm. In turn, *myo*-inositol deficiency caused phosphatidyl-inositol depletion and then poor production of adenosine triphosphate (ATP), leading to reduced Na,K-ATPase activity and protein kinase C (PKC) activity[58,59]. In this process, however, there is no confirmative data of *myo*-inositol depletion in diabetic nerves[60]. In addition, clinical application of *myo*-inositol was not successful[61].

Consistent with the data from human IGT subjects, it was shown that *ob/ob* mice revealed neuropathic changes represented by NCV delay and increased oxidative stress-induced damage[62]. High-fat diet fed mice that showed typical glucose intolerance also showed neuropathic changes[63]. In these mice, postprandial hyperglycemia itself exerted increased flux of the polyol pathway in the peripheral nerve tissues.

The advent of transgenic technology has greatly advanced the polyol pathway story. Transgenic mice that overexpress human AR developed severe neuropathy when they were fed galactose, which is also the substrate of AR[64]. Thus, without hyperglycemia or insulin deficiency, increased flux of the polyol pathway in fact caused peripheral nerve dysfunction and myelinated fiber pathology, similar to those found in diabetic animal models[64]. The study was extended to the STZ-induced diabetic condition in this model, which showed more severe NCV delay and reduced Na,K-ATPase activity with an accumulation of sorbitol and fructose, compared with those in non-transgenic diabetic mice, despite comparable levels of hyperglycemia[65]. The functional changes were accompanied by more severe structural changes in peripheral nerves and alterations of neuropeptide expressions in dorsal root ganglia (DRG)[66]. Concurrently, transgenic mice with hyperglycemia-induced activation of the polyol pathway showed endoneurial reduction of PKC activity with decreased membranous expression of PKCα and a relative increase in PKCβ isoform (Figure 3). The neuropathic changes were improved by giving diabetic transgenic mice ARI. In contrast, studies using targeted mice lacking the AR gene showed that AR-deficient mice were protective against neuropathy through the preservation of glutathione and nicotinamide adenine dinucleotide phosphate (NADPH)[67].

Although these studies confirmed the critical role of AR in diabetic neuropathy, clinical experience of ARI trials[50] showed that the polyol pathway cannot completely account for the development of neuropathy. Indeed, when blood glucose is poorly controlled, severe hyperglycemia can cause neuropathic changes, even in AR-deficient diabetic mice[68]. A pathway independent of AR is yet to be determined and further studies are required for the complete prevention or intervention of the progression of diabetic neuropathy.

The implications of AR in ischemia/reperfusion injury have now revitalized the polyol pathway theory for vascular events, not only in diabetic patients but non-diabetic patients as well (Figure 4). Ischemia/reperfusion causes polyol activation, leading to severe tissue injury against which ARI is preventive[69–75]. In experimental studies, ARI alleviated the pathological lesions in infarction of the brain, the heart, as well as the kidney or retina[71,75,76]. Because diabetic nerves are susceptible to

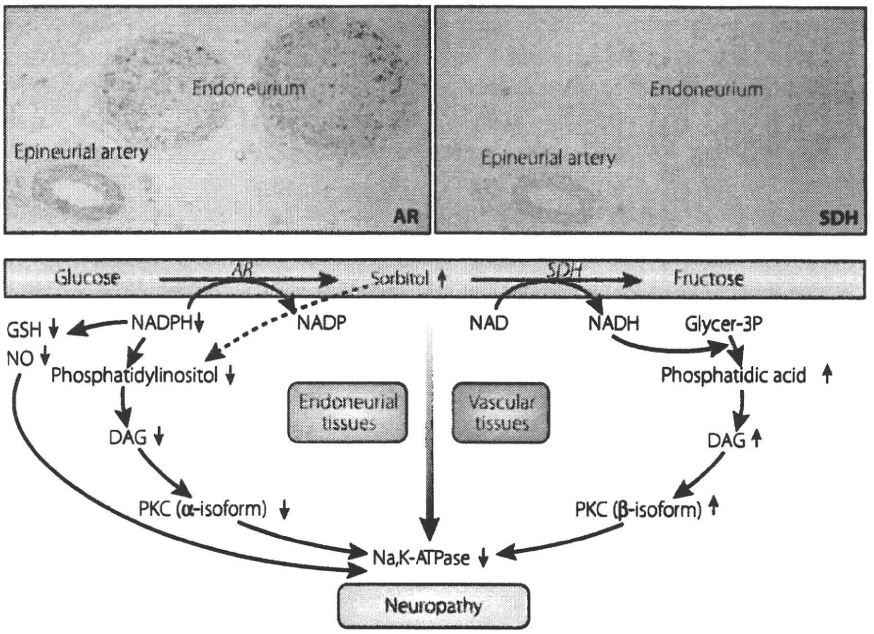

Figure 3 | Tissue-specific regulation of polyol pathway and its metabolic cascade to diabetic neuropathy. Major regulating enzymes of the polyol pathway are differentially expressed in the epineurial artery and endoneurial tissues. Aldose reductase (AR) is strongly expressed in both the endoneurium and the wall of the epineurial artery, whereas expression of sorbitol dehydrogenase (SDH) is equivocal in the endoneurium, but clearly positive for the wall of the epineurial artery (see reference 120, with kind permission from Springer Science + Business Media: Virchows Arch, Vol. 439, 2001, page 48. Enhanced *in situ* expression of aldose reductase in peripheral nerve and renal glomeruli in diabetic patients; Kasajima H, Yamagishi SI, Sugai S, Yagihashi N, Yagihashi S, Figure 2). Hence, hyperglycemia in nerve tissues exerts conversion from glucose to sorbitol by AR, thereby causing the depletion of reduced glutathione (GSH) and nitric oxide (NO) consequent from the overconsumption of nicotinamide adenine di-nucleotide phosphate (NADPH). Concurrently, intracellular *myo*-inositol is depleted to cause phosphatidylinositol (PI) depletion, which further suppresses diacylglycerol (DAG) production and finally protein kinase C (PKC) activity. As a consequence, Na,K-ATPase activity will be reduced to result in functional and structural changes of neuropathy. In contrast, the second portion of the polyol pathway regulated by SDH is activated in the vascular wall in the hyperglycemic condition. As a result of redox changes of NAD/NADH, conversion from glyceraldehyde-3-phosphate (Glycer-3P) to phosphatidic acid will be promoted. Then enhanced synthesis of DAG results in increased PKC activity. In our studies, major isoforms that underwent changes in the diabetic condition are PKCα in the nerve and PKCβ in the epineurial artery (reference 122).

