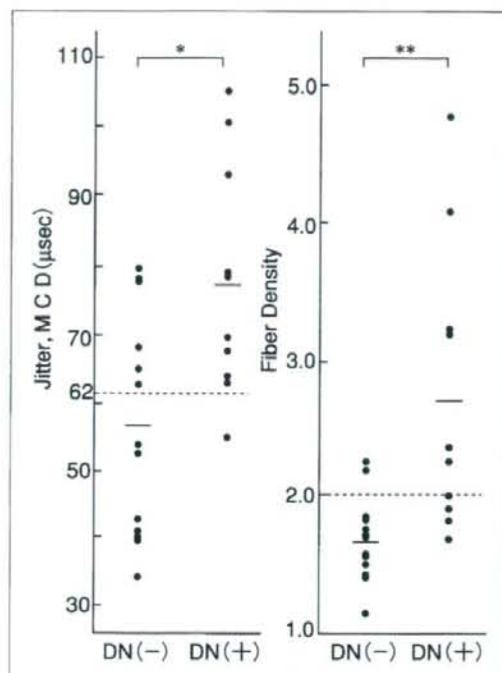

図3 糖尿病性神経障害を有する患者〔DN(+)〕と有さない患者〔DN(−)〕の単一筋線維筋電図によるmean consecutive difference(MCD)とFiber density

それぞれ2つの活動電位の間隔の差の平均と同一神経支配下の筋線維の平均数を示し，両者ともにニューロパチーで増加する．とくに後者は障害後の神経再支配を示す．

* : $P<0.01$, ** : $P<0.05$ by Student's t-test

7testsであろう[15]．これは下肢神経障害スコアNeuropathy Impairment Score of Lower Limbsに伝導速度5項目，振動覚検査および心拍変動による自律神経機能検査を加えて7項目の検査によって評価するもので，検査値の95〜99パーセンタイルに1, 99〜99.9パーセンタイルに2, 99.9パーセンタイル以上に3を配して，総計スコア4.5(健常者の97.5パーセンタイル)以上を神経障害ありとする．これをgold standardとすると，しばしばgold standardとして用いられる神経伝導検査(2神経以上で異常)の感度は81%，特異度は91%となる[16]．「糖尿病神経障害を考える会」では，簡便で信頼性の高い診断基準として簡易診断基準を検討してきたが，実地臨床で使用でき，臨床試験のDN患者のスクリーニングにも有用であることがわかってきた．131例の2型糖尿病患者を対象とするわれわれの集計では，神経伝導検査をgold standardとして感度68%，特異度74%であった[17](図4)．この数字は，実地臨床で求められる簡便さや迅速性などを考慮すると，ある程度満足できる値ではないかと考えられる．

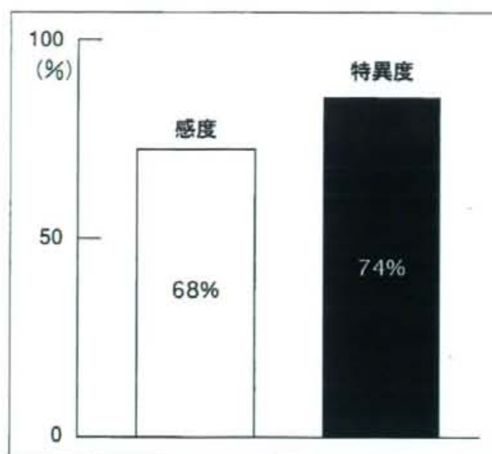

図4 簡易診断基準の感度と特異度

神経伝導検査を至適基準として131名の糖尿病患者を対象として検索した．

2. 診断基準に求められるもの

DPの診断を網膜症や腎症のように1つの物差しで決めることはできない．換言すると，絶対的な物差しがないので，有用な物差しの選択に迷うことになる．結局，万人から支持される基準を確立しようとしても十分なコンセンサスが得られないという現実がある．DNを考える会の提唱する簡易診断基準は，①糖尿病性多発神経障害に基づくと考えられる症状，②アキレス腱反射の低下・消失，③内踝での振動覚低下の3項目のうち2項目以上が陽性(すべて両側性)をDPとするものであり，検証により簡便で有用であることがわかっている．この3項目はいままで診断基準に使用されてきた項目のうちもっとも多用されてきたものを用いている．しかし，客観性には多少欠けるのは否めない．あえて，客観性の高い検査を1つあげるとすると神経伝導検査であろう．また，近年，糖尿病には至らない耐糖能異常の状態でDPとくに疼痛を伴う感覚性神経障害が起こりうることが報告されており[18]，このような病態では神経伝導検査では検出できない痛覚や冷覚を伝える小径線維に病変が起こっているので神経伝導検査では異常を検出

することが困難である．そこで，小径線維の病変を検出する方法として，この線維の終末が分布する皮膚の生検が脚光を浴びており，場合により，診断の一助として試みられる場合もありうる．日常診療では，このような診断基準を根拠にして，原因療法薬を開始する．しかし，この後の治療指針に確立したものはない．これは，有効と判断される治療薬が限られているのが大きな要因であり，また，末梢神経は非可逆性の強い組織であるため，感度・特異度ともに高い確定診断基準による判定に基づく治療開始では手遅れになってしまう可能性がある．いずれにしても，DPが進展するにつれ治療法は，対症療法が主体となっていくが，この際，なんらかの目安となる基準としては病期分類が該当すると思われる．

3．簡易診断基準の長所と短所

本基準のもっとも強調される長所は簡便性であり，その割には診断率が高いことである．神経障害に対する認識はまだ低く無症候性のDPが数多く存在すること，反射手技の再教育など，改めて3項目の内容について検査手技を含めた認識を強化することが望まれる．また，基本的に日常診療で簡易診断基準の成績により治療開始を考慮するなどしても，大きな問題はないと考える．むしろ，末梢神経は非可逆性の強い組織であるため，感度・特異度ともに高い確定診断基準による判定に基づく治療開始では手遅れになってしまう可能性があり，早期診断の見地からは感度優先の基準が望ましい．一方，本基準は診断精度の点で多少低いのは否めない．そのため，臨床研究レベルの有病率の推移や治療効果の判定などには，他の検査を併用して評価することが望ましい．すでに述べた耐糖能異常で発症するとされる"疼痛を伴う感覚性神経障害"のような"小径線維ニューロパチー"をDPの亜型と考えると，このような病型の検出には簡易診断基準は向いてない．もっとも，このような病型に関する検討はわが国では十分実施されていない．

おわりに

DPの検査法や診断基準の開発の究極的な目的は，検査や診断基準を用いて早期に診断してDPの進展を阻止することである．しかし，診断基準は感度・特異度ともに高いことが要求されるものであり，不可逆性の大きい末梢神経では簡易診断基準を用いても，診断しうるときには不可逆になっている可能性が高い．治療効果を優先して考えると感度を優先した早期診断が理にかなっているかもしれない．しかし，現段階では確立した基本的治療薬は開発されておらず，早期診断しても直ぐに根本的治療に生かされるわけではない．

しかし，多くの危険因子が明らかにされており予防的方策が可能である．一方，予防的戦略を有用なものにするためにはDPの自然史に関する深い理解が必要であり，これは検査方法や診断基準に精通することと表裏一体であり，これらの新しい展開に期待したい．

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Diabetes Research and Clinical Practice 77S (2007) S178–S183

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Rationale and usefulness of newly devised abbreviated diagnostic criteria and staging for diabetic polyneuropathy

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Accepted 29 January 2007
Available online 2 May 2007

Abstract

In order to establish a diagnostic criteria for diabetic polyneuropathy (DP) for daily practice, usefulness of the abbreviated diagnostic criteria proposed by Diabetic Neuropathy Study Group in Japan was examined in 131 diabetic patients in admission and outpatient clinic. The prerequisite condition includes: (1) diagnosed as diabetes and (2) other neuropathies than diabetic neuropathy can be excluded. The criteria should meet any of the following three items: (1) sensory symptoms considered to be due to DP, (2) bilaterally decreased or absent ankle reflex and (3) decreased vibratory sensation in bilateral medial malleoli. Using this criteria, sensitivity (68%) and specificity (74%) were obtained by evaluating nerve conduction study as gold standard, suggesting usefulness of the criteria for diagnosis of DP especially for daily practice. Staging of DP is now sought to establish the consensus for the specific therapy for its stage. Thirty-one diabetic patients in admission was evaluated to examine usefulness of the newly devised staging system of DP. Staging was almost consistent between the new staging system and Dyck's staging (gold standard) and nerve function deteriorated with increasing stage, suggesting that usefulness and rationale of this staging system is well substantiated.
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Keywords: Diagnostic criteria; Diabetic polyneuropathy; Staging of diabetic neuropathy; Ankle jerk; Vibration sensation

1. Introduction

Diabetic patients suffer from diabetic microvascular as well as macrovascular complications. Among macrovascular complications, cerebrovascular and cardiovascular diseases are the major causes of death in diabetes and more frequent in diabetic patients than in non-diabetic subjects. Microvascular complications, retinopathy and nephropathy, are the leading causes of blindness and hemodialysis, respectively. Diabetic neuropathy alone and in combination with peripheral vascular diseases is the leading cause of limb amputation. It is also known that diabetic patients with autonomic neuropathy has poor prognosis and sometimes suffer from sudden death associated with cardiovascular autonomic involvement. Thus, diabetic neuropathy, more precisely sensory/autonomic polyneuropathy (DP) [1], extensively disturbs patients' QOL. DP is the most frequent microvascular complication of diabetes and develops earliest among those

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doi:10.1016/j.diabres.2007.01.053

complications. Peripheral nerve lesion tends to become irreversible even at the early stage. Thus, its treatment and care starting as early as possible are essential. For this purpose, early diagnosis is inevitable. However, we do not have simple and useful diagnostic criteria of diabetic neuropathy for daily practice, although excellent research-oriented criteria have been proposed [2–4].

