A total score of the SMON severity was 8.0 ± 4.8 (3.3 ± 2.6 for gait, 2.2 ± 0.9 for sensation and 2.5 ± 2.6 for vision). The living arrangements were as follows: living alone, 11 persons (19.0%); living with a spouse, 13 persons (22.4%); living with a spouse and another family member (son, daughter, daughter-in-law and/or son-in-law), 16 persons (27.6%); living with another family member, 10 persons (17.2%); and other arrangements, 8 persons (13.8%).

The total score and all items of SR-BI of the SMON group were significantly lower than those of the control group (Table I), and the total score and all items, except for preparing meals, washing clothes, and gainful work of SR-FAI were significantly lower than those of the control group (Table II). Table III shows that the total score and all items of SDL of the SMON group were significantly lower than those of the control group. PCS and MCS of SF-36 in the SMON group were 26.3 ± 7.8 and 39.5 ± 11.0, respectively.

Table IV shows the correlation coefficients between SMON severity, SR-BI, SR-FAI, SDL, PCS and MCS. SDL had significant correlations with SMON severity, SR-BI, SR-FAI, PCS and MCS; PCS, with SR-BI and SDL; MCS, with SDL.

Discussion

The number of subjects in this study was not large, but we believe our data reflect the situation for all

Table I. Scores of Self-Rating Barthel Index.

	SMON (*N* = 58)	Control (*N* = 58)
Self-care		
Eating (0–10)	8.8 ± 2.9*	10.0 ± 0.0
Grooming (0–5)	4.4 ± 1.3*	5.0 ± 0.0
Washing or bathing (0–5)	3.7 ± 1.9*	5.0 ± 0.0
Dressing upper body (0–7)	5.6 ± 2.3*	7.0 ± 0.0
Dressing lower body (0–8)	6.0 ± 2.8*	8.0 ± 0.0
Toileting (0–5)	4.2 ± 1.6*	5.0 ± 0.0
Controlling urination (0–10)	5.3 ± 3.5*	10.0 ± 0.0
Controlling bowel movements (0–10)	6.5 ± 3.7*	9.9 ± 0.7
Mobility		
Getting in and out of chairs (0–5)	3.8 ± 1.8*	5.0 ± 0.0
Getting on and off a toilet (0–5)	4.0 ± 1.8*	5.0 ± 0.0
Getting in and out of tub or shower (0–5)	3.3 ± 2.0*	5.0 ± 0.0
Walking 50 m on level ground (0–15)	10.7 ± 5.2*	15.0 ± 0.0
Walking up/down the stairs (0–10)	4.5 ± 4.4*	9.1 ± 2.4
Total score (0–100)	70.8 ± 28.0*	99.0 ± 2.4

The numbers in brackets are theoretical ranges, and measured values are presented as means ± SD.
*$p < 0.05$, Mann–Whitney test, SMON *vs.* control.

Table II. Scores of Self-Rating Frenchay Activities Index.

	SMON (*N* = 58)	Control (*N* = 58)
1. Preparing meals (0–3)	0.9 ± 1.2	1.4 ± 1.4
2. Washing up (0–3)	1.0 ± 1.2*	1.9 ± 1.2
3. Washing clothes (0–3)	1.2 ± 1.3	1.7 ± 1.3
4. Light housework (0–3)	1.2 ± 1.3*	2.3 ± 1.0
5. Heavy housework (0–3)	0.6 ± 1.0*	2.0 ± 1.1
6. Local shopping (0–3)	0.9 ± 1.2*	2.2 ± 1.1
7. Social occasions (0–3)	0.8 ± 1.1*	1.9 ± 1.0
8. Walking outside (0–3)	0.9 ± 1.2*	2.5 ± 0.8
9. Actively pursuing hobby (0–3)	0.7 ± 1.1*	1.6 ± 1.3
10. Driving car/bus travel (0–3)	1.2 ± 1.2*	2.1 ± 1.0
11. Travel outings/car rides (0–3)	0.3 ± 0.7*	0.8 ± 0.8
12. Gardening (0–3)	0.3 ± 0.7*	1.1 ± 1.1
13. Household/car maintenance (0–3)	0.2 ± 0.5*	0.7 ± 1.1
14. Reading books (0–3)	0.8 ± 1.2*	1.3 ± 1.3
15. Gainful work (0–3)	0.2 ± 0.6	0.4 ± 1.0
Total score (0–45)	11.1 ± 11.0*	23.9 ± 7.7

The numbers in brackets are theoretical ranges, and measured values are presented as means ± SD.
*$p < 0.05$, Mann–Whitney test, SMON *vs.* control.