ischemia/reperfusion injury, there emerges a new perspective that ischemia/reperfusion might be involved in the progression or exacerbation of neuropathy to which ARI is effective[77,78].

Glycation and Advanced Glycation End-products

Glycation has long been implicated in the pathogenesis of diabetic neuropathy[30,79,80]. Every component of nerve tissues can be excessively glycated in diabetic nerves. In fact, deposition of advanced glycation end-products (AGE) was shown in human and animal diabetic nerves, in every component of peripheral nerve tissues[30,80]. The deposition was found in the stromal collagens, axoplasms of nerve fibers and Schwann cells, as well as endoneurial vessels[81]. The intensity of AGE deposition detected by carboxymethyllysine immunoreactions correlated well with reduced myelinated nerve fiber density[81]. Hence, AGE was considered to exert injurious processes in the endoneurium through direct toxicity to nerve tissues together with endoneurial microangiopathy (Figure 5). *In vitro*, Schwann cells underwent apoptotic processes with release of tumor necrosis factor (TNF)-α, as well as other inflammatory cytokines, when exposed to a high AGE environment[82]. Axonal cytoskeletons of tubulin and neurofilaments were glycated to stagnate axonal transport, resulting in distal fiber degeneration[30]. Glycation of basement membrane collagen, laminin and fibronectin also caused impairment of regenerative efforts in diabetic nerves[83,84].

Transgenic mice with enhanced expression of the receptor for AGE (RAGE) in endothelial cells showed augmented neuropathic changes in the diabetic condition, exemplified by delayed NCV and more severe structural changes[85]. In this setting, it can be speculated that AGE exerts biological reactions after binding with RAGE expressed on endothelial cells and Schwann cells, leading to the functional and structural phenotype of neuropathy. During this process, intracellular oxidative stress mediated by NADPH oxidase activation might be elicited and then activate transcription of nuclear factor-κB (NF-κB)[86,87]. Bierhaus *et al.* reported that the activation of NF-κB was associated with the alteration of pain sensation in STZ-induced hyperglycemic mice[88]. Diabetic mice lacking the RAGE gene were

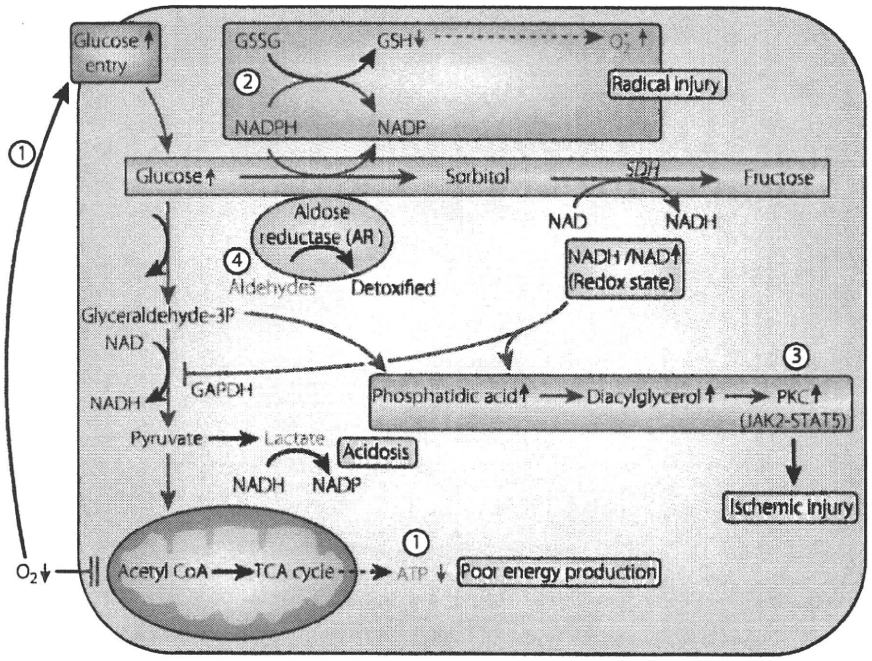

Figure 4 | Implication of aldose reductase in ischemia/reperfusion injury. Recently, a new role of aldose reductase in ischemia/reperfusion and inflammatory injury was proposed. When a cell becomes ischemic, glucose uptake is enhanced to compensate energy depletion (①). However, because mitochondria are impaired to produce ATP as a result of oxygen depletion, surplus glucose enters the collateral pathway to sorbitol and phosphatidic acid. From the former, aldose reductase is activated to cause glutathione deficiency and redox deviation, as in the hyperglycemic condition (②). As a result, free radical injury and protein kinase C (PKC) activation ensue to aggravate ischemic injury (③). Once reperfusion starts, oxygen radicals accumulate aldehydes, which are also substrates of aldose reductase, and enhance radical injury (④) (adapted from reference 69 and modified by the author).

protective against the induction of neuropathy[89]. Thus, these findings support the crucial role of AGE in the development of diabetic neuropathy.

