日本におけるスモン患者調査～高齢化に伴う医療福祉問題～

田中　千枝子[1)]

1）日本福祉大学

要旨：スモン(SMON)（Subacute Myelo-Optico-Neuropathy）は1950年代から70年代にかけて日本で多発した亜急性脊髄・視神経・末梢神経障害をいい、当時の厚生省は11,127人の患者を確認した。スモンは整腸剤キノフォルムを原因とした深刻な薬害事件として社会問題化し、各地での裁判を通じて制度的対応の先駆的事例となり、日本における「薬害の原点」といわれている。国家賠償決着後補償医療・恒久対策として「最後の1人までも救済する」との体制で、医療を中心とした支援が組まれてきた。しかし2,000人強の患者（2010年現在）は平均年齢82歳 となり、高齢化が進行し、かつ6～10歳での発病など若年層（40～50代）も1割弱 存在することで医療福祉ニーズの二極化が生じている。

そこでスモン患者の生活実態と医療福祉ニーズの明確化を目的にする。補償医療と身体障害者福祉の制度体系から、高齢化により介護保険中心に支援システムが移行することによって、従来のニーズとサービスとの間に不一致が生じており、サービス利用の促進が阻害されているとの仮説を立証した。その方法は厚生労働省難病研究班（スモン）により毎年実施されている全国調査（1997年度より毎年）と、2010年度に付加実施した福祉用具利用調査での質的量的データの分析によった。

結果、高齢化に伴い障害の重度化が進行しているにも関わらず、要介護度の認定が混乱しより介護度が軽度にでることにより、当事者の介護保険に対する評価が低下している。介護保険へのアクセスは足踏み状態で円滑に進んでおらず、介護保険を利用していない割合が一般利用者に比べても高い。また同時に利用の必要がないと答える割合も一定程度あり、その群は将来への不安が高い。福祉用具の利用においても、専門職側にスモン特有の制度特典を知らず、また特有の随伴症状に対する器具への配慮・工夫が足りないとの不満が存在した。

キーワード：スモン　薬害患者　高齢化

はじめに

スモン(SMON)（Subacute Myelo-Optico-Neuropathy）は1950年代から70年代にかけて日本で多発した亜急性脊髄・視神経・末梢神経障害をいい、当時の厚生省では死亡を含め11,127人の患者を確認した。スモンは整腸剤キノフォルムを原因とした深刻な薬害事件として社会問題化し、各地で訴訟が起きた。これを契機に1979年に「医薬品副作用被害者救済基金法」が制定され、薬事法の改正で行政の医薬品安全性確保義務が明文化されるなど制度的対応の先駆事例となり、サリドマイドとともに「薬害の原点」といわれている。またその後血液製剤によるHIV/AIDS，B型C型肝炎などに展開し、スモン患者の長期的生活障害の進行に対する医療と福祉の総合恒久対策の在り方は、今後の薬害患者のサポート体制への大きな示唆になると考えられる。

スモン患者は下痢・腹痛などの腹部症状に対して、薬剤キノフォルムが投与されたことにより、

典型例では 2～3週で両下肢に自覚的なしびれ感、下肢の脱力、起立・歩行の不安定が生じ、重症例では両下肢完全麻痺、約20%に視覚障害をきたした。患者の現在では、加齢に伴って本来の障害に加え、白内障、高血圧、四肢関節疾患などの合併症が大きな問題となっている。平成 21年国による賠償の基本となる健康管理手当を受給している患者は2,176名おり、そのうち870名に対する厚生労働省スモン研究班による全国検診・調査（2010年）によると、約96%に異常感覚、約60%に歩行障害、約40%に中等度以上の視覚障害がみられている。障害状況をより詳しく見ていくと、末梢および中枢神経器官、視覚の障害により、疼痛（痛み・痺れ・脱感覚）・鈍化・冷感・熱感などの感覚異常が全患者の9割に存在し、それは発症時の過去と現状の間で変化がない。またその痛みに筋力低下が随伴して生じている。重度の視覚障害は最重度期で6割に見られ、回復しないまま現在も重度障害が4割に見られる。さらにこれらによる 歩行障害は現在8割にみられ、発生当時が5割弱であったことから、高齢化の影響が大きいと考えられ、その半数は歩行不能の重度の障害である。さらに高齢化によるＡＤＬ低下が大きな問題となっているが、スモン患者特有の深部覚の低下および視覚障害により、下肢と体幹の位置感覚がとれないことによる転倒事故がより起きやすい。その結果大腿骨骨折　慢性硬膜下血腫　脊椎圧迫骨折などが生じる。 また視覚障害を持つ中で、白内障の悪化やさらにうつ、骨粗鬆症などの二次的症状も顕著となる。

従来国家賠償決着後補償医療・恒久対策として「最後の1人までも救済する」との体制で医療を中心とした支援が組まれてきた。その柱は 1973 年特定疾患治療研究事業の制定時から指定疾患となり、医療費の助成や健康管理、難病手当であり、補償医療を中心とした対策が立てられてきた。また身体障害者手帳を9割以上が発症当時から所持するなど、障害者福祉法の対象にもなっていた。しかし平均年齢82歳 と高齢化が進行し、かつ6～10歳での発病など若年層（40～50代）も1割弱 存在することで医療福祉ニーズの二極化が生じている。とくに患者の高齢化がすすみ、制度が介護保険中心になってきたことにより、スモン患者の主な生活問題は、制度的には福祉および介護問題に集約されつつある。しかし高齢化、障害の重度化が進行しているにも関わらず、介護保険へのアクセスが円滑ではない可能性がある。高齢化したスモン患者の医療福祉ニーズが現行の制度と不一致を生じている可能性があることが問題となっている。

目的と方法

スモン患者の生活実態と医療福祉ニーズの明確化を目的に、補償医療と身体障害者福祉の制度から、介護保険中心に救済・支援システムが移行することによって、従来のニーズとサービスとの間の不一致が生じているとの仮説を設定した。

その方法は厚生労働省難病研究・スモン研究班により実施されている全国調査（1997年度より毎年）と昨年度付随して実施した福祉用具利用調査での質的量的データの分析によった。厚生労働省は 1997 年より毎年スモン研究班による検診・健康相談事業を実施し、その個々人の身体状況と福祉・介護の状況を詳細なデータとして原則面接により把握してきた。そこで収集されたデータは2010年まで14年にわたり毎年収集・分析され、報告書としてまとめられている。ここでとくに分析対象として抽出したのは、2006年から2010年の5年間（表1、2）のすべての調査に応えた、全国各都道府県在住のスモン患者および家族の調査協力者である。調査協力者は都道府県ごとの事情で抽出選定基準が異なるために、比較対象5年間の調査にすべて該当した対象者をマッチングした。また福祉用具調査は2010年度調査者のうちから、回答のあったものである。

結果と考察

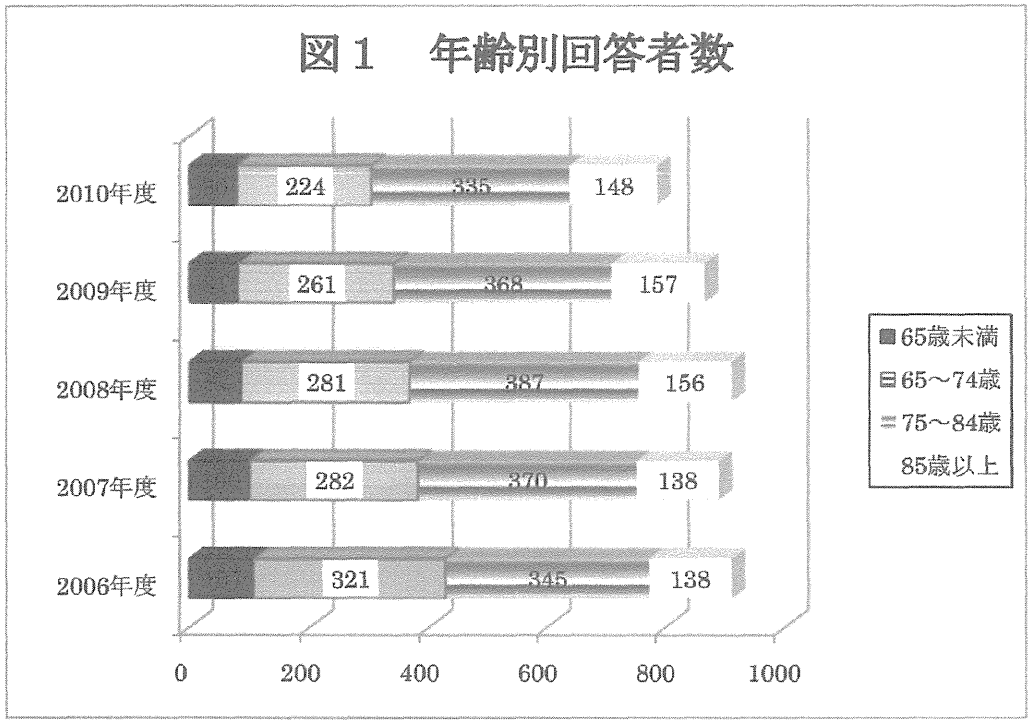
全体の概要

2006年から2010年までの毎年調査の対象者の概要では、各都道府県で毎年選択基準が異なるため、その全体数を表わすわけではないが、2003年に1,000人を切って以降、漸減傾向である。（表1）スモン患者全体では2,000人強であるため、毎年3分の1程度のサンプリング数となっている。また各地保健所で行われる健康診断・調査の際に対面で聴取する事が原則となっているために、病状悪化による入院者など、障害が重度、病状の不安定な対象者は除かれている傾向がある。