Table III. Scores of satisfaction in daily life.

SDL items	SMON (*N* = 58)	Control (*N* = 58)
Physical health (1–5)	1.6 ± 0.9*	3.2 ± 1.2
Mental stability (1–5)	2.2 ± 1.1*	3.7 ± 1.2
Self care (1–5)	2.4 ± 1.3*	4.4 ± 0.8
Ambulatory mobility (1–5)	2.3 ± 1.3*	4.4 ± 1.0
Household work (1–5)	2.1 ± 1.2*	4.1 ± 1.1
Living environment (1–5)	3.1 ± 1.4*	4.3 ± 1.0
Living arrangements with spouse/family (1–5)	3.5 ± 1.2*	4.4 ± 0.9
Hobbies/recreation (1–5)	2.4 ± 1.1*	3.4 ± 1.2
Local/social interaction (1–5)	2.5 ± 1.2*	3.5 ± 1.0
Pension/income (1–5)	2.4 ± 1.3*	3.3 ± 1.2
Work (1–5)	2.8 ± 0.7*	3.1 ± 0.5
Total (11–55)	27.3 ± 8.6*	41.8 ± 7.0

The numbers in brackets are theoretical ranges, and measured values are presented as means ± SD.
*$p < 0.05$, Mann–Whitney test, SMON *vs.* control.

Table IV. Correlation with SDL and SF-36 in patients with SMON.

	SDL	PCS	MCS
SMON severity	−0.387*	−0.204	0.049
SR-BI	0.464*	0.417*	−0.075
SR-FAI	0.442*	0.343	−0.019
SDL	–	0.373*	0.459*

SMON severity, disability scale for SMON; SR-BI, Self-Rating Barthel Index; SR-FAI, Self-Rating Frenchay Activities Index; PCS of SF-36, physical component summary of short form-36; MCS of SF-36, mental component summary of short form-36.
*$p < 0.05$, Spearman's correlation coefficient (with Bonferroni correction for multiple testing).

patients with SMON living at home to some extent. The officially approved number of patients with SMON in Japan is 2504 in April 2006. Because patients with SMON are receiving a special health allowance from the pharmaceuticals and medical devices agency and all such patients are known to receive the payments, the officially approved number of SMON is presumably accurate. Although Kuriyama et al. [11], Fujii and Arakawa [12], Honaga et al. [14] investigated 23, 17 and 7 patients with SMON, respectively, we examined 58 subjects in this study who were thus equivalent to 2% of all patients with SMON in our country.

We have developed SDL in order to evaluate the subjective domains of QOL in patients with SMON, and have studied the similarities and differences between the measurement concepts of SDL and SF-36 in the patients with stroke living at home and the elderly persons living at home [8]. As a result, we demonstrated that SDL could detect the subjective domains of QOL in patients with stroke that were similar to the psychological scales of SF-36.

As mentioned before, there have been few reports on the QOL of patients with SMON, and moreover, there are no reports of research on disease-specific QOL scales for patients with SMON. In the present study, we have investigated the characteristics of disabilities in patients with SMON based on the research to date, and we have examined the similarities and differences between SDL and SF-36. We found that significant correlations were observed between SMON severity and SR-BI, SR-FAI and SDL. It is believed that the subjective domains of QOL in patients with SMON are reduced, along with a decrease in independence of basic ADL and performance of applied ADL, when the SMON severity is severe. In addition, from the fact that correlations were observed between SDL and SMON severity, basic and applied ADL, and PCS and MCS of SF-36, SDL would reflect well the characteristics of disabilities in patients with SMON. On the other hand, no relationship has been observed between SF-36 and SMON severity.

Significant correlations were observed between SMON severity and SDL, but not between SMON severity and SF-36. SDL was originally developed to evaluate the subjective domains of QOL specific to patients with SMON, and the questionnaire consisted of the items related to satisfaction of elderly people living at home and disabilities of patients with SMON. SF-36 is a comprehensive evaluation scale that does not specify the disease or symptoms, and its items are constructed to broadly measure, across eight areas, comprehensive health concepts. It is believed that SDL reflects the disabilities in SMON better than SF-36. Fujii and Arakawa evaluated patients with SMON using Disability Scale for SMON, BI and SF-36, but reported that no correlation with the PCS and MCS of SF-36 could be found. The reason for this was stated as the possibility that QOL of a patient with SMON is not simply correlated with the extent of the disability, and that other factors are involved [12]. However, the results of our research show that SDL, a QOL scale disease-specific to SMON, can detect subtle changes of living conditions which are difficult to accurately determine using SF-36.