After diagnosis of DP, we usually start treatment with antipathogenic drugs. With increasing duration of the disease, the treatment should be changed and basically depend on its stage. Thereby, the satisfactory treatment can be achieved. Thus, staging of DP is very important especially in terms of therapeutic strategy. In addition, for common understanding about the state of DP and for accessing and comparing epidemic data on DP among communities or countries, it is needed to establish the staging of DP. Several staging systems have been introduced and well verified [2–5]. Similar to the diagnostic criteria, however, staging system should be also simplified for practical use.

Taken together, the aim of this study is in an attempt to examine the usefulness of the abbreviated diagnostic criteria and staging for DP for practical use, both of which have been proposed by Diabetic Neuropathy Study Group in Japan [6,7].

2. Materials and methods

2.1. Patient selection and clinical profiles

For the study to construct abbreviated diagnostic criteria, 91 diabetic patients who were in admission and 40 diabetic patients in outpatient clinic in 1999 and for the study to construct staging of DP, 31 diabetic patients who were in admission in 2002 in Divisions of Neurology and Endocrinology and Metabolism of the Department of Medicine, Shiga University of Medical Science [8]. All patients were clinically diagnosed to have type 2 diabetes and had neurological examinations and tests as mentioned below. They were asked to fill out questionnaires consisted 62 items including history of diabetes and vascular events and neuropathic symptoms in accordance with "Neuropathy Screening Instrument Questionnaire" devised by Feldman et al. [9]. After this screening, we had an interview with the patients about their neuropathic symptoms if they were really neuropathic or not.

2.2. Diagnostic criteria for diabetic polyneuropathy

All patients were neurologically examined by one or more neurologists as well as diabetologists.

DP was diagnosed by the diagnostic criteria for DP proposed by Diagnostic Neuropathy Study Group [6] (Table 1). In brief, in the absence of peripheral neuropathies other than diabetic neuropathy in diabetic patients, DP is diagnosed by two or more abnormalities of three neurological examination items. (1) Positive or negative sensory symptoms (bilateral): the patients who have one or more scores in the questionnaire on sensory symptoms were asked if they have bilateral symptoms like numbness, pain, dysesthesia, paresthesia and allodynia in bilateral tips of toes or bottoms of feet; (2) decreased or absent ankle reflex (bilateral); (3) decreased vibratory sensation on bilateral medial malleoli evaluated by C128 Hz tuning fork.

On using this criteria, neuropathic symptoms of DP should exist bilaterally. Unilateral symptoms should be considered mononeuropathy and/or radiculoneuropathy first. Ankle reflex is better examined at a kneeling position. Loss of reflex should be judged only at this position even by reinforcement procedure. A C128 tuning fork was used for evaluation of vibration sense. The time patients feel vibratory sensation is evaluated. Less than 10 s was considered decreased, although this is just a standard and the examiner should keep in mind that the time usually decreases with age.

Table 1
Abbreviated diagnostic criteria for distal symmetric polyneuropathy proposed by Diabetic Neuropathy Study Group in Japan (original version was made in 2004 and revised in 2005)

Prerequisite condition
Must meet the following two items
1. Diagnosed as diabetes
2. Other neuropathies than diabetic neuropathy can be excluded

Criteria
Meet any two of following three items
1. Presence of symptoms considered to be due to diabetic polyneuropathy
2. Decreased vibration in bilateral medial malleoli
3. Decrease or disappearance of bilateral ankle reflex

Notes
1. "Symptoms considered to be due to diabetic polyneuropathy" include following
(1) Bilateral
(2) Numbness, pain, paresthesia or decreased sensation in the tips of toes and bottom of feet
Meet above two items
Exclude symptoms in only upper extremities or only cold sense in the cases with peripheral vascular disease
2. Ankle reflex is examined on standing position on the knees
3. Decreased vibration sense is considered $\leq$10 s by 128 Hz tuning folk
4. Take age into consideration in elderly subjects

Reference item
If either one of following reference items is met, even if above criteria are not met, diabetic polyneuropathy is diagnosed
1. Presence of any abnormality (nerve conduction velocity, amplitude or latency) in two or more nerves in electrophysiological test
2. Presence of clinically apparent diabetic autonomic disturbance (it is desirable to confirm obvious abnormality by autonomic function test)

Table 2
A close correlation between new staging system and Dyck's staging

Dyck's staging	New staging system				
	I	II	III	IV	V
0	11				
1		6			
2			10	2	
3					2

2.3. Staging of diabetic polyneuropathy

Staging of DP was determined by the staging system recently proposed by Diabetic Neuropathy Study Group [7]. An outline of proposed staging of DP is presented in Table 2. The bottom line of this staging is based on the speculative concept that DP undergoes a progressive nerve fiber loss and symptomatologically occurs as sensory neuropathy, accompanied by autonomic neuropathy, and is eventually complicated with motor neuropathy. The stages are divided into five. Stage I is no neuropathy, which does not meet the diagnostic criteria [6]. Stage II is asymptomatic neuropathy in which patients do not have subjective symptoms but meet the diagnostic criteria. At stage III, subjective symptoms are positive and either of ankle reflex or vibration sense is possible to be normal. Overall, patients suffer mainly from sensory neuropathy. Autonomic neuropathy clinically manifests at stage IV. Motor neuropathy appears at stage V (Table 2).

2.4. Nerve function tests

All patients had nerve conduction studies including *F*-waves examination using Sapphire 4ME (Medelec). Most patients had autonomic function tests including coefficients of variation of R–R intervals of the electrocardiogram. In addition, blood pressure was monitored in a supine position was well as 3 min after standing with 1-min intervals using an automatic sphygmomanometer.

3. Results

3.1. Diagnostic criteria for diabetic polyneuropathy

This abbreviated diagnostic criteria is able to diagnose DP approximately 68% in sensitivity and 74% in specificity by using "nerve conduction criteria" [2] as gold standard.

3.2. Staging of diabetic polyneuropathy

Among 31 diabetic patients, 11 patients were classified into stage I, 6 into stage II, 10 into stage III, 2 into stage IV and 2 into stage V (Table 3). Eleven

Table 3
Staging of diabetic polyneuropathy proposed by Diabetic Neuropathy Study Group in Japan

Staging[a]	Conditional criteria		Sensory[b] (hypesthesia[c])	Autonomic[b] (OH, abnormal sweating, severe diarrhea and constipation)	Motor[b] (weakness/ atrophy)	Note 1 (disturbance[d] of QOL)	Note 2 (abbreviated diagnostic criteria)
	Subjective[e] symptoms	ATR (−)/(+/−) vibration ↓					
I (no neuropathy)	(−)~1			(−)		(−)	Does not meet
II (asymptomatic)	(−)	(+)	(−)	(−)	(−)	(−)	Meets
III (symptomatic early)	(+)	(+)[f]	(+)	(−)	(−)	(−)~Mild	
IV (symptomatic middle)	(+)	(+)	(+)	(+)	(−)	Mild~Moderate	
V (symptomatic late)	(+)[g]	(+)	(+)	(+)	(+)	Severe	

The bottom line of this staging is based on the speculative concept that diabetic polyneuropathy undergoes a progressive nerve fiber loss and symptomatologically manifest as sensory neuropathy, accompanied by autonomic neuropathy, and is eventually complicated with motor neuropathy.

[a] Transient sensory symptoms may develop at any stage.
[b] Progresses with increasing stage.
[c] Objective superficial sensation should be tested with monofilament, toothpick, etc.
[d] Definition of the degree in disturbance of QOL is based on SF36.
[e] Bilateral numbness, spontaneous pain, paresthesia, decreased sensation, etc.
[f] May meet one of two items.
[g] No positive symptoms is possible.