年齢別にみると、65才未満の若年スモン患者が1割弱対象となっていることが分かる。また高齢層では、この5年間で前期高齢者層から後期高齢者層へ、その割合の重点が移っている。(図1)

表1 スモン患者調査結果の5年間概要

			2006年度	2007年度	2008年度	2009年度	2010年度
男女比	実数	男	252	250	245	240	237
		女	659	640	666	627	550
		計	911	890	911	867	787
	構成比	男	27.7	28.1	26.9	27.7	30.1
		女	72.3	71.9	73.1	72.3	69.9
		計	100	100	100	100	100
年齢比	実数	65歳未満	107	100	87	81	80
		65～74歳	321	282	281	261	224
		75～84歳	345	370	387	368	335
		85歳以上	138	138	156	157	148
		計	911	890	911	867	787
	構成比	65歳未満	11.7	11.2	9.5	9.30%	10.2
		65～74歳	35.2	31.7	30.8	30.2	28.5
		75～84歳	37.9	41.6	42.5	42.4	42.6
		85歳以上	15.1	15.5	17.1	18.1	18.8
		計	100	100	100	100	100



5年間の変化比較―主観的介護の程度と要介護度および介護保険満足度

5年間連続で調査に協力した患者は210名であった。それを対象にして変化を追う。

介護の必要性では、2006年から5年間の変化で、介護が「毎日必要」が1.4倍以上に増えている。またその変化は「必要時に介護」というグループから回ってきている。介護の重度化は促進されている。（図2）しかしそれに比して同じ対象者の要介護度比較を行うと、その変化は4と5の最重度がやや増加している以外は、変化がほとんどない。（図3）

両結果をマッチングすると、要介護3で毎日介護が占める割合が2006年度8.9%に比して2010年度は 18･8%になり、要介護度が主観的な介護負担感を表わしていないことが分かる。これは2007年度に要介護度の改訂作業が実施されて以来、全体に要介護度が低く出ることが指摘された。その後修正されたものの、介護度が低く出る傾向は変わらなかったと考えられる。

また感覚障害が十分に要介護度に反映されていないのではないかという通常言われている仮説を確認したところ、必ずしもそのような結果は出なかった。

さらに認定された要介護度に対する当事者評価は、5 年間の前後での比較によると、妥当および高いとする評価が 49.8%から 34,6%に明らかに減り、はっきり低いとする不服群が 32.3%から 44.3%に増加した。（図4）この不服群は要介護の度合いそのものに比例したものではなく、全体的な傾向であった。

つまり介護の手間が柔道になってきた実態はあるが、実際の判定としての要介護度とは一致せず、介護保険に対する不信感・不満足感が増大しているしていると考えられる。

図２　介護の必要な程度の変化

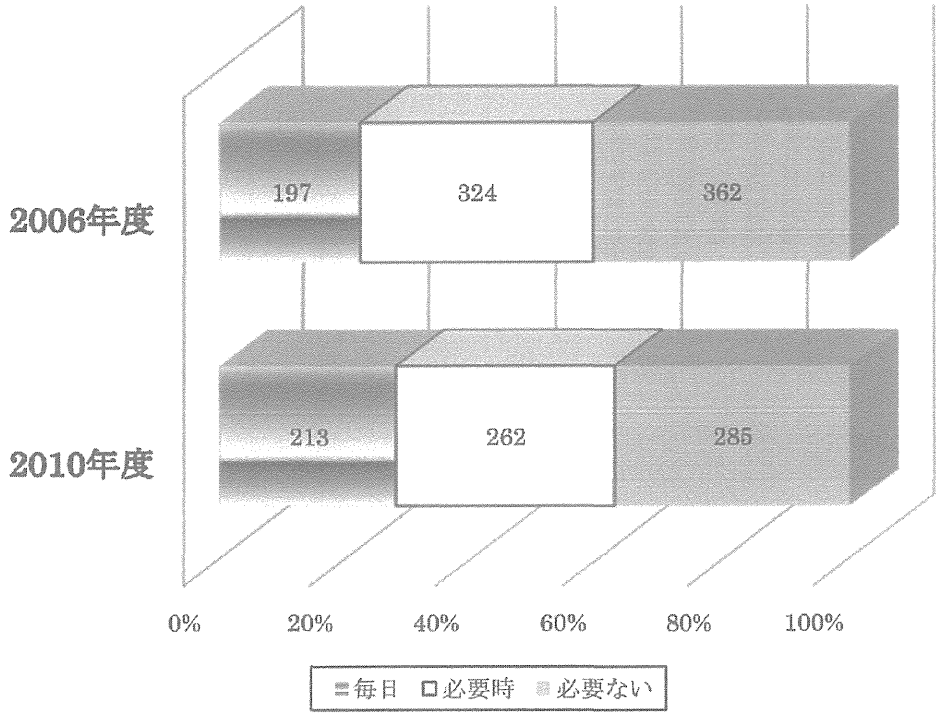
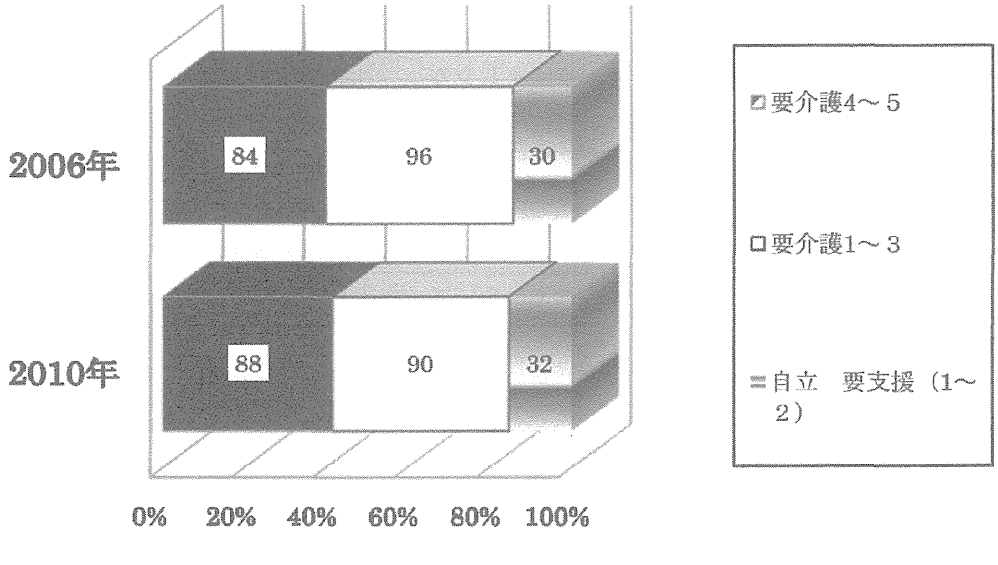
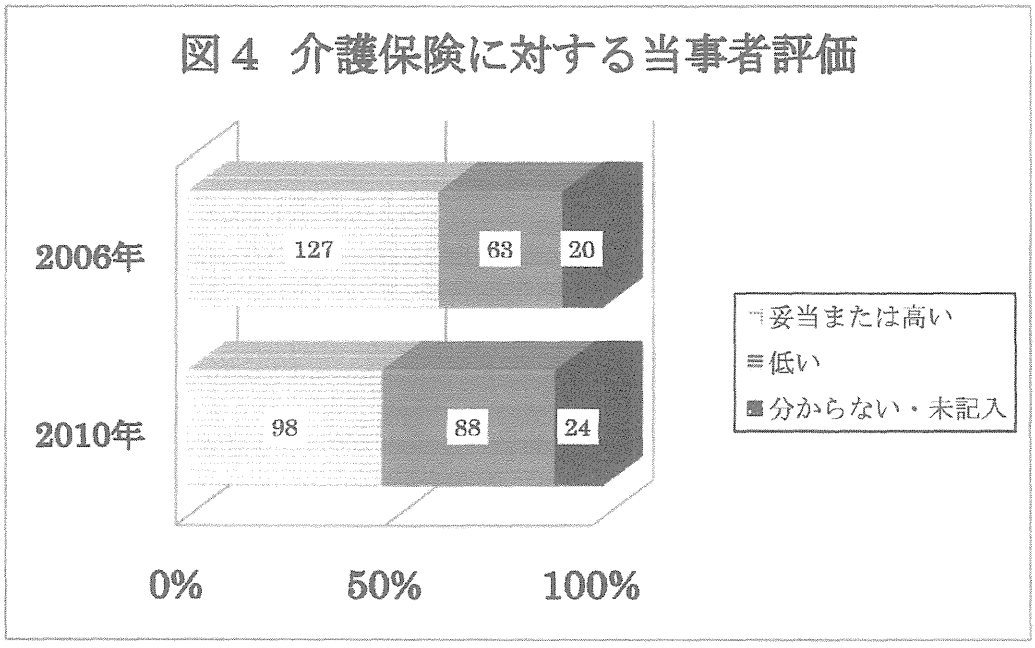