In our previous research which included a comparison between patients with SMON and stroke, patients with SMON showed high values in applied ADL and low values in SDL. Those results also indicated that SDL was able to differentiate the characteristics between patients with SMON and stroke [22].

The limitations of our research can be stated as follows. The first is that 39 non-respondents may have affected the results. The male–female ratio of respondents and non-respondents was about 1:2 in both groups, and there was no disparity. The questionnaires were designed to be anonymous, so there was little information on the non-respondents. Patients with SMON who were bedridden or had very severe disabilities might not have either wanted or be able to respond the questionnaire. However, such patients are less than 5% according to the annual medical checkup administered by the local members of the SMON research committee of the Ministry of Health, Welfare and Labour, and it is unlikely that there was any definite bias toward the respondents. The second is that the values of correlation coefficients with significance were not high. Although the SDL was associated with SMON severity, basic and applied ADL, and QOL, SDL could not explain all of the symptoms and disabilities of patients with SMON, and other factors, including victims of drug poisoning might be involved as Fujii and Arakawa had previously described [12].

In conclusion, the subjective domains of the QOL in patients with SMON were observed to decrease, and SDL was found to closely reflect the characteristics of disabilities in patients with SMON. As a result, SDL is therefore considered to be an appropriate scale for the subjective domains of QOL in patients with SMON.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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LETTERS TO THE EDITOR

キノホルムは特殊な薬ではなかった

〔Clinoquinol was not a special drug.〕

舟川　格

〔Itaru FUNAKAWA, M.D.: 独立行政法人国立病院機構兵庫中央病院神経内科(〒669-1592　兵庫県三田市大原1314)；Department of Neurology, National Hospital Organization Hyogo Chuo Hospital, Sanda, Hyogo 669-1592, Japan.〕

2009年1月19日

拝　啓

スモンは1960年代に多発した整腸剤キノホルムによる神経疾患です[1]．1970年にキノホルムの使用が禁止されてから新たな患者の発生はなくなりました．その結果，若い医師がスモンをもはや過去の病気としてとらえているとしても無理のないことなのかもしれません．しかし，少なくとも神経内科医は多くの患者が今もなお存在していることを真摯に受け止める必要があると思います．

スモンに関する調査研究班ではスモン患者の恒久対策として毎年患者検診を行っています．その折，患者さんから耳にすることはこの疾患が風化するのではないか，という恐れにも似た不安感です．実際，看護学生にアンケート調査を行ったところ完全に風化した病気といえる結果を得ました[2]．

谷崎潤一郎の「瘋癲老人日記」は昭和36年(1961年)11月号から翌年5月号の中央公論に掲載された小説です．この時期はまさにスモンの出現と一致しています．この小説には多くの薬剤が登場しますが，その中に次のような文章があります．「二十六日．昨夜冷奴ヲ食べ過ギタノガ悪カッタト見エテ夜半ヨリ苦シミ出シ二三度下痢スル．エンテロビオフォルムヲ三錠服用シタガマダ止マラナイ．今日一日寝タリ起キタリシテ暮ラス」[3]．エンテロビオフォルムは商品名であり，若い医師にとってキノホルムとは直ちに結びつかないかもしれません．この薬剤については注解があり，そこには次のように記載されています．「スイスのチバ社の下痢止め薬．キノホルムおよびサパミンを含有し，細菌性の下痢に最適」[3]．このように本文や注解をみてもキノホルムは決して医師が構えて処方したような特殊な薬剤ではないことがわかります．また，近所の薬局で簡単に手に入った薬でもありました．つまり，スモンは決して特殊な薬剤によって一部の患者にのみ出現した疾患ではありませんでした．

現在私たちがあまり神経質にならずに使っている薬剤も，一剤だけでなく組み合わせによっては未知なる薬害をひき起こす可能性は十分にあります．検診のたびにスモンは決して風化させてはならない「事件」であると思っておりますので，ご報告させていただきました．

敬　具

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BRAIN RESEARCH

Research Report

Clioquinol inhibits NGF-induced Trk autophosphorylation and neurite outgrowth in PC12 cells