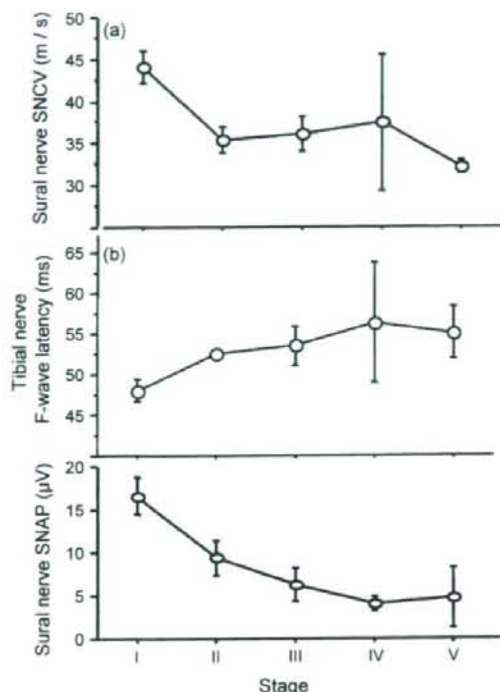

Fig. 1. Nerve function including *F*-wave latency of the tibial nerve and sensory nerve action potential of the sural nerve deteriorates with stage of diabetic polyneuropathy.

patients at stage I all corresponded to stage 0 by Dyck' staging [5]. All of six patients at stage II corresponded to stage I by Dyck's staging. Ten patients at stage III and two patients at stage IV corresponded to stage II by Dyck's staging. Two patients were classified into stage V by new staging and into stage III by Dyck's staging. Thus, two staging systems were closely linked.

3.3. A close association between nerve function and staging

Sural nerve conduction velocity overall decreased with stage, although no difference was demonstrated between stages II and IV (Fig. 1a). Tibial nerve *F*-wave latency increased with stage (Fig. 1b) and sensory nerve action potential of the sural nerve decreased with stage (Fig. 1c) in spite of no difference between stages IV and V.

4. Discussion

The incidence of DP has been variously reported. This variety may have been attributed in part either to relatively small number of patients enrolled in many researches or to different ages of examined patients. However, inconsistency of diagnostic criteria for DP may be most responsible. Among many diagnostic criteria, most reliable criteria may be the ones that have been reported by Dyck and coworkers. The original criteria was substantiated to reflect index of pathology of the sural nerve, which is considered best pathological marker of peripheral neuropathy [2]. Now, the new version has been introduced from a longitudinal study about DP in Rochester, MN, USA [3]. However, each item of a series of their criteria is unlikely to be easily examined in daily practice because of necessity of specific technique or equipment. Although other criteria like the Michigan Diabetic Neuropathy Score [9] may be useful one, we still prefer more simplified criteria just for daily practice. For the purpose of daily use, Diabetic Neuropathy Study Group in Japan have proposed simplified diagnostic criteria for distal symmetric polyneuropathy due to diabetes mellitus [6,7].

In the present study, we demonstrated that abbreviated diagnostic criteria for DP was useful in that satisfactory diagnostic rate was able to be achieved even by simple procedures. Each of three items of the present criteria has been frequently used as a composite item of diagnostic criteria for DP. Pirart used decreased tendon reflexes and vibratory sensation [10]. Partanen et al. used nerve conduction data and neuropathic symptoms [11]. DCCT used objective neuropathic symptoms, abnormal sensation and decreased reflexes [12]. When the composite score (NIS (LL) + seven tests) which has been used in Rochester Diabetic Neuropathy Study and has been well rationalized was used as the gold standard [3], abnormal ankle reflex alone showed 60.3% in sensitivity and 90.5% in specificity and abnormal vibration sensation alone showed 17.2% in sensitivity and 96.4% in specificity [13]. Each test alone showed relatively low sensitivity. We already know the diagnostic usefulness of neuropathic symptoms even when it is used alone especially in terms of sensitivity (unpublished data). Thus, to improve diagnostic usefulness, neuropathic symptoms was added to the item of the criteria. On the other hand, nerve conduction study was taken as gold standard in the present study. It was reported that the minimal criteria of abnormal nerve conduction in two or more nerves showed 81% in sensitivity and 91.2% in specificity [13] when the above-mentioned composite score that has been introduced from long-term experience from clinical trials [3,4] was used as gold standard, suggesting that nerve conduction study that used as the gold standard in the present study is a highly reliable criteria for DP.

The present criteria do not include any neurological examination representative of small nerve fiber function. It may be desirable that neurological examination is able to evaluate both large and small fiber functions. However, two functions usually deteriorate in parallel in DP. In fact, nerve conduction velocity is overall associated with decreased pain sensation and coefficient of variation of R–R interval, suggesting that large fiber function may reflect small fiber function. In addition, neuropathic symptoms, one of the three items of the criteria, may represent part of small nerve fiber function. On the basis of daily practice, the absence of neurological examination showing small nerve fiber function in diagnostic procedure may well be rationalized, although it is necessary to consider the presence of small fiber neuropathy at any time.

We also proposed the new staging system for DP. The staging system about diabetic neuropathy was devised by Dyck and coworkers in 1988 [5]. At the present time it has been modified. In the original staging no neuropathy is referred if one item of the four examinations including neuropathic symptom score, neurological disability score, nerve conduction study, and sensory examination is abnormal. Later, autonomic function was included in the items of criteria. Two or more abnormalities are diagnosed "the presence of neuropathy". The absence of neuropathic symptoms in the setting with two abnormalities corresponds to asymptomatic neuropathy, whereas their presence corresponds to symptomatic neuropathy. Disabling neuropathy is literally disabling neuropathic symptoms. This staging system is really useful if this is appropriately used. However, each examination is not so easily done. Either neuropathic symptom score and neurological disability score is somewhat time-consuming in daily practice and may need some technical skills about neurological examination. Computer-aided sensory examination needs special equipment. This staging system is very useful for research-oriented clinical trials. However, only a few institutes are able to do this. Thus, simple staging system is really requested by many diabetologists and general physicians. For this sense, simple staging system proposed by Boulton et al. [14] may well be useful. However, using this staging, many patients are classified into clinical neuropathy (stage II). This stage may be divided into at least two stages from many aspects. More staging may enable more meticulous management for each stage. This was considered in the present staging system.

The abbreviated criteria for polyneuropathy was used for the framework of the present staging system: the presence or absence of neuropathy was determined by the criteria and asymptomatic neuropathy was diagnosed if two or three items of the criteria were met in the absence of neuropathic symptoms. Time-consuming procedures or examinations requiring special equipment were omitted in view of practical usefulness. QOL was included because it is absolutely necessary for assessment of the disease state. When practically used, this staging system was well correlated with the Dyck's staging as gold standard, suggesting the usefulness and rationale of the present staging system. Especially, the observation that sensory nerve action potential of the sural nerve decreased parallely with stage may justify the underlying concept of this staging that the pathological basis of DP is progressive nerve fiber loss in that the amplitude may reflect number of myelinated nerve fibers. Although we are still seeking to examine the usefulness of these staging systems from every aspect, several institutes have reported its reliability and the data supporting the underlying notion of this staging system that DP initially occurs as sensory neuropathy, accompanied by autonomic neuropathy, and is eventually complicated with motor neuropathy (in personal communication).

In conclusion, the abbreviated diagnostic criteria of DP newly devised by Diabetic Neuropathy Study Group in Japan is able to be easily used and useful for daily practice and for patient selection for clinical trials. Combined with this diagnostic criteria, new staging system of DP is able to be also practically used for managing diabetic patients with DP. However, the final evaluation awaits further nationwide experiences.

Acknowledgements

This paper was presented in 13th Korean–Japan Symposium on Diabetes Mellitus. This study was supported in part by the Research Grants 16B-1 for Nervous and Mental Disorders from the Ministry of Health and Welfare.

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Neuroscience Letters 434 (2008) 266–272

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Isolation of specific peptides that home to dorsal root ganglion neurons in mice

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Received 30 October 2007; received in revised form 17 January 2008; accepted 25 January 2008

Abstract

We isolated peptides that home to mouse dorsal root ganglion (DRG) from a phage library expressing random 7-mer peptides fused to a minor coat protein (pIII) of the M13 phage. An *in vitro* biopanning procedure yielded 113 phage plaques after five cycles of enrichment by incubation with isolated DRG neurons and two cycles of subtraction by exposure to irrelevant cell lines. Analyses of the sequences of this collection identified three peptide clones that occurred repeatedly during the biopanning procedure. Phage-antibody staining revealed that the three peptides bound to DRG neurons of different sizes. To determine if the peptides would recognize neuronal cells *in vivo*, we injected individual GST-peptide-fusion proteins into the subarachnoid space of mice and observed the appearance of immunoreactive GST in the cytosol of DRG neurons with a similar size distribution as that observed *in vitro*, indicating that the GST-peptide-fusion proteins were recognized and taken up by different DRG neurons *in vivo*. The identification of homing peptide sequences provides a powerful tool for future studies on DRG neuronal function *in vitro* and *in vivo*, and opens up the possibility of neuron-specific drug and gene delivery in the treatment of diseases affecting DRG neurons.