Indirect evidence that suggests the role of AGE in neuropathy might be the effects of aminoguanidine on experimental diabetic neuropathy[47,90–92]. This compound was found to inhibit the formation of AGE, concurrently with the improvement of endoneurial blood flow[90], NCV, Na,K-ATPase activity and myelinated fiber structure[91,92]. It should be of note that aminoguanidine effects might also be mediated by its alternate action as an inducible nitric oxide synthase (iNOS) inhibitor or an anti-oxidative function[93].

In our most recent study, we showed that animals given AGE exogenously showed significant NCV delay resembling that found in experimental diabetic neuropathy (Figure 6)[94]. With delayed NCV, nerve Na,K-ATPase activity was reduced and myelinated nerve fibers underwent reduction of fiber size. In this setting, vascular reactions in response to exogenous AGE elicited functional impairment of peripheral nervous systems. In fact, endothelial cells showed a high expression of NF-κBp65 together with swollen and vacuolar changes at the ultrastructural levels. From these findings, AGE action mediated by binding with RAGE causes activation of NF-κB and thereby its downstream signals[88,95,96]. Although preliminary clinical trials of anti-glycation agent, benfotiamime, showed some efficacy for diabetic neuropathy[97], there is still no effective compound that can suppress the AGE formation *in vivo* and improve diabetic neuropathy in humans.

Oxidative Stress

As a cause of diabetic neuropathy, the generation of free radicals is proposed to be a major factor through increased glycolytic process[98,99]. In fact, there are numerous data that showed oxidative stress-induced tissue injury in the peripheral nerve in experimental diabetes[45,63,88,92,95,98]. Based on this background, attempts have been made to inhibit neuropathy with antioxidants[100,101]. In particular, α-lipoic acid has been used for the suppression of oxidative stress in experimental diabetic rats and it was found that it improved NCV delay, nerve blood flow and nerve structure[102–104].

Concurrent with the generation of free radicals during the glycolytic process, mitochondria have a crucial role in cellular death by activation of specific signals and the endonuclease system[105,106]. Hyperglycemia-induced mitochondrial changes include the release of cytochrome C, activation of caspase 3, altered biogenesis and fission, resulting in programmed cell death[105,107]. Excessive entry of glucose causes surplus transport of electrons to generate oxidants in mitochondria, leading to

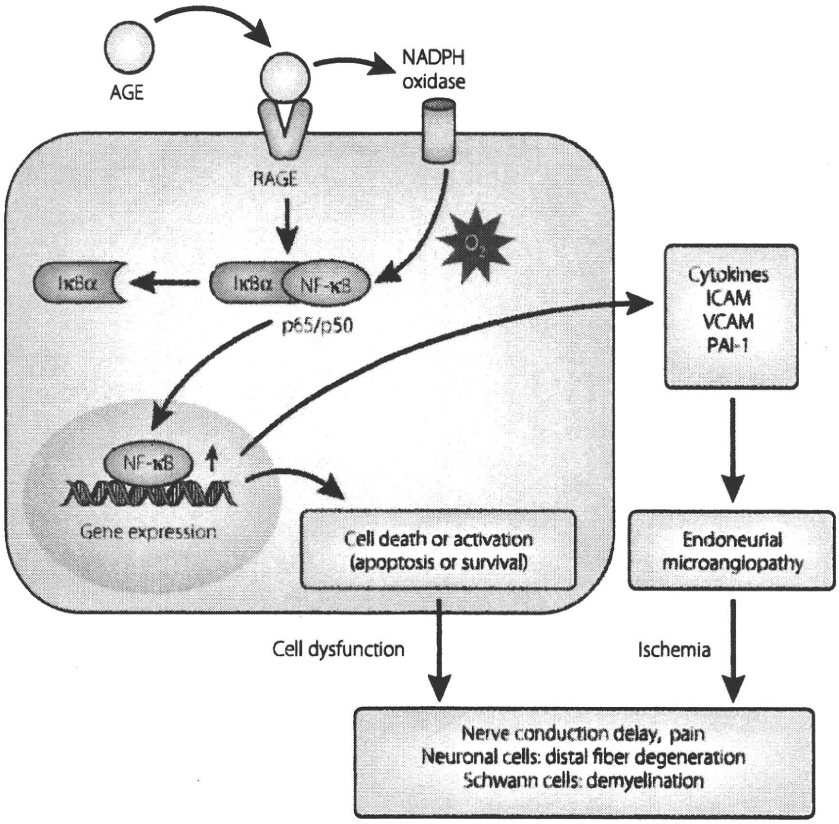

Figure 5 | Advanced glycation end-products (AGE) and receptor for AGE (RAGE) reactions in the pathogenesis of diabetic neuropathy. Nerve tissues, such as Schwann cells, nerve fibers and endothelial cells of vasa nervosum all express RAGE. When AGE bind with RAGE, the reaction generates oxidative stress mainly through the activation of NADPH oxidase. Complexes of IκBα-nuclear factor-(NF)-κB will be separated into each fraction of IκBα and NFκB, the latter of which translocates into the nucleus as a transcription factor to activate genes related to cell death or survival. As a result, both microangiopathic processes and neural dysfunction ensue, resulting in the manifestation of pain or nerve conduction delay.

reduced mitochondrial action potentials (MMP) with poor energy synthesis of ATP[108,109]. Neurotrophic support is also impaired by mitochondrial damage to cause reduced neurotrophin-3 (NT-3) and nerve growth factor (NGF)[108]. It is interesting that a small amount of insulin, that does not alter systemic blood glucose levels, was shown to improve the impaired mitochondrial membrane potential and delayed nerve conduction in STZ-diabetic rats[109].