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ABSTRACT

Clioquinol is considered to be a causative agent of subacute myelo-optico neuropathy (SMON), although the pathogenesis of SMON is yet to be elucidated. To investigate the mechanism of neurotoxicity of clioquinol, we used PC12 cell line and focused on nerve growth factor (NGF) signaling through Trk receptor, which is essential for survival and differentiation of neuronal cells. Clioquinol inhibited NGF-induced Trk autophosphorylation in a dose-dependent manner. This inhibitory activity was further confirmed by the data of the inhibition of NGF-induced mitogen-activated protein kinase (MAPK) phosphorylation, which is located in the down stream of NGF-Trk intracellular signaling pathway. Clioquinol also caused neurite retraction induced by NGF and cell death. NGF-stimulated (differentiated) cells were more vulnerable than naïve cells. These results strongly suggest that clioquinol may cause the perturbation of the intracellular survival pathway by inhibiting Trk-initiated signaling pathway.

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1. Introduction

Clioquinol or 5-chloro-7-iodo-8-hydroxyquinoline was used as an antibiotic for treating diarrhea and skin infection. It has been considered that clioquinol was a causative agent of subacute myelo-optico neuropathy (SMON), which is characterized by subacute onset of sensory and motor disturbances in the lower extremities with visual impairment (Nakae et al., 1973; Tsubaki et al., 1971). Pathological study demonstrated distal dominant axonopathy of the spinal long tracts and optic tracts (Tateishi, 2000). Over 10,000 patients in Japan were affected by SMON before therapeutic use of clioquinol discontinued in clinical practices in Japan. After the ban of the sale of clioquinol in September 1970, there was a drastic disappearance of new cases of SMON though nearly 3000 patients still suffer from its sequelae in 2002 (Konagaya et al., 2004). Even after 30 years of the outbreak of SMON in Japan, the mechanism of neuronal cell damage by clioquinol is yet to be elucidated.

Recently clioquinol reemerged for the treatment of non-infectious indication including malignancy (Chen et al., 2007), Alzheimer's disease (Cherny et al., 2001; Ritchie et al., 2003), and Huntington's disease (Nguyen et al., 2005). Given the potential reintroduction of oral clioquinol medication for these new indications, a clear understanding of clioquinol neurotoxicity is essential to fully appreciate the potential side effects of this drug.

Neurons are non-dividing cells in vivo and nerve growth factor (NGF) is one of the cardinal growth factors to support neuronal survival and differentiation (Thoenen, 1995). Trk is a

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Abbreviations: SMON, subacute mylo-optico neuropathy; NGF, nerve growth factor

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high-affinity NGF receptor which contains tyrosine kinase activity and its activation represents the initial step in the intracellular signal transduction pathway (Kaplan and Miller, 2000). Following ligand binding, the Trk receptor is activated by homodimerization. The degree of tyrosine autophosphorylation correlates well with the biological effects in responsive cells. In this study, to explore the mechanism of neurotoxicity of clioquinol, we focused on NGF-Trk-dependent signal transduction pathway for cell survival and examine the Trk receptor autophosphorylation in a neuronal cell line. The data clearly indicate the potential novel mechanism for the neurotoxicity by clioquinol.

2. Results

2.1. *Morphological observation*

Morphological effects of clioquinol on NGF-induced neurite outgrowth in PCtrk cells were observed under phase contrast microscope. After 24 h in culture, 1 μM of clioquinol-induced neurite retraction in PCtrk cells as shown in Fig. 1. After 48 h in culture, 100 nM and 1 μM of clioquinol also induced neurite retraction (Fig. 1). We further confirmed the effect of clioquinol on these cells by neurofilament expression by western blotting. Western blotting with anti-neurofilament antibody revealed that clioquinol causes decrease of neurofilament expression after 48 h in culture in a concentration-dependent manner. In contrast, β-actin expressions were not changed (Fig. 2A). We could not detect any changes in the expression of neurofilament after 24 h in culture (data not shown). To further confirm the neurite retraction by clioquinol, the length of neurite was measured. At 24 h in culture, the neurites were significantly retracted at 1 μM concentration of clioquinol as shown in the Fig. 2B.

2.2. *Cell viability assay*

To detect the cell death, trypan blue-dye exclusion assay was performed. As shown in Fig. 3, clioquinol-induced cell death in NGF-stimulated PCtrk cells in a concentration-dependent manner. One micromolar of clioquinol increased cell death drastically in NGF-stimulated cells at 24 h incubation (Fig. 3A). In contrast, naïve cells were significantly resistant to the same concentration of clioquinol (Fig. 3A). Most of the NGF-stimulated cells were dead at 1 μM of clioquinol after 48 h incubation. NGF-stimulated cells (differentiated cells) were more vulnerable than naïve cells (undifferentiated cells; Fig. 3B).