図３　要介護度の変化





生活・介護問題へのニーズと介護保険制度・サービス利用

スモン患者の生活・介護ニーズが制度・サービスによってどのくらい充足しているのかについて検討した。図５は介護保険申請比率の 10 年間の比較である。当初はいまだ申請率が 26%程度であったものが、10 年間で倍近くまでになった。しかしここ５年間は 25%前後で微増である。10 年前よりも、介護保険へのアクセス良くなっていると考える。しかし伸び悩んでいる可能性も考えられる。

また一般高齢者の申請率が約 20%であるのに対して、心身に以前からハンディのあるスモン患者は 44%前後と高率である。しかしその数字が妥当であるかについての検討はなされる必要があるであろう。その根拠として、「毎日介護」のグループのうち 75.5%が介護保険自体は申請をしている。24.5%は申請さえもしていない。さらに介護保険申請と実際のサービス利用にも乖離があった。日常生活用具の貸与のみの事例を含んだ介護保険サービスの利用率は 2010 年度現在、申請者に対して 73.2%である。つまり申請しても福祉用具の貸与さえ使わない群が 26.8%存在するということである。スモン患者の特徴は、介護保険の未申請者のうち、67%が将来に向けての強い心配・懸念を表している。（表２）しかしその根源は、８割が介護保険へのニーズではなく、医療サービスが受けられないのではないかという強い不安であった。医療に対する強い信頼と、医療サービスからはぐれることへの不安を同時に持っている。また介護保険のサービスの利用率をみると、在宅主要サービスである訪問看護、訪問介護、ショートステイ等その利用経験は５％の域を脱していない。必要なしおよび無回答の多さが、顕著である。（図６）

これらの重度の介護が必要にもかかわらず、介護保険自体の未申請群と、申請しても利用していない群に関して、ニーズとサービスの不一致を検討する必要がある。

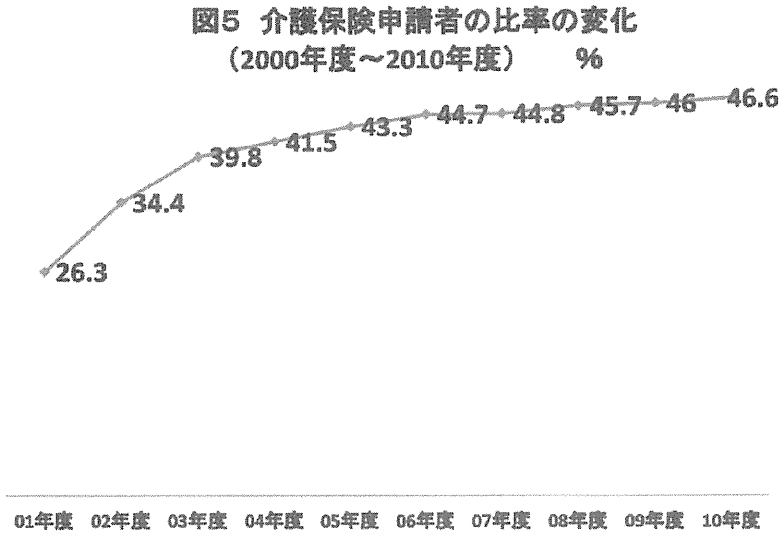
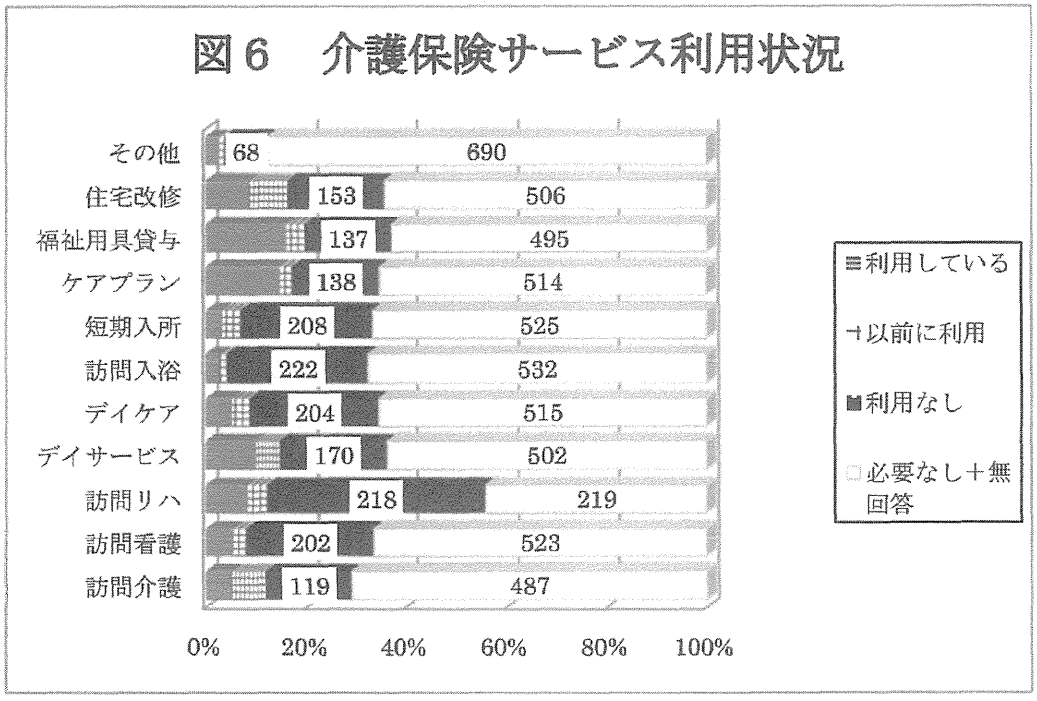


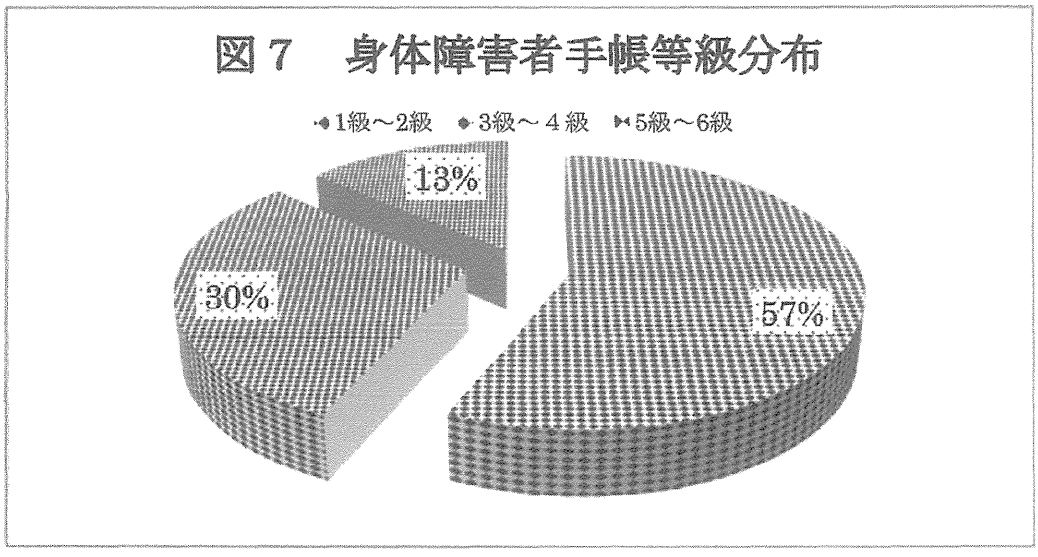
表2 介護保険未申請者の意識

		必要性の有無		心配の強い割合
申請者	374	有	374	210(56.1%)
			—	—
未申請者	413	有	58	33(56.1%)
		無	355	241(67.9%)

生活・介護問題へのニーズと身体障害制度・サービス利用

さらにスモン患者は発症のごく早期から身体障害者手帳を取得する率の高い（9 割以上）集団であった。（図 7）ほとんどが肢体や視覚などの分野の合併で申請されている。1~２級が 57%、3~４級が 30%を占めている。長年の手帳所持によって、サービス利用を実施してきた可能性を考えた。しかしガイドヘルパーの利用は所持者のうち３％となっており、公的サービスの利用が、従来からなされていなかった可能性がうかがえた。また介護保険の枠をオーバーした場合に、身体障害者手帳が使用可能であるが、その枠まで使っているものもいなかった。