As already alluded to, both the polyol pathway and AGE formation produce a large amount of oxidants, and ARI treatment suppresses the oxidative nerve injury[110–112]. In addition to mitochondria, other organelles, such as the Golgi apparatus and endoplasmic reticulum (ER), might also be regarded as an important source of free radicals, resulting in not only apoptosis, but cell death from autophagy[113]. Indeed, nitro-oxidative stress in conjunction with hyperglycemia exerts poly ADP-ribose polymerase (PARP) activation[114], resulting in cellular dysfunction and cell death, which can be prevented by PARP inhibitor[115]. Serum from type 2 diabetic patients accelerates neuroblastoma cell death by increased autophagic processes with activation of cell death signals[116]. α-Lipoic acid was found to be beneficial to some extent to alleviate neuropathic symptoms in diabetic patients[117]. However, to confirm whether this compound is in fact effective to inhibit the progression of the disease, further confirmation is required.

PKC Activity

PKC is central in nerve function and a key in the pathogenesis of diabetic neuropathy[118,119]. However, the alterations are complicated in nerve tissues and their supportive endoneurial vascular system, as the major enzymes of collateral glycolytic pathway are different between these two tissues[120] (Figure 3). Such inhomogeneous tissue composition might explain the inconsistent findings on PKC activity in diabetic nerves. Nakamura *et al.* did not find any significant change of PKC activity in the homogenized whole peripheral nerve tissues in STZ diabetic rats, although PKC-β specific inhibitor improved NCV delay and nerve blood flow[121]. In contrast, in our studies on STZ-induced diabetic mice, we separated the tissues into endoneurium and epineurium for the measurement of PKC activity, the latter of which is rich in microvessels[122]. We found that the former showed decreased PKC activity with significantly decreased

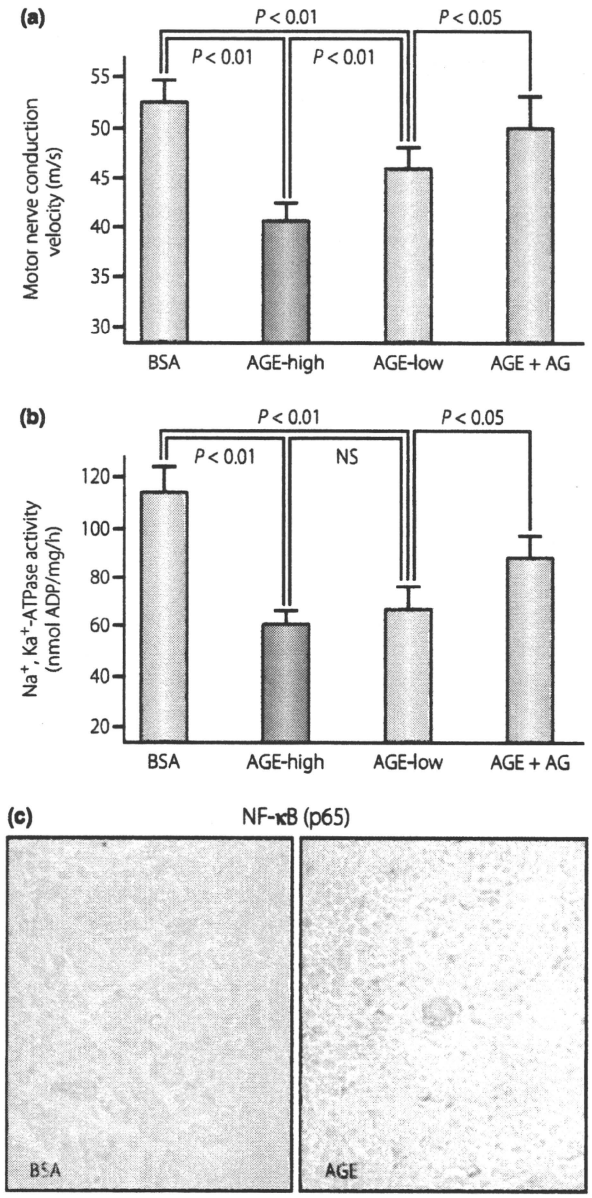

Figure 6 | Neuropathy in normal rats given exogenous advanced glycation end-products (AGE). When AGE were given exogenously, normal rats showed neuropathic changes, similar to those found in experimental diabetic animals. Rats given AGE showed (a) a significant delay of motor nerve conduction velocity and (b) suppression of nerve Na,K-ATPase activity, whereas no effects were detected in bovine serum albumin (BSA)-treated rats. Such suppression was corrected by co-treatment with aminoguanidine, an inhibitor of glycation and nitric oxide. (c) On the sections, AGE-treated rats showed strong expression of nuclear factor-κB on the nuclei of endothelial cells of microvessels and Schwann cells (quoted from reference 94).

membranous expression of the PKC-α isoform, as we already stated earlier about polyol pathway, whereas the latter showed increased PKC activity with enhanced expression of PKC-β (Figure 3). The results of epineurial tissues were consistent with the changes in other systemic vascular tissues. In keeping with this finding, hyperglycemia caused reduced PKC activity in cultured Schwann cells exposed to high glucose[123].

Hence, the application of PKC-β-specific inhibitor is expected to be useful for the treatment of diabetic vascular complications. Experimental studies showed beneficial effects of PKC-β-specific inhibitor on neuropathic changes in STZ-induced diabetic rats[121,124,125]. Despite extensive efforts, however, clinical trials were not successful due, in part, to the high improvement rate in the placebo group[126]. Other isoforms of PKC were also implicated in the causation of diabetic neuropathy and inhibitors for these isoforms have been explored[127,128].