2.3. *Western blotting analysis of Trk autophosphorylation*

A mature form of Trk and its precursor form were recognized as 140 and 110 kDa, respectively. The immunoblot analysis with α-PY revealed a single band at 140 kDa corresponding to Trk (Fig. 4). To examine the effects of clioquinol on NGF-induced Trk autophosphorylation, PCtrk cells were incubated in the presence of clioquinol at various concentrations (from 10 nM to 10 μM) followed by the stimulation with NGF for 5 min. The cells were lysed with lysis buffer. Then, the cell-free lysates were immunoprecipitated with α-Trk and the immunoprecipitates were immunoblotted with α-PY. As shown in the Fig. 4, the inhibition of NGF-induced Trk autophosphorylation became evident at 100 nM clioquinol and it was further decreased in a concentration-dependent manner.

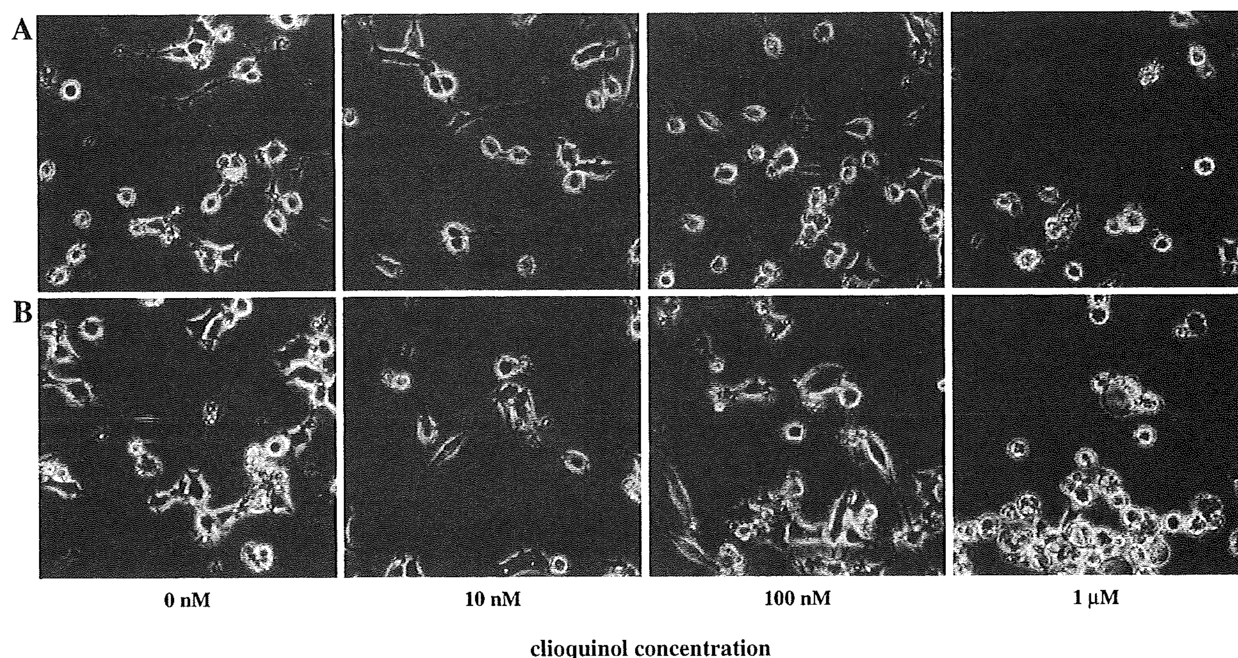

Fig. 1 – Morphological effects of clioquinol on NGF-induced neurite outgrowth. Representative photomicrographs of the cells cultured with various concentration of clioquinol for 24 h (A) and 48 h (B). Note the neurite retraction in (A) 1 μM and (B) 100 nM and 1 μM of clioquinol.