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Keywords: DRG; Phage display; Neuropathic pain; Biopanning; Neuropathy

Phage display is a powerful technology to identify peptide sequence motifs that target a particular tissue or cell type in the body [2,8,23]. Coupling such peptides to drugs and genes would enable their targeted delivery to specific cells and tissues *in vitro* and *in vivo* [8,18,21,25]. A commonly used platform is the combinatorial filamentous M13 phage library that displays short random peptides fused to a minor coat protein (pIII) [23], which can be used to isolate specific cell type-binding peptides by a procedure called biopanning [18]. In this study, we have applied the technology to identify peptide motifs that recognize, and are specifically taken up by, neurons in the dorsal root ganglion (DRG) in mice.

DRG neurons are potential target cells for the treatment of diseases of the peripheral sensory nervous system [22]. For instance, neuropathic pain is a common symptom in various disorders, including metabolic abnormalities, malignancies, physical injuries, toxins and poisons, and hereditary diseases [22], and is the cause of much morbidity and misery. Although various pharmaceutical agents, anesthetics, surgical operations, or procedures such as transcutaneous electrical nerve stimulation, have been used to treat the symptoms of neuropathic pain [16], such palliative treatments are mostly non-targeted and of limited efficacy [16]. DRG neurons are the primary afferent neurons and can be classified into two broad groups: large and small neurons. Large neurons are thought to be involved mainly in proprioception, while most small neurons are involved in

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doi:10.1016/j.neulet.2008.01.062

nociception [26]. Although both neuronal populations have also been subclassified by biochemical and histological methods, such as lineage tracing or immunostaining of different markers (neurotransmitters, cell surface carbohydrates), much work is needed before the physiological functions of the different neuronal subtypes are clearly defined [4,9,14,19], a deficiency that limits our understanding of various pathophysiological processes [14], as well as our ability to develop novel therapeutics for disease processes affecting the peripheral sensory nervous system [5].

In this study, we have isolated three peptides that recognize specific, defined sizes of DRG neurons. These peptides will be powerful tools for research into the structure and function of subpopulations of DRG neurons, and for developing new therapies for diseases involving specific subtypes of DRG neurons.

Adult C57BL/6 mice aged 8–12 weeks were used in all experiments, and were housed in an animal room with a 12-h light and 12-h dark cycle in an illumination-controlled facility. All experiments were conducted with the approval of the Research Center for Animal Life Science at Shiga University of Medical Science. A phage library expressing random 7-mer peptides fused to a minor coat protein (pIII) of the M13 phage, at a complexity of about 1.3×10^9 independent sequences, was purchased from New England BioLabs (The Ph.D.-C7C Phage display Peptide Library kit, Beverly, MA). Rabbit anti-fd bacteriophage antibody was purchased from Sigma–Aldrich Corp. (St. Louis, MO). Donkey FITC-labeled anti-rabbit IgG was purchased from Chemicon (Temecula, CA). The three synthetic peptides, DRG1: SPGARAF, DRG2: DGPWRKM, DRG3: FGQKASS, were supplied from Yanaihara Institute (Shizuoka, Japan). Various cell lines; mouse neuroblastoma cells (Neuro-2a), human embryonic kidney epithelial cells (HEK-293), opossum kidney cells, were purchased from American Type Culture Collection (Manassas, VA) for use in a subtractive panning protocol. These cultured cells were maintained in DMEM medium with 10% fetal calf serum (FCS, Invitrogen, Gaithersburg, MD) with antibiotics at 37 °C in 5% CO_2.

Mouse DRG neurons were isolated from lumbar segments L1–L5 of C57BL/6 mice using an enzymatic procedure described previously [24]. The neurons were purified with 30% percoll (MP Biomedicals, Solon, OH), washed twice with Ham's F12 medium supplemented with 10% fetal calf serum (Invitrogen), and then plated on poly-L-lysine (Sigma)-coated 10 cm dishes for *in vitro* biopanning or on 8-well Lab-Tek Chamber Slides (NUNC, Naperville, IL) for *in vitro* phage binding. The cells were allowed to attach at 37 °C for 12 h in 5% CO_2.

The *in vitro* phage display library screening protocols described by Hong and Clayman [6] were employed with minor modifications. Briefly, after washing isolated DRG neurons three times with PBS, the culture medium was changed FCS-free DMEM medium. Then, 1×10^{10} plaque-forming units (pfu) of the M13 phage library were added to isolated DRG neurons (1×10^6/well), and incubated at 37 °C in 5% CO_2 for 10 min. After washing three times, the bound phages were recovered by grinding the cells with protease inhibitor in DMEM medium. Aliquots were diluted appropriately and infected *E. coli* ER2738, mixed with Agarose Top, and incubated in LB/IPTG/X-gal plates at 37 °C overnight. After titrating plaque-forming units, phages in the aliquot were further amplified by infecting *E. coli* with them in a shaker at 37 °C for 4.5 h. Phages from the *E. coli* were purified, and $1 \times 10^{10-11}$ pfu were added to isolated DRG neurons again. In all, we carried out five such rounds of screening with DRG neurons. To select more specific phages for DRG neurons, a subtractive panning protocol [17] against irrelevant cell lines (Neuro-2a, HEK-293, and opossum kidney cells from American Type Culture Collection, Manassas, VA) was performed twice. These cultured cells were maintained in DMEM medium with 10% fetal calf serum (FCS, Invitrogen, Gaithersburg, MD) with antibiotics at 37 °C in 5% CO_2. To avoid the selection of polystyrene and poly-L-lysine-specific peptides, a subtractive panning protocol against poly-L-lysine culture dishes was also performed twice [1].

In vitro phage binding to DRG neurons was carried out on cultured neurons in 8-well chamber slides following three washes with PBS to clear the cell surface ($n = 1 \times 10^5$ cells/well). Cells were incubated at 37 °C for 1 h with either the three selected phages or control library phage at 2×10^9 pfu/well (1×10^{10} pfu/ml), with or without synthetic peptides (10^{-4} or 10^{-6} M; DRG1: SPGARAF, DRG2: DGPWRKM, DRG3: FGQKASS from Yanaihara Institute, Shizuoka, Japan), which had seven amino-acid sequences homologous to the selected phages. Subsequently, unbounded phages and/or peptides were removed by washing the chamber slides three times with PBS. To detect phages bound to the cells, the cultured cells were fixed with 4% paraformaldehyde and incubated for 1 h with rabbit anti-fd antibody (diluted 1:1000 in PBS with 3% donkey serum, Sigma–Aldrich Corp., St. Louis, MO), washed three times with PBS, and subsequently incubated with FITC-conjugated donkey anti-rabbit IgG diluted 1:1000 for 1 h (Chemicon, Temecula, CA). After three washes, the cells were treated with propidium iodine (PI) for nuclear staining, and then observed under a confocal laser-scanning microscope (LSM510, Carl Zeiss, Jena, Germany). For quantitative analysis, we photographed ten randomly selected fields in each well and measured diameters (long and short) of the cells positive for phage immunostaining and PI staining. The mean cell size was calculated as the mean of the long and short diameter, and cell-size differences were evaluated in different groups.

To evaluate their binding to DRG cells *in vivo*, GST-fusion proteins of three selected peptides were synthesized using the pGEX4T-1 vector kit (Amersham, Piscataway, NJ) according to the manufacturer's instructions. Briefly, oligonucleotides for C-DRG1-C, C-DRG2-C, and C-DRG3-C were synthesized cloned into the pGEX4T-1 vector, and transformed in *E. coli*. The synthesized GST-fusion proteins were pull down by Glutathione Sepharose 4B (Amersham), and purified. The purities and reactions to anti-GST antibodies (Amersham) were confirmed by gel-electrophoresis stained by Coomassie Blue and anti-GST immunoblotting.

Under intraperitoneal pentobarbiturate anesthesia (0.1 mg/g BW), 10 μl (500 μg/ml) of purified GST-fusion proteins (GST-DRG1, GST-DRG2, GST-DRG3, or GST alone) were injected into the subarachnoid space of 8-week-old female C57BL/6 mice ($n = 3$ each). After 1 h, the mice were sacrificed

and transcardially perfused with cold saline followed by 4% paraformaldehyde, 0.5% glutaraldehyde and 0.3% picric acid in 0.1 M PBS (pH 7.4). The L5 DRGs were then isolated and cut sectioned (10 μm) for fluorescence immunohistochemistry. After blocking with 10% horse serum, the sections were incubated overnight at 4 °C with goat anti-GST antibody (Amersham) diluted 1:1000 and rabbit anti-neurofilament L (NF-L, non-phosphorylated form, Chemicon, Temecula, CA) antibody diluted 1:100 in PBST. The sections were incubated for 1 h with Alexa Fluor 488-labeled anti-goat IgG and Alexa Fluor 555-labeled anti-rabbit IgG (Molecular Probes, Eugene, OR) at room temperature, mounted with VECTASHIELD mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories Inc., Burlingame, CA), and observed under a fluorescence microscope (Axioplan 2 imaging and Axiocam, Carl Zeiss, Jena, Germany). For quantitative analysis, we took pictures of randomly selected fields and measured diameters (long and short) of the cells positive for GST, NF-L and DAPI. The mean cell size was calculated as the mean of the long and short diameters, and cell-size differences were evaluated in the three different groups.