結論と課題

スモン患者は40年もの長い間、健康管理手当てと補償医療、身体障害者手帳の制度を中心にサービスを組み立ててきた。また身体障害者手帳も、ガイドヘルパーなどスモンならではの障害を支援するサービスが設定されているのも関わらず、十分な利用状況とはいえなかった。長い間の身体障害者生活の中で、家族介護を中心とした在宅サポート体制が整って安定していた方が多いと考えられる。しかも高齢化に伴い、介護保険優先のシステムの中で、要介護度が障害の進行に伴って上昇しないことで、介護保険への評価も低くなっている。そして身体障害者の制度にもな

じみがない状態で、介護保険の必要性は感じないが、将来への強い不安を抱く集団が3割~4割存在する。補償医療でほとんどの今までのニーズを賄ってきた体験が障害となって、介護や福祉のサービスに向けて利用の動機づけをとりにくい状況にある。

一方で記述式の福祉用具調査では、スモンの特典を活かした保健・福祉サービス利用のやり方が存在するにもかかわらず、利用されていなかった。また周囲の専門職が知らないために、その利用が制限されるようなこともあった。地域の各専門職はスモンという疾患およびその制度を知らないまま、その制度的特典を還元できていないことも散見され、利用者に不利益をもたらすことにもなった。

そこで介護保険や福祉サービス利用に困難のある事例を直接分析することによって、利用の抑制をかけているものを詳細に明らかにすること、また利用の促進に向けて、患者・家族会を中心に、厚生労働省やかくち保健行政と連携しながら、啓発活動としての勉強会や講演会を実施すること。さらにスモン患者のサービス利用について、適切なアドバイスを与えられるような相談職員の研修を実施する事等を考えてきたい。

≪参考文献≫

小長谷正明「スモン―薬害の原点―」『医療』2008　国立医療学会　vol.63 №4　PP227-234

泉博「空前の薬害訴訟『スモンの教訓』から何を学ぶか」丸ノ内出版 1996

スモンの会全国協議会編「薬害スモン全史」第1巻　被害実態篇　労働旬報社　1981-1986

スモンの会全国協議会編「薬害スモン全史」第2巻　裁判篇　労働旬報社　1981-1986

スモンの会全国協議会編「薬害スモン全史」第3巻　運動篇　労働旬報社　1981-1986

川瀬清他編　「ノーモアスモン：スモンの恒久対策の確立と薬害根絶のために」新日本医学出版社　1980

下山瑛二　「健康権と国の法的責任：薬品・食品行政を中心とする考察」岩波書店　1979

高野哲夫　「スモン被害：薬害根絶のために」三一書房　1979

亀山忠典他編　「薬害スモン」大月書店　1977

宝月誠編　「薬害の社会学―薬と人間のアイロニー―」世界思想社　1986

高橋秀臣　「スモン訴訟の真相」行政通信社　1979

スモン損害賠償研究会　「スモンと損害賠償」勁草書房 1986

Changes of Ultrasonic Bone Assessment in Subacute Myelo-Optico-Neuropathy (SMON) Patients

—Longitudinal Findings in 4-8 Years—

Katsumi Yamanaka[1], Takatoshi Ujihira[2], Shizuyo Inaba[3] and Nakako Fujiwara[4]

ABSTRACT

Objective: Decreases of ultrasonic bone assessment in subacute myelo-optico-neuropathy (SMON) patients were measured over a period of 6 years. The decreases themselves and factors related to this were discussed. **Method**: A longitudinal analysis was carried out on data collected on 35 female SMON patients. We evaluated stiffness (St) which was calculated on the calcaneus using an ultra sonic bone densitometer on two occasions between 1994 and 2003. The decreases in St from the first examination to the second were analyzed. **Result**: The mean decrease in St and Standard Deviation (SD) was 10.9±6.9. The mean decrease of St and SD per year was 1.97±1.37. The largest decrease (13.4) of St with statistical significance was recognized in the moderate, severe group compared to the slight group in the SMON disturbance severity categories. Age, height, Barthel index and a history of bone fracture seemed to bear a relationship to the decrease of St. **Conclusion**: Our results suggest that the decrease of ultrasonic bone assessment in SMON patients was higher than in healthy people and such a decrease was associated with the SMON disturbance severity.

INTRODUCTION

Subacute myelo-optico-neuropathy (SMON) due to 5-chloro-7-iodo-8-hydroxyl-quinoline (clioquinol, chinoform) broke out in Japan in the years around 1950-1960. SMON patients ordinarily started with abdominal symptoms (abdominal pain, diarrhea *etc.*) before the onset of neurological symptoms. The cardinal signs are an acute or sub-acute onset of bilateral ascending parasthesia and dysesthesia of lower extremitas. Other major signs are the impairment of deep sensation, weakness in the lower limbs, bi-lateral impairment of vision, greenish discoloration of the tongue and feces and sphincter disturbances. There are no significant laboratory findings in the blood and cerebral spinal fluid. After withdrawal from

Key words : Ultrasonic bone assessment, Subacute myelo-optico-neuropathy (SMON), Chinoform, SMON disturbances severity, Barthel index

[1] Department of Public Health, School of Nutritional Sciences, Nagoya University of Arts and Sciences, [2] Division of Health Promotion, Health Bureau, Nara Prefecture, Japan, [3] Health Department, Health & Welfare Bureau City of Nagoya, Japan, [4] School of Nursing & Health, Graduate School of Nursing & Health, Aichi Prefectural University

medication the disease comes to a standstill. In severe cases, sensory or motor disorders remain in the lower half of the body. In mild cases, these disorders gradually improve within months or years. Sensory disorders tend to persist for longer periods. Visual disturbances improve, but in some cases, blindness remains[1]. The Ministry of Health and Welfare recognized SMON as a drug-adverse disease in 1960. Since 1960, the Ministry has formed a SMON research committee and has supported SMON patients by examining their health condition. The total number of patients reached over 9,000 according to a national survey in 1970. In 1986 (since the end of the SMON outbreak) there were around 4,000 patients according to a national survey. These patients suffered from superficial sensory disorder, dysethesia and paresthesia in all cases and motor and gait disturbances in half the cases in 1996[2]. In Aichi Prefecture, there were around 400 patients in 1960. Since 1960, the Aichi Prefecture SMON research committee has examined SMON patients at the request of the Aichi Prefecture SMON patient group. An examination was held once every three or four years in Aichi Prefecture. Between 1990 and 1993, it was clear that the rate of SMON patients with fractures was higher in elderly persons who live in residential areas and many patients with fractures showed a low mineral bone mass[3~5]. Due to these results and those from the Aichi Prefecture SMON patient group, bone mineral assessment (stiffness by ultrasound) has been added to the examinations since 1994. As there has been no report on the lowering of stiffness changes of SMON patients in any cohort study, we would like to report it here.

METHOD

1 Background of This Study and Participants

A general examination of SMON patients has been conducted since 1960. The measurement of ultrasonic bone assessment was added to this in 1994 when there were 161 SMON patients in Aichi Prefecture. Eighty eight patients did not have ultrasonic bone assessments in the examination due to the following reasons: impaired leg movement, they were under doctor consultation, examination deemed not comprehensive enough and they thought there was little benefit from such an examination. Seventy three (8 male and 65 female) had a bone mineral assessment test once in the first survey between 1994 and 1997. In 2001, the second survey began and from a total of 144 SMON patients (losing 17 people either from moving out of Aichi Prefecture or death) 65 (7 male and 58 female) SMON patients took the second survey. Male patients were excluded from the subjects due to the low number of participants. A female patient who was under 50 years old was excluded from the subjects due to hormonal influence. Finally, there were 35 female patients who took the ultrasonic bone assessment measurement test in both surveys.

2 Ethics

All participants provided written informed consent. This study was approved by the Ethics Committee of the Nagoya University of Arts and Sciences.

3 Ultrasonic Bone Assessment Measurements

We evaluated the index stiffness (St) which was calculated from the speed of sound (SOS in m/s) and broadband ultrasonic attenuation (BUA in dB/MHz) on the os calcis using an ultrasonic bone densitometer (A-1000 Achilles of Lunar Corp)[6]. All measurements were made using the same equipment (A-1000) and the same version's software. In the measurement environment, the temperature 30℃ was maintained. Before the measurements, we corrected the values of measurements using the same phantom. We measured St in the first survey (St 1) and St in the second survey (St 2). Then changes from St 1 to St 2 were calculated. Decreases of St were defined as St decrease. From St we also calculated a percent age-adjusted St (St Percent) which shows the percentage of the average stiffness (St) of a healthy Japanese female in the same age group.