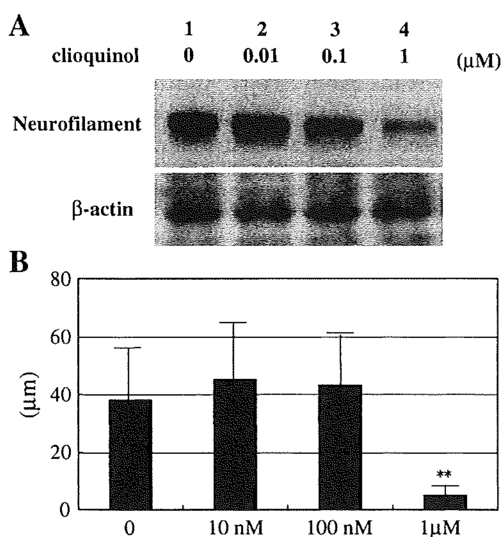

Fig. 2 – (A) Neurofilament expression in NGF-stimulated cells by western blotting. The cells were stimulated with 50 ng/ml of NGF and were cultured with various concentrations of clioquinol for 48 h. Cell lysates were applied for western blotting with anti-neurofilament antibody or anti-β-actin antibody. Note the decrease of neurofilament expression in higher concentration of clioquinol in contrast to the stable expression of β-actin. (B) NGF-stimulated cells were cultured with different amount of clioquinol for 24 or 48 h. Neurite retraction by clioquinol was quantitated by measurement of neurite length manually in phase contrast micrographs. Each 100 cells from three independent wells were evaluated. Bars and error bars represent mean±SEM. Statistical analysis by Student's t-test was performed, $^{**}p<0.01$.

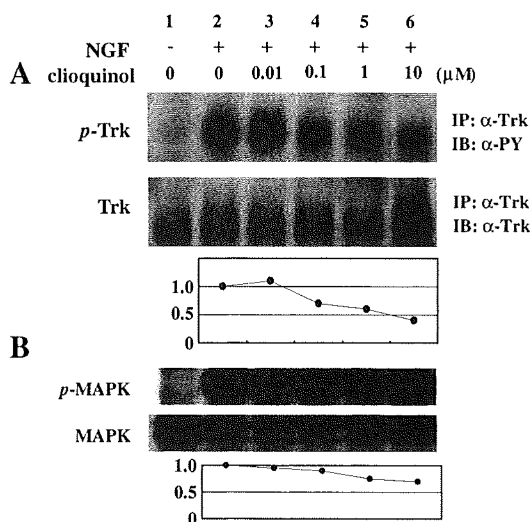

Fig. 4 – Concentration-dependent effects of clioquinol on Trk and MAPK phosphorylation. Cells were incubated with clioquinol for 1 h at various concentrations, then the cells were stimulated with 50 ng/ml of NGF for 5 min. Cell-free lysates were immunoprecipitated with α-Trk. Trk immunoprecipitates were subjected to SDS–PAGE and were immunoblotted with α-PY or α-Trk (A). Cell-free lysates were also subjected to SDS–PAGE and immunoblotted with anti-phosphospecific MAPK antibody (B) and anti-MAPK antibody (B), respectively. The cells in lane 1 were not stimulated with NGF and the cells from lane 2 to 6 were stimulated with NGF (50 ng/ml) for 5 min. Small graphs showed relative ratio of Trk autophosphorylation or MAPK phosphorylation. Phosphorylated Trk or MAPK at each concentration of clioquinol was adjusted with total Trk protein or MAPK protein, respectively, and was compared as a ratio.

To further confirm the inhibition of NGF-induced Trk autophosphorylation by clioquinol, MAPK (mitogen-activated protein kinase) phosphorylation response to NGF was also examined. MAPK is a serine/threonine kinase, which is located in the downstream of Trk signal transduction pathway and a key molecule to convey a signal to the nucleus. As shown in Fig. 3, NGF treatment caused MAPK phosphorylation (lane 2).