BLAST (http://www.ncbi.nlm.nih.gov/blast/) searches of the mouse database with the phage peptide sequences were carried out to identify homologies with proteins of interest, including neuronal growth factors, cytokines, hormones, and cell adhesion molecules.

Data were analyzed by ANOVA for multiple comparisons and shown as mean ± S.D. Significance was assigned at $p < 0.05$.

We used an *in vitro* biopanning protocol, and isolated 113 phage plaques in five rounds of screening with DRG neurons and four rounds of negative selection, two against irrelevant cell lines, and two against poly-L-lysine-coated culture plates. Of the 113 plaques, 5 displayed an identical 7-amino-acid sequence (DRG1), 4 shared another 7-mer sequence (DRG2), and 3 others displayed a third shared sequence (DRG3); 17 other sequences (DRG4–DRG20) were each shared by 2 different plaques. The sequences of the other 67 clones were all different from one another (Table 1). We further analyzed the three-phage clones, DRG1, DRG2 and DRG3, since these three clones were appeared at least three times by binding to DRG neurons *in vitro*; these phage clones were designated DRG-p1, DRG-p2 and DRG-p3, respectively.

We analyzed the binding of the three different clones to isolated DRG neurons maintained in culture (Fig. 1). DRG-p1 bound to 38% of the DRG neurons (83/219), DRG-p2 to 43% (100/231), and DRG-p3 to 29% (69/235), while the control phage library bound to 8% (45/539) of the DRG neurons. The addition of peptides (10^{-4} M) with identical amino-acid sequences blocked the staining with anti-phage antibodies by 97% for DRG-p1, 94% for DRG-p2, and 94% for DRG-p3, while adding 10^{-6} M peptide blocked staining by 47% for DRG-

Table 1
Amino-acid sequence of DRG-specific 7-mer peptides and the numbers of phage plaques with identical amino-acid sequences

Name	Sequence	Counts	Name	Sequence	Counts	Name	Sequence	Counts
DRG1	SPGARAF	5	DRG30	HTTSSLY	1	DRG59	HTGPFGL	1
DRG2	DGPWRKM	4	DRG31	MGQNLRF	1	DRG60	LSTSSKK	1
DRG3	FGQKASS	3	DRG32	NLQLAPD	1	DRG61	TPPSPRT	1
DRG4	TGFQSGS	2	DRG33	SSFRGAT	1	DRG62	PALSHST	1
DRG5	DSSRTRL	2	DRG34	LHKSHLL	1	DRG63	TPSWSKK	1
DRG6	DFIRTQA	2	DRG35	APPELRL	1	DRG64	STPAVPP	1
DRG7	LKHTNEA	2	DRG36	HRTIASG	1	DRG65	NLNAHHK	1
DRG8	QGAHNNN	2	DRG37	TESIGDK	1	DRG66	QHQKQGY	1
DRG9	NPHKAPN	2	DRG38	APDETER	1	DRG67	NKTTNIM	1
DRG10	NPSLQAP	2	DRG39	KGLPPGH	1	DRG68	TSASLSS	1
DRG11	PPWSSPK	2	DRG40	PSGTPSY	1	DRG69	RSSPPNT	1
DRG12	AQSHNKL	2	DRG41	SNRSPLM	1	DRG70	SPPRPTG	1
DRG13	LPTSKKM	2	DRG42	TIGQSYR	1	DRG71	VNTPERH	1
DRG14	NHLKNPA	2	DRG43	SPTEGTP	1	DRG72	TPQYPKL	1
DRG15	TFSIGEK	2	DRG44	PLSGAPW	1	DRG73	PTLLPHQ	1
DRG16	QAIQNST	2	DRG45	DAPTHMH	1	DRG74	NNANYRL	1
DRG17	HNTNAQH	2	DRG46	TDFRSRV	1	DRG75	GPHFHQS	1
DRG18	TPSLPQT	2	DRG47	LVLPPLA	1	DRG76	PAMNSVK	1
DRG19	NMPTQRS	2	DRG48	SSSPARL	1	DRG77	GTTPTST	1
DRG20	PVRSPAV	2	DRG49	TATNTRT	1	DRG78	HNSTRGS	1
DRG21	SSQAPQS	1	DRG50	DGAGTWV	1	DRG79	DDSGPLR	1
DRG22	DAQKNMN	1	DRG51	EKHLAPR	1	DRG80	NMHPTAT	1
DRG23	GLQLSQT	1	DRG52	PLTPLGF	1	DRG81	HQNWRHT	1
DRG24	SASNTQY	1	DRG53	MTPFMGS	1	DRG82	PSTKYHS	1
DRG25	EGHLVSQ	1	DRG54	DSPGWPH	1	DRG83	PLRLAHQ	1
DRG26	SDPGNYM	1	DRG55	GERHSLT	1	DRG84	QMPGNNL	1
DRG27	ALDNVPH	1	DRG56	TTAVALR	1	DRG85	LATPLRN	1
DRG28	PTKQHAK	1	DRG57	NGLHVQR	1	DRG86	LNGLKAA	1
DRG29	TELQRHN	1	DRG58	TLSPRSA	1	DRG87	TVSSHRA	1

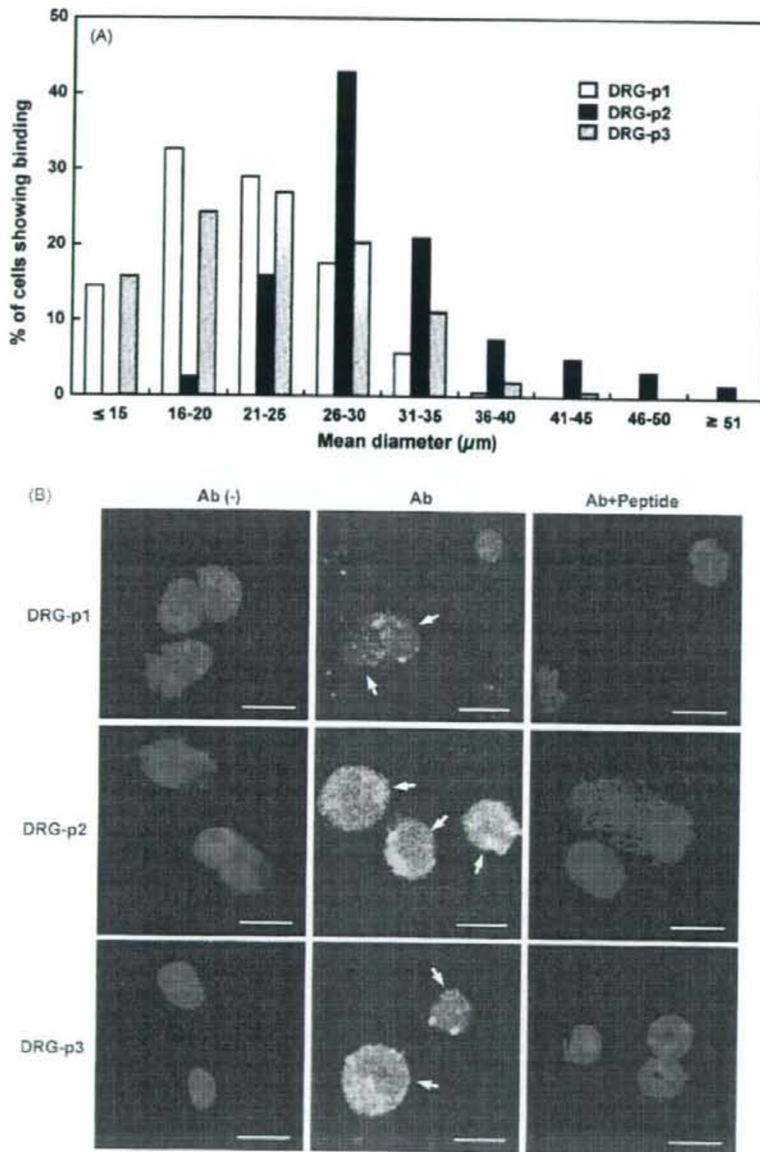

Fig. 1. Binding of three different phages to cultured-DRG neurons. The cells were analyzed by a confocal laser-scanning microscopy. (A) Cell-size distribution of the DRG neurons positive for DRG-p1, DRG-p2 or DRG-p3. (B) Bindings of the 3 different phage clones to mouse DRG neurons. Positive reactions stained with anti-fd antibody (Ab) are green. Ab (−) is without antibody and Ab + peptide is antibody plus peptides (10^{-4} M) corresponding to each phage. Nuclei were stained with PI (red). Bars, 20 μm.

p1, 54% for DRG-p2, and 53% for DRG-p3, as compared with similarly bound cultures stained with antibody alone. As each peptide was only capable of competing for the binding of the phage with an identical 7-amino-acid sequence (data not shown), the phage-neuron binding was concluded to be sequence specific (Fig. 1(B)).