Table 1 Mean age, stiffness and percentage-adjusted stiffness of SMON patients in Survey 1 and Survey 2

	Survey 1	Survey 2
Number of patients	35	35
Mean age (SD)	66.8 (7.6)	73.2 (7.4)
Mean stiffness (SD)	60.2 (9.7)	48.9 (11.0)
Mean percent age-adjusted stiffness (SD)	85.1 (11.9)	73.7 (15.0)

SD : standard deviation

4 Anthropometric Measurements

Besides ultrasonic bone assessment measurements, we obtained body indices (height, weight and body mass index (BMI)), SMON disturbances severity, Barthel index (BI) [7], Diseases of spine or joint and history of bone fractures in the last three years. Six factors were divided into two groups. These were; Age: under 65 years old, and 65 years old and over. Height: 150 cm or more, less than 150 cm. Weight: 45 kg or more and less than 45 kg. BMI: 21 and over and less than 21. Bone fracture: absent, present. The SMON disturbance severity (Severity) was dependent on the total level of disturbances, these were sensory disturbances, dysesthesia, motor dysfunction and visual disturbances and these were examined in the patients during the routine SMON examination. SMON Severity is usually classified into 5 types: extremely slight, slight, moderate, severe, extremely severe. In this study the two groups (one group as extremely slight, slight and the other group as moderate, severe, extremely severe) were used for analysis. BI is the most widely used general disability measurement[7]. BI is ranked according to a total score (0 to 100) from 10 items. Total score 0 means complete dependence and 100 means complete independence for all 10 items. 95 means there is dependence for one item and less than 95 means there is dependence for 2 items or more. BI was divided into the two groups (score 100 and less than 100).

5 Data Analysis

Mean St and mean percent age-adjusted St were compared between Survey 1 and Survey 2 in **Table 1**. Mean St in Survey 1, mean St in Survey 2 and mean St decrease were shown in **Table 2** respectively. The Mann-Whitney test (non-parametric test in independent samples) was used to compare differences of St and St decrease between each category of the 8 factors. The data was calculated using SPSS (Statistical Package for the Social Sciences) 16.0 J for Windows. The level of significance was set at $p < 0.05$.

RESULT

1 Participants' Period from Survey 1 to Survey 2

The number of participants according to the period of time from Survey 1 to Survey 2 was as follows: 15 people for 8 years, 8 people for 7 years, 3 people for 5 years and 9 people for 4 years. The mean number of years for which participants were followed for was 6.5 years.

2 Participants' Age

The number of participants followed up from Survey 1 to Survey 2 was 35. The mean age and standard deviation (SD) of participants was 66.8±7.6, the age range was between 50 to 81 years old and the number of participants in each age group was as follows; 5 were in the 50-59 year old group, 16 were in 60-69 year old group and 14 were in the 70-85 year old group for Survey 1. The mean age and SD of participants was 73.2±7.4, the range was between 58 to 87 and the number of participants according to the age groups was as follows; 2 were in the 50-59 year old group, 7 were in 60-69 year old group and 26 were in 70-85 year old group for Survey 2 (**Table 1**).

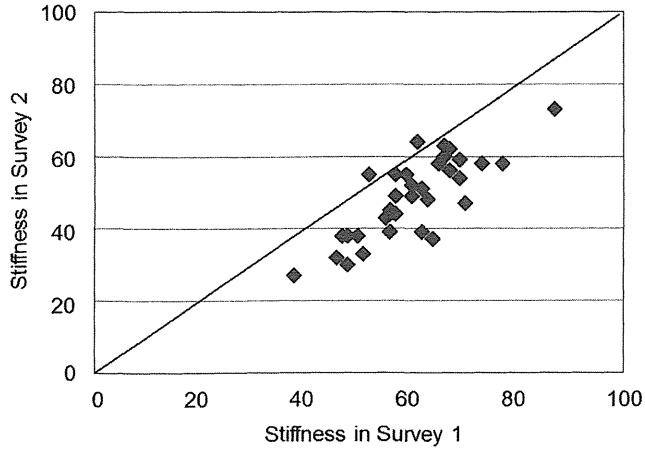


Fig.1 Scattergram of stiffness of SMON patients in Survey 1 and Survey 2 ($n=35$)

3 St in Survey 1 and Survey 2

The mean St 1 and SD was 60.2±9.7 and the mean percent age adjusted Stiffness (St percent) in Survey 1 and SD was 85.1±11.9. The mean St 2 and SD was 48.9±11.0 and the mean St percent in Survey 2 and SD was 73.7 ± 15.0 (**Table 1**). The number of participants with a decrease of ST was 33 and there were 2 participants with an increase in ST. The mean decrease of St and SD from Survey 1 to Survey 2 was 11.3±6.7. For the 33 participants with a decrease of St, the range of a decrease of St was between 2 and 27 as shown in **Fig.1**, and, the mean decrease of St and SD in a year was 1.9±1.4. The relationship of St between Survey 1 and Survey 2 is shown in **Fig.1**.

4 Factors Related to Stiffness

Eight factors relating to St were analyzed. These 8 factors were divided into two groups (categories). The mean St in each category of the 8 factors was shown in **Table 2**. The largest decrease (13.4) of St was seen in the severe, moderate group (Severity-factor). The smallest decrease (7.6) of ST was seen in the slight group (Severity-factor). The difference in decrease for the categories across the 8 factors was the highest in the Severity category.

1) Age

The St in the group (65 years old and over) was significantly lower than in the under 65 year old group in both Survey 1 and Survey 2. The decrease of St in the group (65 years old and over) was higher than in the under 65 year old group, however the decrease was not statistically significant between the two age groups.

2) Height

The St for the group (150cm or more) was higher than in the group (less than150 cm) for both surveys. The decrease of St for the group (150cm or more) was higher than in the group (less than 150 cm); however the decrease was not statistically significant between the two height groups.

3) Weight

The St for the group (45kg or more) was significantly higher than in the group (less than 45 kg) for both surveys. The decrease of St for the group (45 kg or more) was lower than in the group (less than 45 kg) however, the decrease was not statistically significant different between the two weight groups.

4) BMI

The St for the group (21 and over) was significantly higher than in the group (less than 21) for both surveys. The decrease of St in the two groups was approximately similar.

Table 2 Mean stiffness of Survey 1 and Survey 2 and decrease of St from Survey 1 to Survey 2 according to 8 factors

Factors	Categories	Number	Mean stiffness (St)		Mean St decrease
			Survey 1	Survey 2	
Age	<65 years	13	**67.1±8.5	**56.5±7.7	9.3±8.7
	≧65 years	22	56.1±8.0	44.5±10.4	11.6±6.7
Height	≧150cm	15	62.5±12.1	49.8±12.5	12.7±6.5
	<150cm	20	58.5±7.3	48.3±10.1	10.2±6.8
Weight	≧45kg	18	*63.9±8.9	*53.1±11.0	10.8±7.7
	<45kg	17	56.3±9.2	44.6±10.6	11.7±5.6
BMI	≧21	18	*64.0±8.9	*53.0±9.9	11.0±7.6
	<21	17	56.2±9.1	44.6±10.9	11.5±5.8
Severity	Slight	13	60.2±7.2	52.6±7.7	**7.6±4.9
	Moderate, Severe	22	60.2±11.1	46.8±12.3	13.4±6.8
Barthel index	100	16	63.0±8.9	52.9±9.7	10.1±5.2
	<100	19	57.8±9.9	45.6±11.2	12.3±7.7
Disease of spine or joint	−	16	57.4±9.6	47.0±12.1	10.4±8.2
	+	19	62.6±9.4	50.6±10.1	12.0±5.2
History of bone fracture	−	20	61.3±9.9	49.3±11.5	12.6±11.4
	+	15	58.7±9.6	48.4±10.8	10.3±6.0

**: There was a significant ($p<0.01$) difference of St or St decrease between the groups using the Mann-Whitney test.

*: There was a significant ($p<0.05$) difference of St decrease between the groups using the Mann-Whitney test.

5) Severity

The St was the same in the two groups in Survey 1. However, the St in the moderate, severe group was lower than the slight group in Survey 2. The decrease of St in the moderate, severe group was significantly higher than the slight group.

6) Barthel index

The St in the group with a Barthel index of 100 was higher than in the group with a Barthel index of less than 100 in both surveys. The decrease of St in the group with a Barthel index of 100 was lower than in the group with a Barthel index of less than 100, however, the decrease was not statistically significant different between the two Barthel index groups.

7) Diseases related to spine or joints

The St in the group with the disease was higher than in the group without the disease in both surveys. The decrease of St in the group with the disease was higher than the group without the disease, however, the decrease was not statistically significant different between the group of diseases of spine or joint (absent) and (present).

8) History of bone fractures

The St in the group without a history of bone fractures was higher than in the group with a history of bone fractures. The decrease of St in the group without bone fracture was higher than in the group with a history of bone fractures. However, the decrease was not statistically significant different between the

group of history of bone fracture (−) and (+).