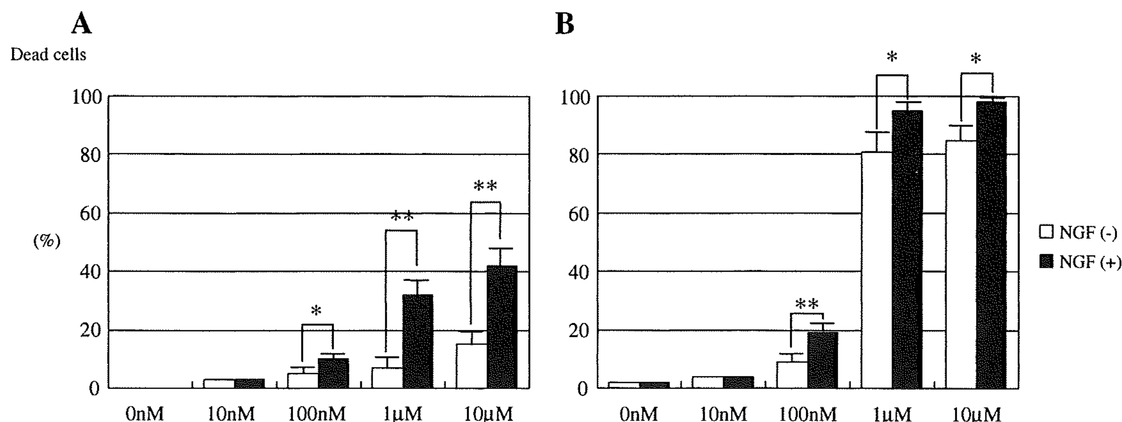

Fig. 3 – Cell viability assay by trypan blue staining. Cells cultured with or without NGF were incubated with various concentrations of clioquinol for 24 or 48 h. Dead cells were counted by trypan blue staining. Each experiment was performed in triplicate. Bars and error bars represent mean±SEM. Note the NGF-stimulated cells are significantly vulnerable to clioquinol. Statistical analysis by Student's t-test was performed, $^{*}p<0.05$; $^{**}p<0.01$.

The addition of clioquinol in culture medium caused a reduction of MAPK phosphorylation response similar to the Trk autophosphorylation response (lanes 3–6).

3. Discussion

Extensive epidemiological studies demonstrated that intake of clioquinol causes SMON and the prohibition of clioquinol as a medicine in 1970 dramatically decreased the number of new cases of SMON in Japan (Konagaya et al., 2004). In contrast to the epidemiological success, pathogenic mechanism of clioquinol neurotoxicity is still unknown even after 30 years of discontinuation of clioquinol. In this study, to elucidate the pathogenic mechanism of clioquinol, we focused on the NGF-Trk-mediated signal transduction pathway and demonstrated that clioquinol inhibits autophosphorylation of Trk receptor on a neuronal cell line. Clioquinol inhibited Trk autophosphorylation in a concentration-dependent manner and it was further confirmed that the phosphorylation of MAPK, which is a key molecule located in the downstream of NGF-Trk signal transduction pathway, was also inhibited by clioquinol. Under microscope, clioquinol caused neurite retraction in NGF-stimulated cells. In addition, clioquinol caused cell death both in NGF-stimulated and non-stimulated cells. However, NGF-stimulated cells (differentiated cells) were more vulnerable to clioquinol than non-stimulated cells (undifferentiated naïve cells).

In this study, 1 μM was a critical concentration of clioquinol toxicity to PCtrk cells. It is compatible with the previous observation in the animal experiments of SMON, i.e., plasma levels of clioquinol were approximately 0.5 to 5 μg/ml (1.7 to 17 μM) (Matsuki et al., 1997). Recently clioquinol has been the focus of attention as a potential drug for malignancy, Alzheimer's disease, and Huntington's disease (Chen et al., 2007; Cherny et al., 2001; Nguyen et al., 2005; Ritchie et al., 2003). For these new indications, further understanding of clioquinol neurotoxicity is necessary to avoid potential side effects of this drug.

Heretofore, the molecular mechanisms of clioquinol-induced neurotoxicity have been proposed. Among various potential mechanisms of clioquinol neurotoxicity postulated, metal ion chelating activity of clioquinol, notably Zn^{2+}, Cu^{2+}, Co^{2+}, and Fe^{3+}, is one of the most attractive mechanism because based upon this chelating activity, clioquinol is started to use as a therapeutic drug for malignancy, Alzheimer's disease, and Huntington's disease, vice versa. Clioquinol, in the presence of zinc, is reported to be converted to a potent mitochondrial toxin (Arbiser et al., 1998). Since clioquinol has been shown to cause increased systemic absorption of zinc in humans, it is likely that clioquinol-zinc chelate was present in appreciable levels in patients with SMON and may be the ultimate causative toxin of SMON (Arbiser et al., 1998). Clioquinol can form powerful lipophilic chelates with divalent cations, and this characteristic has led to the speculation that clioquinol is able to function as a carrier of heavy metals to the CNS which could cause toxicity in nerve tissue (Ohtsuka et al., 1982; Yagi et al., 1985; Yassin et al., 2000). Clioquinol injection in mice resulted in a rapid loss of synaptic zinc. Immediate early gene transcription factor, c-Fos, was induced in the hippocampal region and other parts of telencephalon and subsequently cell death was observed in these areas (Ismail et al., 2008).