We then analyzed the cell-size distribution of the DRG neurons that bound to individual phage clones. Interestingly, DRG-p1 and DRG-p3 staining occurred mainly in small-sized neurons with a mean diameter of 22.0 ± 5.3 μm (for DRG-p1) and 23.2 ± 6.7 μm (for DRG-3p), while DRG-p2 staining occurred mainly in large-sized neurons with a mean diameter

of 30.9 ± 7.0 μm (Fig. 1(A)). There was a significant difference between the mean diameter of the cells positive for DRG-p1 and that for DRG-p2 ($p < 0.001$), and of the cells positive for DRG-p2 and for DRG-p3 ($p < 0.001$), but no difference between the cells positive for DRG-p1 and DRG-p3.

To examine the DRG-targeting of peptides *in vivo*, we injected individual GST-peptide-fusion proteins (5 μg each) or GST alone into the subarachnoid space in C57BL/6 mice, isolated the DRGs, and used anti-GST immunostaining to detect the neuron-associated GST-peptide-fusion proteins. Additionally, we double-immunostained the cells against NF-L (non-phosphorylated form) antibodies, and found that essentially all (99%) cells positive for GST-peptide-fusion proteins were also positive for neurofilament (Fig. 2(B)). In DRG cells from control mice injected with GST, no anti-GST staining was observed (Fig. 2(B)). Furthermore, 39% (368/949) of the NF-L-positive neurons were positive for GST-DRG1, 25% (214/848) for GST-DRG2, and 14% (147/1081) for GST-DRG3. Cell-size analysis showed that GST-DRG1- and GST-DRG3-positive cells were mainly small-sized neurons, whereas GST-DRG2-positive cells were mainly large-sized DRG neurons (Fig. 2(B)). The mean diameter of the cells positive for GST-DRG1 was 21.6 ± 5.2 μm, GST-DRG2 33.5 ± 7.4 μm, and GST-DRG3 22.7 ± 6.6 μm. There was a significant difference in mean diameter between GST-DRG1-positive cells and GST-DRG2-positive cells ($p < 0.001$), and between GST-DRG2-positive and GST-DRG3-positive cells ($p < 0.001$), but no size difference between GST-DRG1-postive and GST-DRG3-positive cells. Therefore, the cell-size specificity of the three different peptides in phages *in vitro* (Fig. 1(A)) was confirmed by the *in*

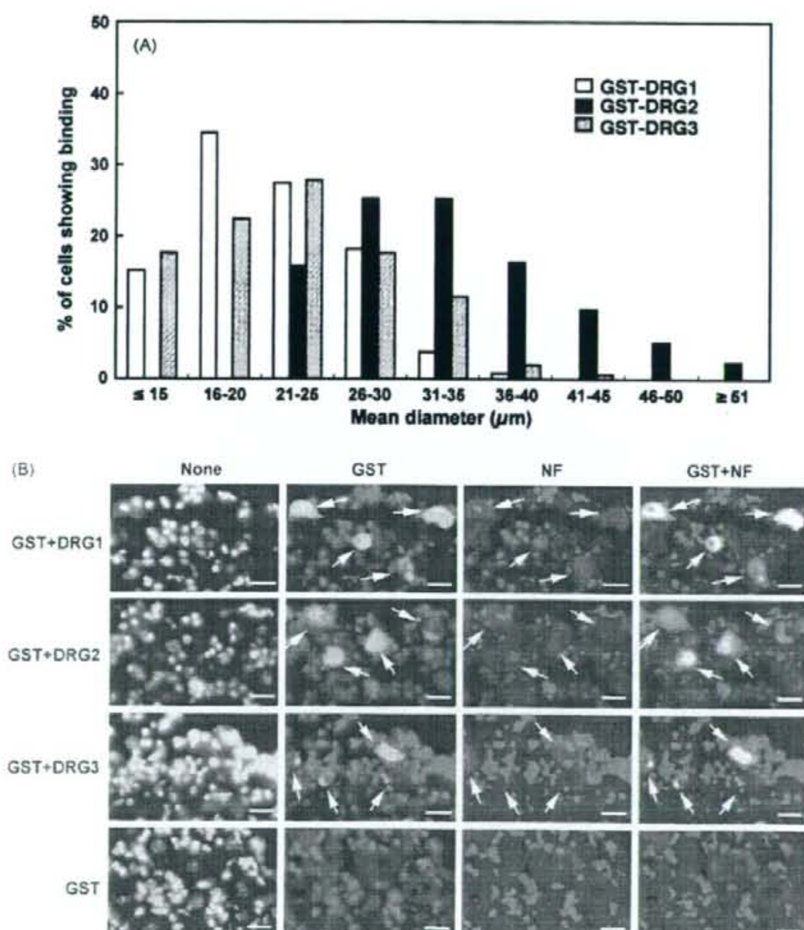

Fig. 2. Fluorescence microscopy of *in vivo* binding of GST-peptide-fusion proteins to DRG neurons. (A) Cell-size distribution of the DRG neurons positive for GST-DRG1, GST-DRG2 or GST-DRG3. (B) Double immunofluorescence staining of DRG-neurons with anti-GST (green) and anti-neurofilament L (NF-L, non-phosphorylated form, red). Nuclei were stained with DAPI (blue). None indicates no primary antibody. Arrows indicated the cells positive for both GST and NF-L. Bars, 20 μm.

vivo-binding experiment. Moreover, as most of the GST-positive immunoreactivity was detected in the cytoplasm of the DRG neurons (Fig. 2(B)), and as anti-GST antibodies used in this experiment were specific for recombinant GST-peptide-fusion proteins synthesized in *E. coli*, and did not react with mouse endogenous GST, the bound GST-peptide-fusion proteins appear to have been internalized into the cytosol of the targeted neurons. In contrast, we examined the GST immunoreactivities in the central nervous system (brain and spinal cord), and obtained no specific staining (data not shown).

We searched for possible endogenous candidates in the mouse protein database that might bind to the DRG, "receptors" for the three 7-amino-acid peptides and found the same DRG1 7-amino-acid sequence in the extra cellular matrix protein TM14 (NP_077199.2) [3]. However, six out of the seven amino-acids were located in the putative signal peptide but not in the putative binding domain in TM14. The mouse neuronal differentiation-related protein (NDRP, NP_001096649.1) has the same 7-amino-acid sequence as that in DRG2 [7]. NDRP is expressed in the sensory neuronal system including in DRG neurons, though our current knowledge of its function is limited. We failed to find any peptide sequence in the database similar to that in DRG3.

We used the *in vitro* screening of a C7C peptide library displayed on M13 filamentous phages against isolated mouse DRG neurons to isolate phage clones that encompassed three different 7-amino-acid peptides that displayed specific binding for these neurons *in vitro*. Immunohistochemical analysis following the injection of GST-peptide-fusion proteins *in vivo* demonstrated that the three peptides bind to DRG neurons with different sizes; furthermore, these fusion proteins were internalized into the cytoplasm of the targeted neurons.

The Ph.D.-C7C Phage display Peptide Library kit expresses random 7-amino-acid peptides fused to the minor coat protein (pIII) of the M13 phage. By sequence analysis, only 113 phage plaques were represented in the bound peptide, consistent with a very efficient selection process (Table 1). The final binding studies allowed us to identify three phages (DRG1, DRG2, or DRG3) that occurred repeatedly. We believe that the efficient identification of these clones was the result of multiple rounds of positive and negative selection using the biopanning protocol [12]. We cannot exclude the possibility that some of the other 84 clones (Table 1) might represent true DRG-binding peptide-phages, but we decided to pursue the three best-represented clones at this time for practical reasons.

The three clones that we analyzed further showed interesting specificities to the targeted neurons: DRG-p1 and DRG-p3 to small-sized neurons and DRG-p2 to large-sized neurons. Moreover, the size specificity of target neurons for individual peptides was confirmed by *in vivo* binding experiments: GST-DRG1 and GST-DRG3 recognized small-sized neurons, and GST-DRG2 recognized large-sized neurons. Since both DRG1 and DRG3 recognized small sized neurons, two peptide ligands might bind to the same cell populations. Therefore, we performed double staining of isolated DRG neurons after the incubation with DRG-p1 and GST-DRG3 by antibodies against phage and GST, respectively. Double positive staining was obtained in 51% of DRG-p1-positive neurons, and was in 71% of GST-DRG3-positive neurons, respectively (data not shown). The result indicates that both DRG1 and DRG3 recognized different targeting molecules, but may bind to the same cell populations in specific subpopulations in small-sized neurons. Histologically, mammalian sensory DRG neurons have been classified into two major types: large-light and small-dark cells on the basis of their staining characteristics seen under light microscopy [11]. Clinically, peripheral sensory neuropathy (including neuronopathy) has been classified into two types based on fiber size: large fiber sensory neuropathy and small fiber sensory neuropathy [16,22]. The symptom typically found in large fiber sensory neuropathy is an ataxic gait, and that in small fiber sensory neuropathy mainly pain [16,22]. Neuronal size is directly related to axonal characteristics [10,11,22]. Thus, each targeting motif included in DRG1, DRG2, DRG3, and, potentially, in some of the other 84 peptides sequences, may be useful tools for pathophysiological analysis such as a cell-identification marker, or for the generation of gene-delivery constructs for sensory neuropathies [13,15,20]. Future studies will involve the isolation and identification of the DRG neuronal cell surface proteins that bind to the three peptides.