DISCUSSION

Quantitative ultrasound (QUS) was introduced for indirect measurement of bone quality[8,9] and is considered as an alternative technique to provide information on bone in order to identify subjects with a high risk of bone fragility[10]. The Stiffness (St) index is measured using the "Achiless" ultrasound bone densitometer (Lunar). The value of St was shown in several papers[6,11,12]for clinical purposes. St was highly correlated with the spine, femoral neck and total body bone mineral density (BMD) using dual X-ray absorptiometry (DXA)[6,11]. Calcaneal SOS and BUA also correlated well with calcaneal BMD[12]. Several studies concerning osteoporosis, BMD, SOS and BUA in SMON patients were reported. The first study[3] showed that there were no significant differences in the prevalence of osteoporosis between SMON patients and a normal control group, however, more advanced osteoporotic changes were observed in the severely affected SMON cases. This was followed by reports[13,14] that female SMON patients had lower St than healthy females when using an ultrasound bone densitometer. The SMON patients with a history of bone fractures had lower St than patients without a history[13]. From the results showing that SMON patient's St Percent was 80% when compared to a person of the same age in this study, it could be considered that SMON patients had an osteoporotic condition, compared to healthy people. However, any St decrease or BMD decrease in SMON patients in cohort studies are poorly known. St decrease from Survey 1 to Survey 2 in this study was 1.9 (3.1%) per year. This value of 1.9 was higher than 0.56 per year in healthy Japanese females using Achiless[6] and also higher than 0.82 per year in an average Japanese female group in another study using Achiless[11] The decrease (3.1%) in this study was higher than a 1.01% decrease after 23 months in nursing home residents using Achilles in a previous report[15].

The difference in St between categories was calculated in 8 factors in this study. The most prevalent difference was observed in age. This result consisted of previous reports, which clarified negative associations between age and St or age and BMD[6,11,12,16~21]. This study showed that the higher St was observed in the higher height, higher weight and higher BMI group. This result was also consistent with previous papers which showed a positive correlation between BMD and height, BMD and weight, and BMD and BMI. The results above mentioned showed that SMON patients had the same health condition as healthy people.

There are many reports which show there is a relationship between insufficient physical activity and a low BMD[17,22~25]. SMON patients have largely limited physical activity in their daily lives due to the physically handicapped aspect of SMON. In this study low physical activity is considered to cause low St in these SMON patients with severe or moderate SMON disturbance, low BI and present diseases related to the spine or joints.

SMON patients can easily fall which leads to broken bones, because they are handicapped in their general mobility and have a low St. Patients with a history of bone fractures had a low mean St in both surveys, however, they had a low mean St decrease in this study. This reason is not clear in this study.

Physically handicapped people with spinal cord injuries or cerebral palsy were reported to have a low St.[26,27] There was no contra-indication to these reports that the SMON patients had a low St.

A low St and a higher decrease of St in SMON patients are crucial if we are to consider their health condition. Currently, the national survey and health examination of SMON patients are conducted annually in Japan. The measurement of bone mass density should be added to routine examinations to prevent osteoporosis and bone fractures.

REFERENCES

1) The National Institute of Health: Reviews on SMON. Japanese Journal of Medical Science & Biology 1975;28: 1-293.
2) Iida M, Matumoto A, Itoh H, Chida K, Sobue G, Saida K, et al. Analysis on clinical examination of SMON patients in 1995 In: Iwashita H, editor. Annual report of SMON research committee (1995). Chikugo: SMON Research Committee:1996;17-22.
3) Ando H, Yamada T, Kachi T, Tamura T, Harada A. Osteoporosis in subacute myelo-optico-neuropathy in 1989. In : Ando K, editor Annual report of SMON research committee (1989). Ohbu : SMON Research Committee : 1990;235-8.
4) Ando H, Yamada T, Kachi T, Tamura T, Sobue I. Osteoporosis in subacute myelo-optico-neuropathy (2nd report) in 1990. In : Ando K, editor Annual report of SMON research committee (1990). Ohbu :SMON Research Committee : 1991; 182-4.
5) Ando H, Kachi T, Yamada T. Risk factors for fractures in subacute myelo-optico-neuropathy in 1992. In: Ando K, editor. Annual report of SMON Research Committee (1992), Ohbu : SMON Research Committee;1993: 203-7.
6) Yamazaki K, Kushida K, Ohmura M, Sana M, Inoue T. Ultrasound bone densitometry of the os calcis in Japanese women. Osteoporos Int 1994;4:220-5.
7) Mahoney FT, Barthel DW. Functional evaluation: the Barthel index. Maryland State Medical Journal 1965; 14: 61-5.
8) Njeh CF, Boilvin CM, Langton CM. The role of ultrasound in the assessment of osteoporosis: A review. Osteoporos Int 1997;7:7-22.
9) Malavolta N, Mule' R, Frigato M. Quantitative ultrasound assessment of bone. Aging Clin Exp Res 2004;16: 23-8.
10) Gregg EW. The epidemiology of quantitative ultrasound: a review of the relationships of bone mass, osteoporosis and fracture risk. Osteoporos Int 1997;7:89-99.
11) Yamazaki K, Kushida K, Ohmura M, Sana M, Sato Y, Inoue T. Ultrasound bone densitometry of the os calcis. Ther Res 1992;13:585-93.
12) Yuh I, Yamamoto I, Ohnaka Y, Takada M, Nakajima K, Masuda K, et al. Clinical evaluation of ultrasonic measurement of the calcaneal bone. Nihon Houi Kaisi 1993;53:1340-6.
13) Yamanaka K, Fujiwara N. Factors related to ultrasonic bone density in subacute myelo-optico neuropathy (SMON) patients. Annual Report of Institute of Health and Nutrition, Nagoya University of Arts and Sciences 2007;19-25.
14) Yamada M, Takayanagi T, Omae R. Bone mineral density of patients with subacute myelo-optico-neuropathy (SMON) in 1993. In: Iida M editor. Annual report of SMON research committee (1993). Suzuka; SMON Research Committee 1994;158-61.
15) Krieg MA, Thiebaud D, Burckhardt P. Quantitative Ultrasound of bone in institutionalized elderly women: A Cross-sectional and longitudinal study. Osteoporos Int 1996;6:189-95.
16) Jones G, Nguyen T, Smbrook P, Kelly PJ, Eisman JA. Progressive loss of bone in the femoral neck in elderly people: longitudinal finding from the Dubbo osteoporosis epidemiology study.BMJ 1994;309:691-5.
17) Uusi-Rasi K, Sievänen H, Pasanen M, Oja P, Vuori I. Maintenance of body weight, physical activity and calcium intake helps preserve bone mass in elderly women. Osteoporos Int 2001;12:373-9.
18) Dennison E, Eestell R, Fall CHD, Kellingray S, Wood PJ, Cooper C. Determinants of bone loss in elderly men and women: A prospective population-based study. Osteoporos Int 1999;10:384-91.
19) Toyokawa S, Nishikawa H, Ueji M, Motegi K, Kano K. Structural equation modeling of the relationship of bone mineral density and its risk factors in Japanese women. Environ Health Prev Med 2001;6:41-6.
20) Flohr F, Lutz A, App EM, Matthys H, Reincke M. Bone mineral density and quantitative ultrasound in adults with cystic fibrosis. Eu J Endocr 2002;146:531-6.
21) Yoshimi I, Aoyagi K, Okano K, Yahata Y, Kusano Y, Moji K, et al. Stiffness index of the calcaneus measured by

quantitative ultrasound and menopause among Japanese women: The Hizen-Oshima study. Tohoku J Exp Med 2001;195:93-9.
22) Uusi-Rasi K, Sievänen H, Vuori I, Pasanen M, Heinonen A, Oja P. Maintenance of body weight, physical activity and calcium intake with bone mass and size in healthy women at different ages. J Bone Miner Res 1989;13:133-42.
23) Uusi-Rasi K, Sievänen H, Pasanen M, Oja P, Vuori I. Association of calcium intake and physical activity with bone mass and size in premenopausal and postmenopausal women: a peripheral quantitative computed tomography study. J Bone Miner Res 2002;17:544-52.
24) Cussler EC, Going SB, Houtkooper LB, Stanford CA, Blew RM, Flint-Wagner HG, et al. Exercise frequency and calcium intake predict 4-year bone changes in postmenopausal women. Osteoporos Int 2005;16:2129-41.
25) Heinonen A, Oja P, Sievanen H, Pasanen M, Vuori I. Effect of two training regimens on bone mineral density in healthy perimenopausal women: A randomized controlled trial. J Bone Miner Res 1998;139:483-90.
26) Chow YW, Inman C, Pollintine P, Sharp CA, Haddaway MJ, el Masry W, et al. Ultrasound bone densitometry and dual energy X-ray absorptiometry in patients with spinal cord injury: a cross-sectional study. Spinal Cord 1996;34:736-41.
27) Hartman C, Brik R, Tamir A, Merrick J, Shamir R. Bone quantative ultrasound and nutritional status in severely handicapped institutionalized children and adolescents. Clin Nutr 2004;23:89-98.