Proinflammatory Processes

There is emerging evidence that nerve tissues in diabetes undergo a pro-inflammatory process that presents symptoms and enhances the development of neuropathy[129,130]. Indeed, diabetic nerves contain macrophages, occasionally lymphocytes and release increased TNF-α or interleukins (IL) in humans and animals[129,131,132] (Figure 7). Inhibition of cytokine release or macrophage migration was associated with the improvement of NCV delay and structure in STZ-diabetic rats treated with *N*-acetylcysteine[133] or pioglitazone[134]. The arachidonic acid pathway is activated to increase in cyclooxygenase (COX)-2 concentrations in the peripheral nerves of STZ diabetic rats in which inhibition of COX-2 corrected nerve blood flow and NCV delay[135]. To further confirm this data, COX-2 gene-deficient mice were protective for NCV delay and neuropathic deficits after STZ-induced hyperglycemia[136]. The pro-inflammatory condition activated the stress-kinase, mitogen-activated protein (MAP)-kinase, in diabetic nerves, which was also suppressed by treatment with pioglitazone[134]. Thus, MAP-kinase is considered to be a potential target for a new treatment of diabetic neuropathy[137,138]. In this process, NF-κB is activated to lead the cell to cell death or proliferation[139,140]. Because a pro-inflammatory reaction is induced by the polyol pathway hyperactivity or increased AGE formation as well, it should be clear to what extent the pro-inflammatory process is a single initiating or influential factor for the development of neuropathy. Ischemia reperfusion might also accelerate the inflammatory processes to which diabetic nerves are susceptible[77,78].

With increasing information about the role of inflammation, approaches to suppress the pain symptoms or neuropathy itself are now carried out with the specific target of cytokines or cell signals[141–143].

Cellular and Trophic Factors

The lack of neurotrophins plays an important role in the pathogenesis of diabetic neuropathy[144–149]. In fact, the production of NGF was suppressed in the skin and substitution of NGF ameliorated neuropathic changes of small fibers and autonomic pathology in diabetic animals[150,152]. NT-3, brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF)

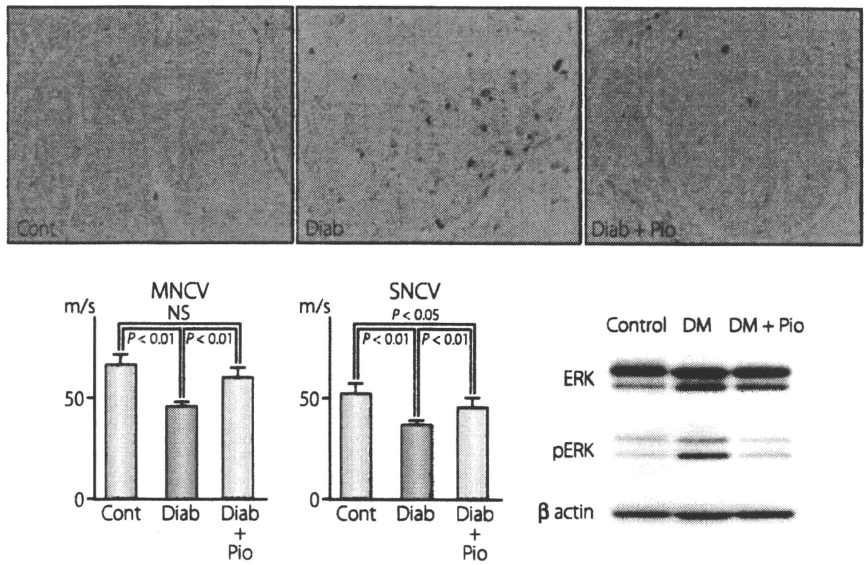

Figure 7 | Pro-inflammatory reactions and experimental diabetic neuropathy. In the sciatic nerve of STZ-induced diabetic rats, there were many macrophages stained positive for ED1 (upper center). Migration of macrophages was inhibited when diabetic rats were treated with pioglitazone (upper right). Pioglitazone treatment also corrected the delay of motor nerve conduction velocity (MNCV) and sensory nerve conduction velocity (SNCV), and activation of extracellular signal-regulated kinase (ERK), one of mitogen activated protein kinases (MAPK) (adapted from reference 134).