We also tried to determine the size of the targeted protein using the whole homogenate of DRG tissues from mice, and found some specific bands by Western blotting analysis (data not shown). Since those bands may be proteins targeted by the peptides, DRG1, DRG2, and DRG3, and should be clarified in the future studies.

In conclusion, we have used *in vitro* phage display technology to isolate and identify three different peptides that bind to DRG neurons *in vitro* and *in vivo*. We further showed that the peptides recognize neurons of two different sizes. While additional studies are needed to characterize the potential binding proteins and their receptors, the identification of three DRG neuron-specific peptides provides a powerful tool that will facilitate future research into the structural basis of neuronal cellular subpopulations and the generation of molecular delivery systems targeting different DRG neurons *in vivo*.

Acknowledgments

We thank M. Koike, H. Urabe, Y. Katayama, K. Matsumura, T. Yamamoto, Y. Mori, R. Fujiwara, T. Watanabe, F. Kimura, and A. Yamaguchi for technical support. This work was supported by a Grant-in-Aid (#18390100 to H. Kojima and #18590934 to H. Yasuda) for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan, by the President's Discretionary Fund from Shiga University of Medical Science (#1515503L to H. Kojima), and by USA National Institutes of Health grant (HL-51586 to L. Chan).

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DOI: 10.1111/j.1464-5491.2008.02490.x

Original Article: Complications

Stratified analyses for selecting appropriate target patients with diabetic peripheral neuropathy for long-term treatment with an aldose reductase inhibitor, epalrestat

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Accepted 15 May 2008

OnlineOpen: This article is available free online at www.blackwell-synergy.com

Abstract

Aims The long-term efficacy of epalrestat, an aldose reductase inhibitor, in improving subjective symptoms and nerve function was comprehensively assessed to identify patients with diabetic peripheral neuropathy who responded to epalrestat treatment.

Methods Stratified analyses were conducted on data from patients in the Aldose Reductase Inhibitor—Diabetes Complications Trial (ADCT). The ADCT included patients with diabetic peripheral neuropathy, median motor nerve conduction velocity ≥ 40 m/s and with glycated haemoglobin (HbA_{1c}) ≤ 9.0%. Longitudinal data on HbA_{1c} and subjective symptoms of the patients for 3 years were analysed (epalrestat $n = 231$, control subjects $n = 273$). Stratified analyses based on background variables (glycaemic control, grades of retinopathy or proteinuria) were performed to examine the relationship between subjective symptoms and nerve function. Multiple logistic regression analyses were conducted.

Results Stratified subgroup analyses revealed significantly better efficacy of epalrestat in patients with good glycaemic control and less severe diabetic complications. In the control group, no improvement in nerve function was seen regardless of whether symptomatic benefit was obtained. In the epalrestat group, nerve function deteriorated less or improved in patients whose symptoms improved. The odds ratio of the efficacy of epalrestat vs. control subjects was approximately 2 : 1 (4 : 1 in patients with HbA_{1c} ≤ 7.0%).

Conclusion Our results suggest that epalrestat, an aldose reductase inhibitor, will provide a clinically significant means of preventing and treating diabetic neuropathy if used in appropriate patients.

Diabet. Med. 25, 818–825 (2008)

Keywords aldose reductase inhibitor, diabetic peripheral neuropathy, good condition of blood glucose level, polyol pathway

Abbreviations ADCT, Aldose Reductase Inhibitor—Diabetes Complications Trial; AEs, adverse events; ARI, aldose reductase inhibitor; HbA_{1c}, glycated haemoglobin; MFWL, minimum F-wave latency; MNCV, motor nerve conduction velocity; VAS, visual analogue scale; VPT, vibration perception threshold

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Introduction

Various drugs have been developed to treat diabetic neuropathy [1], including aldose reductase inhibitors (ARIs). ARIs suppress the activity of aldose reductase, a rate-limiting enzyme involved in the polyol pathway, which is enhanced in diabetic neuropathy. The effects of ARIs on diabetic neuropathy and diabetes-related complications have been investigated in animal and clinical studies [2,3]. Clinical efficacy of ARIs in diabetic neuropathy has been reported in terms of nerve function, subjective symptoms and histopathological findings of neural tissue [2–4]. Generally, parameters for nerve function, such as nerve conduction velocity and vibration perception threshold (VPT) are used as primary variables of efficacy of ARIs [5–9]. We previously reported the results of the 3-year Aldose Reductase Inhibitor—Diabetes Complications Trial (ADCT), which demonstrated the clinical efficacy of epalrestat, an ARI, in Japanese diabetic neuropathy patients with median motor nerve conduction velocity (MNCV) as the primary endpoint [10]. Stratified analyses of the ADCT data suggested that the effects of epalrestat on median MNCV were most evident in patients with better glycaemic control and without retinopathy or nephropathy [10].

Subjective symptoms may be more important to patients than nerve function. In ADCT, the efficacy of epalrestat was investigated by analysis of subjective symptoms such as numbness of upper and lower extremities, sensory abnormalities of lower extremities and cramp [10].

Here, we report additional analyses of ADCT data [10], in which stratified analyses of subjective symptoms were performed to identify patients likely to experience better responses to epalrestat. We determined the correlation between amelioration of subjective symptoms and nerve function to clarify the significance of ARIs in the treatment of neuropathy. Furthermore, we carried out logistic regression analysis using a comprehensive clinical assessment parameter based on nerve function and subjective symptoms and performed quantitative analysis of the efficacy of epalrestat adjusted for background variables.

Patients and methods

ADCT was conducted at 112 medical facilities in Japan between 1997 and 2003. The protocol was approved by the Institutional Review Board of each medical facility and all patients gave informed consent.

The ADCT methodology has been reported previously [10]. Patients had mild diabetic peripheral neuropathy based on subjective symptoms, no foot ulcers and neurological dysfunction [at least two parameters: MNCV (indispensable) and VPT or Achilles tendon reflex, etc.]. Patients were enrolled if they had a median MNCV $\geq$ 40 m/s (seemingly reversible) and stable glycaemic control [glycated haemoglobin (HbA_{1c}) $\leq$ 9.0%, with $\pm$ 0.5% variation in the previous 3 months]. Subjects were excluded if the primary cause of the neurological disorder was not diabetes (alcoholic neuropathy, carpal tunnel syndrome, sequelae of cerebrovascular disease, etc.), if they had peripheral arterial disease (ankle brachial pressure index of $\leq$ 0.8) or severe hepatic or renal disorder, if they were participating in other interventional studies, or if they were receiving other experimental medications for diabetic neuropathy, prostaglandin E_1 preparations or any other medication that affects symptoms of diabetic neuropathy. Patients were randomized to either the epalrestat or control groups; details of the randomization method were described previously [10]. Epalrestat (50 mg) was administered orally three times daily before each meal (150 mg/day). Both groups continued conventional therapy (diet treatment, oral glucose-lowering agents, insulin and anti-hypertensive agents). With the exception of rescue medication, new medication to aid neuropathy control was prohibited.

Study endpoints and measures of outcome

The primary endpoint was change from baseline to study end in median MNCV in the patient's non-dominant arm. This arm was chosen to avoid any bias as a result of possible lower limb impairment caused by the Japanese lifestyle (a tendency to sit straight). Secondary endpoints included changes from baseline to study end in minimum F-wave latency (MFWL) of the median motor nerve and VPT. VPT was measured using a 128-Hz tuning fork by measuring the time in seconds during which the patient felt vibrations. Changes of subjective symptoms of diabetic neuropathy were assessed using a 100-mm visual analogue scale (VAS). For a general measure of symptoms, the mean score was calculated for 10 symptoms (spontaneous pain in upper and lower extremities, numbness of upper and lower extremities, paraesthesia or hyperaesthesia of lower extremities, dizziness, cramp, coldness, abnormal sweating and constipation) and four symptoms in the lower extremities (spontaneous pain, numbness, paraesthesia or hyperaesthesia and cramp). The mean VAS of each symptom ranged from 20 to 30. The symptom score using in this study is the mean VAS of 10 symptoms per patient, although many values were zero. The mean symptom score at the beginning of the study in the control and epalrestat groups was 8.2 and 9.3, respectively.