〈2010年8月3日 受稿〉

Toxicology 299 (2012) 55–59

Contents lists available at SciVerse ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

Clioquinol induces DNA double-strand breaks, activation of ATM, and subsequent activation of p53 signaling

Masato Katsuyama[a,b,*], Kazumi Iwata[b], Masakazu Ibi[b], Kuniharu Matsuno[b], Misaki Matsumoto[b], Chihiro Yabe-Nishimura[b]

[a] Radioisotope Center, Kyoto Prefectural University of Medicine, Kyoto 602-8566, Japan
[b] Department of Pharmacology, Kyoto Prefectural University of Medicine, Kyoto 602-8566, Japan

ARTICLE INFO

Article history:
Received 13 April 2012
Received in revised form 11 May 2012
Accepted 14 May 2012
Available online 22 May 2012

Keywords:
Clioquinol
SMON
ATM
p53
Neurotoxicity

ABSTRACT

Clioquinol, a $Cu^{2+}/Zn^{2+}/Fe^{2+}$ chelator/ionophor, was used extensively in the mid 1900s as an amebicide for treating indigestion and diarrhea. It was eventually withdrawn from the market because of a link to subacute myelo-optic neuropathy (SMON) in Japan. The pathogenesis of SMON, however, is not fully understood. To clarify the molecular mechanisms of clioquinol-induced neurotoxicity, a global analysis using DNA chips was carried out on human neuroblastoma cells. The global analysis and quantitative PCR demonstrated that mRNA levels of $p21^{Cip1}$, an inhibitor of cyclins D and E, and of GADD45α, a growth arrest and DNA damage-inducible protein, were significantly increased by clioquinol treatment in SH-SY5Y and IMR-32 neuroblastoma cells. Activation of p53 by clioquinol was suggested, since clioquinol induced phosphorylation of p53 at Ser15 to enhance its stabilization. The phosphorylation of p53 was inhibited by KU-55933, an inhibitor of ataxia-telangiectasia mutated kinase (ATM), but not by NU7026, an inhibitor of DNA-dependent protein kinase (DNA-PK). Clioquinol in fact induced phosphorylation of ATM and histone H2AX, a marker of DNA double-strand breaks (DSBs). These results suggest that clioquinol-induced neurotoxicity is mediated by DSBs and subsequent activation of ATM/p53 signaling.

1. Introduction

Clioquinol (5-chloro-7-indo-8-quinolinol) was used extensively as an amebicide for treating indigestion and diarrhea in the mid 1900s. Later it was withdrawn from the market because its use was epidemiologically linked to the incidence of subacute myelo-optic neuropathy (SMON) in Japan (Cahoon, 2009). SMON is characterized by subacute onset of sensory and motor disturbances in the lower extremities and visual impairment (Nakae et al., 1973; Tsubaki et al., 1971). Although pathological studies demonstrated axonopathy of the spinal cord and optic nerves (Tateishi, 2000), the underlying mechanisms of clioquinol toxicity are yet to be elucidated. In PC12 cells, clioquinol suppressed nerve growth factor-induced Trk autophosphorylation and neurite outgrowth (Asakura et al., 2009). In cultured dorsal root ganglion neurons, clioquinol induced mechanical hyperalgesia and cold allodynia via activation of TRPA1 (Andersson et al., 2009). When injected into young rats, it attenuated dentate gyrus long-term potentiation (Takeda et al., 2010).

Recently, clioquinol has been reevaluated as a prototype for metal-protein-attenuating compounds that decrease oxidative stress and deposits of metalloproteins in patients with Alzheimer disease (Adlard et al., 2008; Cherny et al., 2001), Parkinson disease (Kaur et al., 2003), and Huntington disease (Nguyen et al., 2005). Especially for Alzheimer disease, the effectiveness of clioquinol and its derivative PBT2 was verified by phase II clinical trials (Faux et al., 2010; Lannfelt et al., 2008; Ritchie et al., 2003). These beneficial effects appeared to be mediated by prevention of protein aggregation via chelation of metal ions. Clioquinol was also reported to inhibit the aging-associated mitochondrial enzyme CLK-1 (Wang et al., 2009). Another line of investigation, however, demonstrated that cytotoxicity of clioquinol was mediated by oxidative stress (Benvenisti-Zarom et al., 2005), inhibition of the 20S proteasome (Mao et al., 2009), or induction of the cytoplasmic clearance of X-linked inhibitor of apoptosis protein (XIAP) (Cater and Haupt, 2011). These cytotoxic effects were also thought useful for cancer therapy (Ding et al., 2005).

Clioquinol has been recognized conventionally as a Cu^{2+}/Zn^{2+} chelator (Cherny et al., 2001; Choi et al., 2006). It also works as a Fe^{2+} chelator (Kaur et al., 2003; Wang et al., 2009). Furthermore, it was recently shown to work as a $Cu^{2+}/Zn^{2+}/Fe^{2+}$ ionophore that

* Corresponding author at: Radioisotope Center, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Kamigyo-ku, Kyoto 602-8566, Japan. Tel.: +81 75 251 5381; fax: +81 75 251 5381.
E-mail address: mkatsuya@koto.kpu-m.ac.jp (M. Katsuyama).

0300-483X/$ – see front matter © 2012 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.tox.2012.05.013

Table 1
Sequences of primers used in quantitative PCR.

Gene		Primer sequences
$p21^{Cip1}$	Sense	5'-aggggacagcagaggaaga-3'
	Antisense	5'-ggcttcctcttggagaagatcag-3'
GADD45α	Sense	5'-ccacattcatctcaatggaag-3'
	Antisense	5'-cagggagattaatcactggaa-3'
HPRT	Sense	5'-agactttgctttccttggtca-3'
	Antisense	5'-aggctttgtattttgcttttc-3'

drives these metal ions into the cell (Andersson et al., 2009; Ding et al., 2005). Thus, the characteristics of clioquinol and its effects on neuronal cells have been controversial. To clarify the molecular mechanisms underlying clioquinol-induced neurotoxicity, we carried out a global analysis using DNA chips in human neuroblastoma cells and demonstrated that clioquinol activates ATM and subsequent p53 signaling.

2. Materials and methods

2.1. Materials

Clioquinol, KU-55933 (InSolution ATM Kinase Inhibitor), U0126, and MnTBAP were purchased from Merck (Darmstadt, Germany). NU7026 was obtained from Cayman Chemicals (Ann Arbor, MI). SB239063 was purchased from Alexis Biochemicals (San Diego, CA). Antibodies against phospho-p53 (Ser15) and p53 (DO-1) were purchased from Cell Signaling Technology (Danvers, MA) and Medical and Biological Laboratories (Nagoya, Japan), respectively. The antibody against β-actin was obtained from Sigma (St. Louis, MO). Antibodies against phospho-ATM (Ser1981), ATM, histone H2AX (phospho-Ser139), and histone H2AX were purchased from Epitomics (Burlingame, CA).

2.2. Cell culture

Human SH-SY5Y cells, purchased from European Collection of Cell Cultures, were cultured in Ham's F12: Eagle's medium with Earle's salts (1:1) supplemented with non-essential amino acids and 15% fetal bovine serum (FBS). IMR-32 cells from the JCRB Cell Bank were cultured in Eagle's medium with Earle's salts supplemented with non-essential amino acids and 10% FBS.

2.3. Cell proliferation assay

SH-SY5Y or IMR-32 cells were grown in 96-well plates in the presence or absence of clioquinol for 24 h. Cell proliferation was quantitated using a CyQuant Direct Cell Proliferation Assay Kit (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions.

2.4. Microarray analysis

SH-SY5Y cells were grown in the presence or absence of 50 μM clioquinol for 24 h. Total RNA isolated using an RNeasy Plus Mini kit (Qiagen, Hilden, Germany) was prepared for labeling and hybridization to a human Oligo chip 25k (Toray, Tokyo, Japan) using standard methods. The GEO database accession code for the microarray data obtained is GSE32173.

2.5. Quantitative PCR

Total RNA was reverse transcribed to first-strand cDNA using a ReverTra Ace qPCR RT Kit (TOYOBO, Osaka, Japan), and quantitative PCR was performed in a StepOnePlus Real-Time PCR System (Life Technologies) using THUNDERBIRD SYBR qPCR Mix (TOYOBO). Gene expression was quantified using standard curves that were generated using serially diluted plasmid reference samples and normalized to the expression level of hypoxanthine phosphoribosyltransferase (HPRT). The specificity of the PCR products was confirmed by gel electrophoresis and a dissociation curve analysis. Primer sequences are shown in Table 1.

2.6. Western blotting

Whole cell lysate was prepared essentially as described previously (Fan et al., 2005; Katsuyama et al., 2005). Briefly, cells were lysed in a buffer containing 1% Triton, 0.5% sodium deoxycholate, 10 mM Tris–HCl (pH 6.8), 150 mM NaCl, 1 mM EDTA, protease inhibitor cocktails (Nacalai Tesque, Kyoto, Japan), 1 mM NaF, 20 mM β-glycerophosphate, and 1 mM Na_3VO_4. The lysate was centrifuged and the supernatant was used as whole cell lysate. Aliquots containing equal amounts of protein (10 μg) were subjected to SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). Hybridization of antibodies and washing were carried out essentially as described previously (Fan et al., 2005; Katsuyama et al., 2005). Representative autoradiographs from three experiments are shown in the figures.