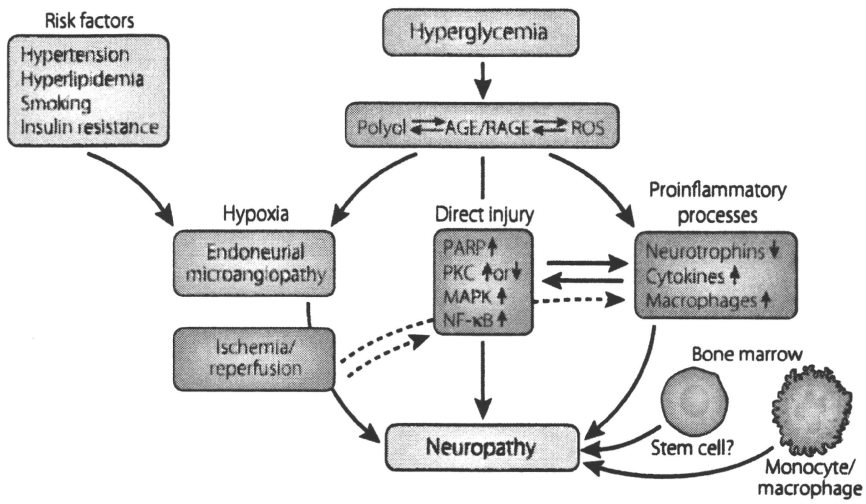

Figure 8 | Summary of pathogenetic mechanisms of diabetic neuropathy. Long-term hyperglycemia causes downstream metabolic cascades of polyol pathway hyperactivity, advanced glycation end-products (AGE)/receptor for AGE (RAGE) reactions and increased reactive oxygen species (ROS). They compromise both endoneurial microvessels and neural tissues themselves through activation of poly-ADP-ribose polymerase (PARP), alterations of protein kinase C (PKC) and an increase in mitogen-activated protein kinase (MAPK), as well as activation of nuclear factor-(NF)-κB, resulting in functional and structural changes of peripheral neuropathy. Metabolic aberrations in the nerve elicit pro-inflammatory reactions, inducing release of cytokines, suppression of neurotrophins and migration of macrophages, and promote the development of neuropathy. Recently, cellular factors derived from the bone marrow were found to produce chimeric cells in peripheral nerves of diabetic animals to elicit nerve injury. There is also the possibility that other cellular components from the bone marrow have an influence on the nerve pathology in diabetes. In addition, ischemia/reperfusion might also accelerate nerve injury, in part mediated by inflammatory reactions. Risk factors represented by hypertension, hyperlipidemia, smoking and insulin resistance are also important contributors to the development of neuropathy.

were also decreased in the muscle tissues in diabetic patients[153]. NT-3 was shown to protect the NCV delay and perception threshold in diabetic animals[154], but the results were not always positive[155,156]. Unfortunately, application of NGF in a clinical trial did not succeed in the correction of neuropathy, in part because of the emergence of pain[157]. Efforts have now been

made to more efficiently deliver or produce trophic factors at the target tissues by introducing gene therapy or cell transplantations[59,159–162].

Recent studies have shown a new insight into the pathogenesis of neuropathy. In diabetic nerves, there were chimeric cells that were a combination of resident Schwann cells or neuronal cells and migrated proinsulin-producing cells derived from bone marrow[163]. Although the significance of such chimeric cells is yet to be known, they eventually undergo apoptotic cell death, thus injuring the constitutive cells, leading to neuropathic changes. Much remains to be further investigated to confirm such intriguing cells and to clarify their significance.

Direction of Treatment

Based on the proposed mechanisms of neuropathy so far (Figure 8), efforts have been continuously made to develop effective means for the treatment of neuropathy. However, to date, there are only a few agents available in limited countries; ARI (epalrestat) in Japan and α-lipoic acid (thioctic acid) in Germany. Other agents, such as benfotiamine as an anti-glycation agent, PKC-β-inhibitor (ruboxitaurine) or NGF were unsuccessful at the final stage of randomized clinical trials. Nevertheless, there are still ongoing trials that we hope will be successful in future. Very recently, it was shown that autonomic neuropathy in the bone marrow impaired activation and migration of endothelial precursor cells (EPC), which might determine the fate of vascular complications[164]. It also becomes clear that the vagus nerve conveys signals for regeneration of islet β-cells[165], which might be disturbed in diabetic patients. These novel findings reinforce the importance of diabetic neuropathy for patient care and direction of treatment in diabetes. In particular, early inhibition of causative factors is extremely important not only to halt, but to reverse, the lesions. However, once the lesions are developed, as stated earlier, a variety of factors are exerted to accelerate the neuropathy. In this setting, the combination of several inhibitors might be required.

Neuropathy has long been regarded merely as a disorder of the most distal portion of the body. Effects of hyperglycemia on the nervous system have now been shown to be a much more serious condition. Neuropathy itself is an important trigger for systemic abnormalities in diabetic patients. Much more investigation on the nerve changes in the pancreas, liver and related organs is required for a better understanding of the whole body in diabetic patients and to develop effective treatment of this disease.

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Article: Treatment

Efficacy and safety of pregabalin for treating neuropathic pain associated with diabetic peripheral neuropathy: a 14 week, randomized, double-blind, placebo-controlled trial

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Abstract

Aims To evaluate the efficacy, safety and pharmacokinetics of pregabalin in treating neuropathic pain associated with diabetic peripheral neuropathy in Japanese patients.

Methods A randomized, double-blind, placebo-controlled, multicentre 14 week clinical trial was conducted. Japanese patients with diabetic peripheral neuropathy ($n \doteq 317$) were randomized to receive placebo or pregabalin at 300 or 600 mg/day. The primary efficacy measure was a change of mean pain score from baseline to end-point from patients' daily pain diaries.

Results Significant reductions in pain were observed in patients treated with pregabalin at 300 and 600 mg/day vs. placebo ($P < 0.05$). Improvements in weekly pain scores were observed as early as week 1 and were sustained throughout the study period (300 and 600 mg/day difference from placebo at study end-point, –0.63 and –0.74, respectively). Pregabalin produced significant improvements in weekly sleep interference scores, the short-form McGill Pain Questionnaire, the Medical Outcomes Study–Sleep Scale, the 36-item Short-Form Health Survey scale, and the Patient and Clinical Global Impression of Change. Patient impressions of numbness, pain and paraesthesia were also significantly improved. Regarding treatment responders, 29.1 and 35.6% of patients treated with 300 and 600 mg/day, respectively, reported ≥50% improvement in mean pain scores (vs. 21.5% for placebo). Pregabalin was well tolerated; somnolence (26%), dizziness (24%), peripheral oedema (13%) and weight gain (11%) were the most common adverse events and generally were reported as mild to moderate.

Conclusions Pregabalin was effective in reducing pain and improving sleep disturbances due to pain, and was well tolerated in Japanese patients with painful DPN.

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Keywords clinical trial, diabetes mellitus, peripheral neuropathic pain, pregabalin, sleep

Abbreviations CGIC, Clinical Global Impression of Change; CL_{cr}, creatinine clearance; MOS, Medical Outcomes Study; PGIC, Patient Global Impression of Change; SF-36, 36-item Short-Form Health Survey; SF-MPQ, Short-Form McGill Pain Questionnaire.