The response to therapy was determined using a general assessment of subjective symptoms and nerve function. Patients were rated as responders if any of the following changes were observed over the 3-year study period: $\geq$ 1 m/s increase in median MNCV [11], $\geq$ 5% decrease in MFWL [12], $\geq$ 50% increase of time in VPT [13], or $\geq$ 50% decrease in the mean score for 10 symptoms [14].

Statistical analysis

As for ADCT [10], efficacy analyses were performed in patients who had data for at least 1 year, using the last-observation-carried-forward (LOCF) method [15,16].

Statistical methods included χ^2-tests for nominal scale, Mann–Whitney *U*-tests for ordered categorical scale, two-sample *t*-tests for comparison of mean values between groups and two-way repeated ANOVA for changes in glycaemic control. Multiple logistic regression analysis was conducted using the defined parameters of efficacy. Normalization for the multiplicity of stratified analyses was not performed. All analyses were carried out using SAS Version 8.02 (SAS Institute, Cary, NC, USA). $P < 0.05$ was considered statistically significant.

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Table 1 Patient characteristics at baseline

Patient characteristics	Control (n = 273)	Epalrestat (n = 231)	P-value
Sex			
Male	161 (59.0)	132 (57.1)	
Female	112 (41.0)	99 (42.9)	0.678*
Age (years)	61.5 ± 9.0	61.0 ± 10.0	0.541†
Duration of diabetes (years)	12.5 ± 8.0	13.2 ± 9.1	0.408†
Duration of neuropathy (years)	3.3 ± 3.6	3.7 ± 4.9	0.363†
HbA_{1c}			
HbA_{1c} before and after treatment			
0 years	7.0 ± 0.1	7.2 ± 0.1	
3 years	7.2 ± 0.1	7.3 ± 0.1	0.122‡
Change over 3 years			
< 7.0%	71 (26.0)	51 (22.1)	
7.0% ≤ HbA_{1c} < 9.0%	156 (57.1)	141 (61.0)	0.470§
≥ 9.0%	46 (16.8)	39 (16.9)	

Data are means ± standard deviation (SD) or n (%).
P-values were calculated using *χ^2- tests, †two-sample t-test, ‡ANOVA and §Mann–Whitney U-test. Duration of neuropathy refers to the mean patient-reported duration of neuropathy symptoms.
HbA_{1c}, glycated haemoglobin.

Results

Patients

Patient clinical characteristics are provided in Table 1. There were no significant differences between the two groups.

In ADCT [10], the control and epalrestat groups included 305 and 289 patients, respectively. Of these, 31 and 55 patients withdrew after < 1 year, respectively; the reasons for withdrawal were a change in hospital (12 patients in each group), co-morbid illnesses (seven in each group), amelioration of symptoms (two epalrestat recipients), adverse events (20 epalrestat recipients), deterioration in symptoms (seven control subjects) or other (five control subjects, 14 epalrestat recipients). Both amelioration of symptoms and adverse events were observed only in the epalrestat group, resulting in a higher withdrawal rate in this group. Additionally, 59 and 53 patients had insufficient data for the primary efficacy analysis, primarily because of the unavailability of an electromyogram or a problem with the measuring technique. Thus, the primary efficacy analysis included 215 and 181 patients in the control and epalrestat groups, respectively.

This analysis included 273 patients in the control group and 231 patients in the epalrestat group, for whom data were available on symptom change and glycaemic control. The correlation between subjective symptoms and median MNCV was analysed in 214 patients in the control group and 179 patients in the epalrestat group.

Changes in glycaemic control

The changes in HbA_{1c} observed in the two treatment groups are shown in Table 1. No significant differences between epalrestat recipients and control subjects in glycaemic control were observed at baseline or over 3 years of treatment.

Stratified subgroup analyses of symptoms

Stratified subgroup analyses were performed to examine the relationship between changes in symptom score for 10 symptoms and glycaemic control, grade of retinopathy and grade of proteinuria (Fig. 1).

In both groups, the mean symptom score improved significantly from baseline at years 1, 2 and 3 (Fig. 1a). The improvement was most evident in the epalrestat group and differences between the two groups were significant at years 1, 2 and 3 ($P = 0.019$, $P = 0.002$ and $P = 0.009$, respectively; Fig. 1a).

HbA_{1c}

In patients with HbA_{1c} < 7.0%, the mean symptom score improved significantly in both groups at year 1, 2 and 3. The improvement was most evident with epalrestat and significant between-group differences were observed at years 1 and 2 ($P = 0.042$ and $P = 0.049$, respectively). In patients with HbA_{1c} ≥ 7.0% and < 9.0%, the control group showed significant improvement in the mean symptom score at year 1, whereas epalrestat recipients showed significant improvement at years 1, 2 and 3. Improvements in the mean symptom score were significantly greater with epalrestat than control at years 2 and 3 ($P = 0.009$ and $P = 0.027$, respectively). In patients with HbA_{1c} ≥ 9.0%, the control group showed no significant improvement in the mean symptom score at any time points, whereas the epalrestat group showed significant improvement at years 2 and 3. There were no significant between-group differences at any time points (Fig. 1a).

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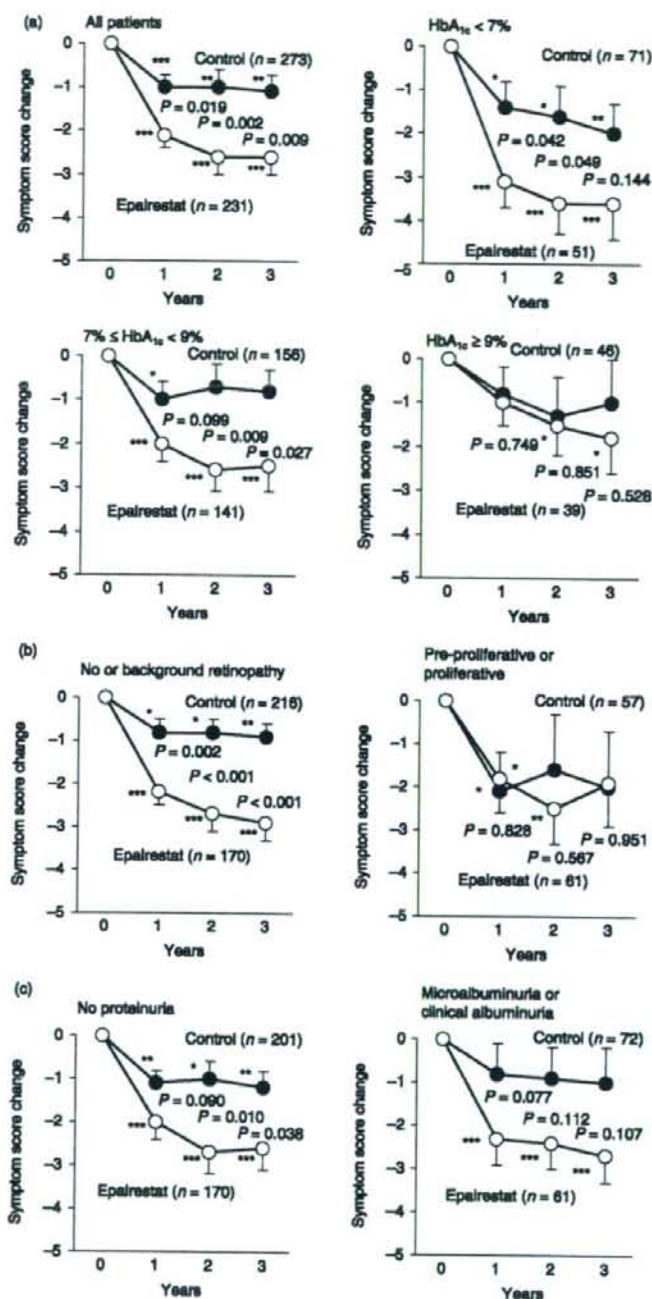

FIGURE 1 Effects of epalrestat on symptom score according to glycated haemoglobin (HbA_{1c}) over 3 years (a), baseline level of retinopathy (b) and baseline level of proteinuria (c). ○, epalrestat group; ●, control group. Data are reported as means ± standard error (SE). P-values were calculated using the two-sample t-test. $^{*}P < 0.050$, $^{**}P < 0.010$ and $^{***}P < 0.001$ were calculated vs. baseline using the paired t-test. P-values are stated for between-group differences.

Grade of retinopathy

In patients with no or background retinopathy at baseline, the mean symptom score improved significantly in both groups at years 1, 2 and 3. The improvement was significantly greater in the epalrestat group at years 1, 2 and 3 ($P = 0.002$, $P < 0.001$ and $P < 0.001$, respectively). In patients with pre-proliferative or proliferative retinopathy, the control group showed significant improvement only at year 1, whereas the epalrestat group

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