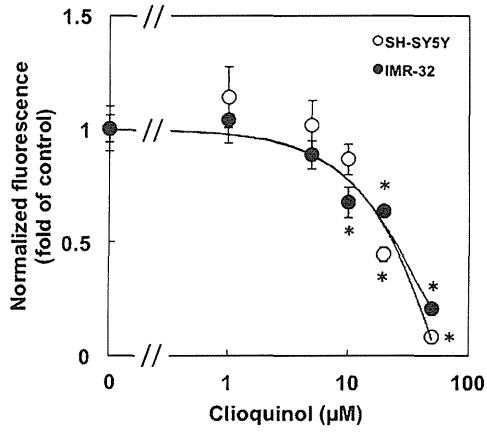


Fig. 1. Clioquinol suppressed proliferation of SH-SY5Y and IMR-32 neuroblastoma cells. SH-SY5Y and IMR-32 cells were grown in 96-well plates in the presence or absence of clioquinol for 24 h. Cell proliferation was quantitated using a CyQuant Direct Cell Proliferation Assay Kit. Open circles, SH-SY5Y; closed circles, IMR-32. *$P < 0.05$ vs. control ($N = 8$).

2.7. Statistical analysis

Values were expressed as the mean ± SE. The statistical analysis was performed using Student's *t* test. For multiple treatment groups, one-way ANOVA followed by Bonferroni's *t* test was applied.

3. Results

3.1. Clioquinol suppressed proliferation of SH-SY5Y and IMR-32 human neuroblastoma cells

We first examined whether clioquinol affects the proliferation of neuroblastoma cells using the CyQuant Direct Cell Proliferation Assay Kit. As shown in Fig. 1, treatment with clioquinol for 24 h significantly suppressed proliferation of SH-SY5Y and IMR-32 cells at concentrations higher than 10–20 μM.

3.2. Clioquinol increased levels of mRNA for $p21^{Cip1}$ and GADD45α

A global analysis with DNA chips was carried out using SH-SY5Y cells grown in the presence or absence of 50 μM clioquinol for 24 h. Among approximately 25,000 genes, 2429 were up-regulated in their expression and 2727, down-regulated, by clioquinol (GEO database accession code: GSE32173). Notably, the expression of $p21^{Cip1}$, an inhibitor of cyclins D and E, and that of a growth arrest and DNA damage-inducible protein, GADD45α, were up-regulated by treatment with clioquinol. Both of these proteins are dependent on p53, a tumor suppressor. Up-regulation of the mRNA expression for these proteins was verified by quantitative PCR in SH-SY5Y and IMR-32 cells (Fig. 2).

3.3. Clioquinol induced phosphorylation of p53 at Ser15

The up-regulation of $p21^{Cip1}$ and GADD45α expression suggests that p53 is activated by clioquinol. We therefore examined whether phosphorylation at Ser15, an indicator of the activation of p53, is enhanced by clioquinol treatment. As shown in Fig. 3A, treatment with clioquinol for 24 h markedly induced phosphorylation of p53 in SH-SY5Y cells at concentrations higher than 10 μM. In IMR-32

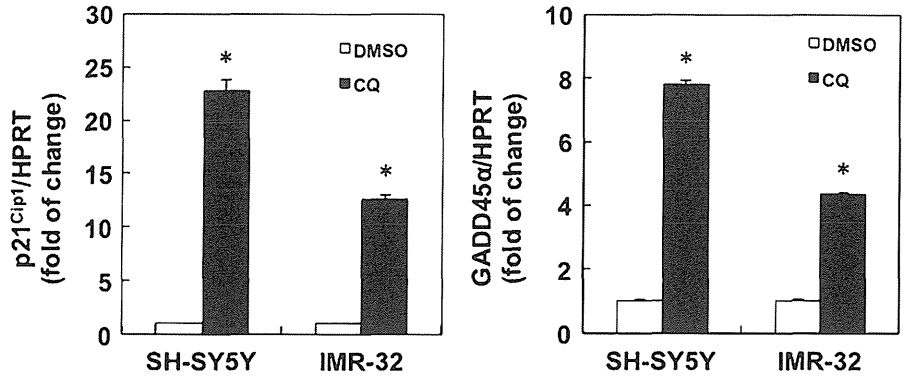


Fig. 2. Clioquinol increased the expression of mRNAs for $p21^{Cip1}$ and GADD45α. Cells were grown in the presence or absence of 50 μM clioquinol for 24 h. Transcript levels were measured by quantitative PCR and normalized to the level of hypoxanthine phosphoribosyltransferase (HPRT) mRNA. $^*P < 0.05$ vs. DMSO ($N = 3$).

cells, phosphorylation of p53 was induced even at 5 μM. Concomitantly, total p53 levels were also increased by clioquinol treatment at higher concentrations. This may be attributable to its stabilization by phosphorylation at Ser15 and inhibition of the interaction between p53 and MDM2 (Kruse and Gu, 2009). Time course experiments indicated that phosphorylation of p53 was induced within 24 h (Fig. 3B).

3.4. *Clioquinol-induced phosphorylation of p53 was inhibited by KU-55933, an inhibitor of ataxia telangiectasia mutated (ATM) kinase*

Phosphorylation of p53 at Ser15 was reported to be catalyzed by ATM, ATM-Rad3-related kinase (ATR), DNA-dependent protein kinase (DNA-PK), p38 MAP kinase, and extracellular signal-regulated protein kinase (ERK) (Kruse and Gu, 2009; She et al., 2000). Therefore, we next examined which kinase is involved in the activation of p53. As shown in Fig. 4, KU-55933, a specific inhibitor of ATM, suppressed phosphorylation of p53 at Ser15 induced by clioquinol treatment. On the other hand, NU7026, an inhibitor of DNA-PK, did not affect the phosphorylation. SB239063, a p38 inhibitor, and U0126, a MEK inhibitor that suppresses ERK activation, were also ineffective against the phosphorylation of p53

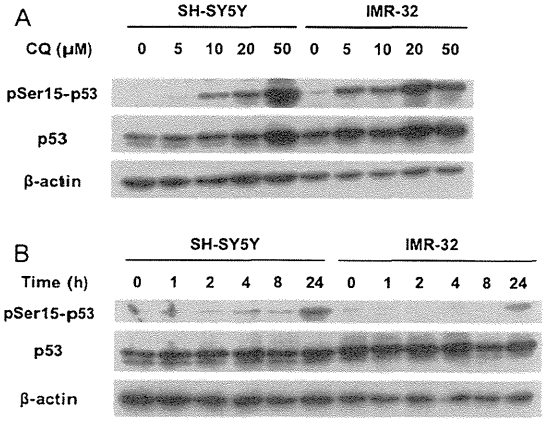


Fig. 3. Clioquinol induced phosphorylation of p53 at Ser15. (A) A dose-dependent increase in p53 phosphorylation. Cells were grown in the presence or absence of clioquinol for 24 h and whole cell lysate was isolated. (B) The time course of p53 phosphorylation. Cells were grown in the presence of clioquinol (20 μM for SH-SY5Y, 10 μM for IMR-32). A representative blot of three independent experiments is shown.

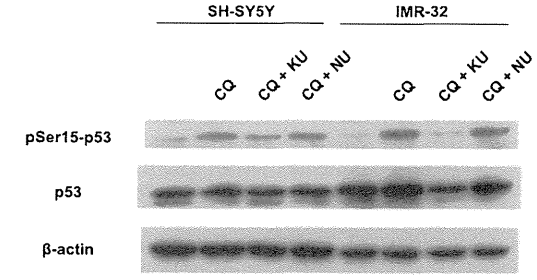


Fig. 4. Clioquinol-induced phosphorylation of p53 was suppressed by KU-55933, an inhibitor of ATM. Cells were grown in the presence or absence of clioquinol and 10 μM KU-55933 or NU7026 for 24 h. The experiment was repeated three times and a representative result is shown.

(data not shown). These results suggest that clioquinol-induced activation of p53 is mediated by ATM.

3.5. *Clioquinol induced phosphorylation of ATM*

We next examined whether clioquinol activates ATM. As shown in Fig. 5, clioquinol induced phosphorylation of ATM within 24 h in both cell lines, indicating that ATM is activated by clioquinol treatment.

3.6. *Clioquinol induced phosphorylation of histone H2AX*

It is well known that ATM is activated by DNA double-strand breaks (DSBs) induced by ionizing radiation (Bakkenist and Kastan, 2003). We therefore examined whether clioquinol induces DSBs. As shown in Fig. 6, clioquinol markedly induced phosphorylation of histone H2AX, a marker of DSBs, at 4 h in SH-SY5Y cells. Phosphorylation of histone H2AX was also detected in IMR-32 cells at 2 h, though to a much lesser extent. These results suggest that clioquinol-induced DSBs are involved in the activation of ATM.

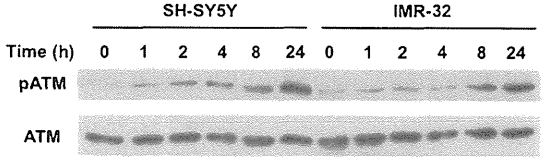


Fig. 5. Clioquinol induced phosphorylation of ATM. Cells were grown in the presence of clioquinol for the period indicated and whole cell lysate was isolated. The experiment was repeated three times and a representative result is shown.