Introduction

The number of patients with diabetes mellitus increases yearly in Japan. The estimated prevalence was 6.8 million in 2000 and is expected to grow to 8.9 million in 2030, which places Japan among the top 10 countries for prevalence of patients with diabetes [1]. Consequently, an increase in patients with vascular and neurological complications is expected.

Diabetic peripheral neuropathy is one of the most common complications associated with diabetes and consists of several distinct clinical entities categorized as sensory, focal and multifocal or autonomic [2,3]. Painful neuropathic symptoms are associated not only with discomfort but also with sleep interference, weight loss, anxiety, depression and an overall decrease in quality of life [4–9], the impact of which increases with pain severity [4]. In addition, a large proportion of patients

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with diabetic peripheral neuropathy report limited activity and lost productivity at work [4].

There is a need for pharmacological treatment options that can relieve the painful symptoms of diabetic peripheral neuropathy. Drugs such as antidepressants, antiepileptics, topical treatments and opioids have been used to date [10,11]. In addition to these, epalrestat (aldose reductase inhibitor) and mexiletine (anti-arrhythmic) have been approved to treat symptoms of this condition in Japan. Despite these advances, the pharmacological treatment of painful diabetic peripheral neuropathy continues to be a challenge for the physician.

Pregabalin binds with high affinity to the $\alpha_2\delta$ site and has analgesic, anxiolytic and anticonvulsant activities. Pregabalin has become one of the first-line treatments for painful diabetic peripheral neuropathy in many Western countries, as several Phase 3 clinical studies have confirmed its efficacy and safety [12–17].

The present study was undertaken to evaluate the efficacy, safety and pharmacokinetics of pregabalin for painful diabetic peripheral neuropathy in Japanese patients. As the efficacy and safety profile of pregabalin was considered similar between Japanese and Western patients with postherpetic neuralgia [18,19], this study included doses similar to those in previous studies of diabetic peripheral neuropathy conducted in the West.

Patients and methods

Study population

Patients included men and women ≥18 years of age diagnosed with Type 1 or Type 2 diabetes mellitus at least 1 year previously and diagnosed with painful distal, symmetrical, sensorimotor polyneuropathy due to diabetes. Patients were enrolled if they had a score of ≥40 mm on the visual analogue scale of the short-form McGill Pain Questionnaire (SF-MPQ) and had evaluated and recorded pain for at least 4 of the previous 7 days in the daily pain diary prior to treatment initiation, with the mean score being ≥4 on an 11-point (0–10) numeric rating scale. Patients excluded were those diagnosed with a malignant tumour within the past 2 years, those whose creatinine clearance (CL_{cr}), calculated according to the Cockcroft & Gault formula [20], was ≤0.5 ml/s (≤30 ml/min), and those who had pain or skin conditions that may affect the evaluation of pain.

Study design

The study consisted of a 1 week baseline phase, a 13 week treatment phase and a 1 week down-titration phase. Patients were classified into two groups based on CL_{cr} ($0.5 < CL_{cr} \leq 1.0$ ml/s or $CL_{cr} > 1.0$ ml/s) and then randomized to one of three treatment groups (placebo, pregabalin 300 or pregabalin 600 mg/day). Patients randomized to 600 mg/day with $0.5 < CL_{cr} \leq 1.0$ ml/s received 300 mg/day. The stratified randomization based on CL_{cr} stratum was centrally organized using a validated web-based system.

During the 1 week titration phase, the initial dose was 75 mg twice daily and was slowly increased until the target dose was reached. Patients then took pregabalin 150 or 300 mg or placebo twice daily during the 12 week fixed-dose treatment phase. Patients who did not enter the long-term open-label extension concluded dosing following a 1 week dosage taper phase. The study was approved by the institutional review boards and conducted in compliance with the Declaration of Helsinki, International Conference on Harmonization Good Clinical Practices guidelines and Japanese regulations at 62 sites from October 2007 to March 2009 (first subject, first visit to last subject, last visit), and registered under the Clinical Trials Registry number NCT00553475. All patients provided written informed consent.

Efficacy and safety assessments

The primary efficacy end-point was the change from baseline in mean weekly pain score from the patient's daily pain diary at week 13 [study end-point (last observation carried forward)] using an 11-point numeric rating scale.

Secondary efficacy end-points included weekly mean pain scores, responder rates (defined as ≥50% reduction in mean pain score from baseline to end-point), SF-MPQ completed at each clinic visit, and weekly mean sleep interference scores using an 11-point numeric rating scale. Additional efficacy measures included Medical Outcomes Study (MOS)–Sleep Scale, 36-item Short-Form Health Survey (SF-36) [21], patient impression of subjective symptoms (numbness, pain and paraesthesia), Patient Global Impression of Change (PGIC) and Clinical Global Impression of Change (CGIC).

Adverse events were recorded from the first dosing of the study drug to the last visit of the study. Physical examinations and laboratory assessments were conducted at multiple times throughout the study. Plasma concentrations of pregabalin were assessed by blood samples at weeks 8 and 13 of the treatment phase.

Population pharmacokinetic analysis

Blood samples were collected from 154 patients (n = 298). Plasma pregabalin concentration samples were assayed using a validated liquid chromatography/tandem mass spectrometry. The lower limit of quantification was 0.157 μmol/l. The analysis was performed using non-linear mixed effects modelling methodology as implemented in NONMEM (version 5; ICON Development Solutions, Ellicott City, MD, USA). A one-compartment model with first-order absorption and elimination (NONMEM subroutine ADVAN2) was fitted to pregabalin concentrations.

Statistical analyses

A sample size of 308 patients (n = 132 for both placebo and 300 mg/day groups, and n = 44 for 600 mg/day group) was

targeted. Differences against placebo in the change from baseline in mean weekly pain score were estimated at −1.0 for 300 mg/day and −1.6 for 600 mg/day with common standard deviation of 2.4 based on the results of Western studies [14,16,17]. To detect the difference between placebo and 300 mg/day with at least 90% power, 130 patients for each group were required. In addition, to show the superiority of 600 mg/day to placebo with almost 90% probability together with detection of the difference between placebo and 300 mg/day groups, 3:3:1 randomization ratio was selected.

All patients who received at least one dose of study drug with baseline and at least one postbaseline efficacy measurement were included in the efficacy analyses. The primary comparison was pregabalin 300 mg/day vs. placebo with respect to change from baseline in mean weekly pain score. An analysis of covariance (ANCOVA) model with treatment and CL_{cr} stratum as factors and baseline mean weekly pain score as a covariate was used for the comparison. The key secondary analysis was to compare 600 mg/day with placebo. Significance level for testing the treatment effect was 0.05. No adjustments were made for multiplicity, since all comparisons, except for a single primary comparison, were considered secondary and were used to support the findings of the primary analysis. The same ANCOVA model as the primary analysis was used for secondary analyses of continuous data. The Cochran–Mantel–Haenszel chi-square test was used for secondary analyses of categorical data.

All patients who received at least one dose of study drug were included in the safety analyses. Safety end-points were summarized descriptively by treatment group, and no inferential analyses were conducted.

Results

Patient disposition/demographics

In total, 317 patients were randomized to groups of placebo (n = 136), 300 mg/day (n = 136) or 600 mg/day (n = 45). The analysis set for efficacy and safety included 314 patients. Three of the randomized patients discontinued the study before taking study drug owing to protocol violations. Patients were well matched on demographic and baseline characteristics (Table 1) [22]. Eighty-four per cent of patients (placebo, n = 119; 300 mg/day, n = 114; 600 mg/day, n = 32) who were treated completed the study, while 16 patients (11.8%) in the placebo group, 20 patients (14.7%) in the 300 mg/day group and 13 patients (28.9%) in the 600 mg/day group withdrew from the study. The most common reason for discontinuation was treatment-related adverse events, which occurred in six patients (4.4%) in the placebo group, 10 patients (7.5%) in the 300 mg/day group and 12 patients (26.7%) in the 600 mg/day group. In the placebo group, seven patients (5.2%) discontinued owing to lack of symptom improvements.

Table 1 Demographic characteristics

	Placebo (n = 135)	Pregabalin 300 mg/day (n = 134)	Pregabalin 600 mg/day (n = 45)
Women, n (%)	32 (23.7)	32 (23.9)	13 (28.9)
Age (years)			
Mean (SD)	61.3 (9.6)	61.3 (10.3)	62.2 (10.3)
Range	35–85	35–85	41–78
Weight (kg)			
Mean (SD)	64.9 (12.8)	65.9 (12.7)	65.3 (13.1)
Range	41–104	31–113	37–105
Estimated CLcr at screening (ml/s)			
Mean (SD)	1.62 (0.62)	1.65 (0.58)	1.55 (0.46)
Diabetes type, n (%)			
Type 1	5 (3.7)	6 (4.5)	1 (2.2)
Type 2	130 (96.3)	128 (95.5)	44 (97.8)
HbA_{1c} values (%) (JDS)*			
Mean (SD)	7.2 (1.1)	6.9 (1.1)	7.2 (1.1)
Range	4.9–10.2	4.6–10.1	4.7–9.6
HbA_{1c} values (%) (NGSP)†			
Mean (SD)	7.6 (1.1)	7.3 (1.1)	7.6 (1.1)
Range	5.3–10.6	5.0–10.5	5.1–10.0
HbA_{1c} values (mmol/mol)‡			
Mean (SD)	59 (12)	57 (12)	60 (12)
Range	34–92	31–91	32–86
Duration of Type 1 diabetes (years)			
Mean (SD)	12.5 (8.9)	13.8 (9.1)	15.7
Median	9.9	12.7	15.7
Range	3.3–23.9	4.5–26.9	—
Duration of Type 2 diabetes (years)			
Mean (SD)	14.0 (9.1)	13.3 (10.1)	14.5 (9.6)
Median	13.1	11.1	12.5
Range	1.2–40.6	1.1–56.6	1.0–40.0
Duration of peripheral neuropathic pain (years)			
Mean (SD)	4.2 (3.1)	4.3 (3.6)	4.5 (3.9)
Median	3.4	3.1	2.9
Range	1.0–19.5	1.1–20.8	1.1–15.4
Baseline mean pain score			
Mean (SD)	6.1 (1.4)	6.0 (1.4)	6.1 (1.3)
Median	6.0	5.9	6.1
Range	4.0–9.4	4.0–9.9	4.0–9.3

CL_{cr}, creatinine clearance; HbA_{1c}, glycated haemoglobin; JDS, Japan Diabetes Society; NGSP, National Glycohemoglobin Standardization Program.

* HbA_{1c} values (%) measured using the standard substance by JDS, and these are 0.4% lower than those by NGSP.

† The value for HbA_{1c} (%) is estimated as an NGSP equivalent value (%) calculated by the formula HbA_{1c} (%) = HbA_{1c} (JDS) (%) + 0.4%, considering the relational expression of HbA_{1c} (JDS) (%) measured by the previous Japanese standard substance and measurement methods for HbA_{1c} (NGSP) [22].

‡ HbA_{1c} values (NGSP equivalent value) are converted to the HbA_{1c} values (mmol/mol) (International Federation of Clinical Chemistry and Laboratory Medicine, IFCC).

Primary efficacy measure

Treatment with pregabalin at 300 and 600 mg/day resulted in statistically significant reductions in mean pain scores from baseline to study end-point compared with placebo (Fig. 1a). The difference from placebo was −0.63 [95% confidence interval