What is the molecular mechanism for the strong inhibition of clioquinol on Trk-mediated intracellular survival pathway? Zinc itself has been known to alter the conformation and to inhibit the biological activity of neuropeptides related to neuronal survival, including NGF (Ross et al., 1997). However, in the present experimental conditions, zinc was free in the culture medium. Therefore, zinc-related mechanism for the inhibition of Trk autophosphorylation seems unlikely. Trk has an intrinsic tyrosine kinase activity, which is located in the cytoplasmic domain. The initial step of the intracellular signal transduction of NGF is believed to be activated by the autophosphorylation on tyrosine residues. Although the clear molecular mechanism of clioquinol-induced impairment of the Trk receptor function remains to be elucidated, Trk-initiated intracellular signaling pathway of NGF is an essential pathway for neuronal survival and differentiation in vivo. The evidence that differentiated cells stimulated with NGF were more vulnerable than undifferentiated cells in this study is compatible with the prominent post-mitotic neuronal cell damage in SMON. Therefore, it is quite reasonable to assume that the failure of this important cell survival pathway would result in the fatal impacts on the normal biology of the neurons. Thus, clioquinol-induced impairment of Trk autophosphorylation response in vitro culture system would implicate the new possible molecular mechanism for the development of the pathological states observed in patients with SMON. Moreover, this hypothesis suggests a new therapeutic strategy employing NGF for the treatment of many patients suffering from intractable residual symptoms such as pain and neuronal dysfunctions.

4. Experimental procedures

4.1. *Cell culture*

PC12 cells transformed with human *trk* complimentary DNA (PCtrk cells) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum, 5% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin (Mutoh et al., 2000). Clioquinol (5-chloro-7-iodo-8-hydroxyquinoline) was purchased from Sigma (St. Louis, MO) and was dissolved in 100% DMSO at a final concentration of 10 mM as a stock solution. The stock solution was further diluted for various experiments. To exclude the cellular toxicity of DMSO to PCtrk cells, the vehicle containing equivalent amount of DMSO was examined. We confirmed that cells were not affected by DMSO up to 0.2% concentration.

4.2. *Neurite retraction*

We examined the effect of clioquinol on NGF-stimulated neurite retraction of PCtrk cells. PCtrk cells were stimulated with 50 ng/ml of NGF in serum-free medium overnight, then the neurite-extended cells were cultured with 50 ng/ml of NGF and various concentration of clioquinol (from 10 nM to 10 μM) for 48 h. The cells were observed under phase contrast

microscope. To examine the effect on neurite-extended PCtrk cells, neurofilament expressions were determined by western blotting. Western blotting with anti-neurofilament antibody (Biomol International, Plymouth Meeting, PA) or anti-β-actin antibody (Cell Signaling Technology, Danvers, MA) was performed to further confirm the effect of clioquinol on neurite-extended cells. Detailed procedures of western blotting were described in section 4.3.

Furthermore, to quantitate the morphological effects on neurite-extended cells, phase contrast micrographs took from typical areas of these cultures and the length of neurites was measured manually. Each 100 cells from three independent wells were evaluated. Statistical analysis by Student's t-test was performed.

4.3. *Trk autophosphorylation and MAPK phosphorylation*

Cells were preincubated with serum-free medium for 1 h at 37 °C, and the cells were incubated in the presence of clioquinol at various concentrations (from 10 nM to 10 μM) for 1 h. Then, the cells were stimulated with 50 ng/ml of NGF for 5 min to examine the effect of clioquinol on NGF-induced Trk autophosphorylation. After stimulation, the cells were collected with chilled phosphate-buffered saline and lysed with lysis buffer (20 nM HEPES, pH7.2/1% Nonidet P-40/10% (vol./vol.) glycerol/50 mM NaF/1 mM phenylmethylsulphonyl fluoride (PMSF)/1 mM Na_3VO_4/10 μg of leupeptin per ml). The cell-free lysates were normalized for protein (1 mg/ml) and immunoprecipitated with anti-Trk antibody (α-Trk; Santa Cruz Biotech Inc, Santa Cruz, CA). The Trk immunoprecipitates were separated by sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS–PAGE) under reducing conditions on 7–14% gradient acrylamide gels, which was followed by blotting on polyvinylidene difluoride (PVDF) membranes. Tyrosine phosphorylation of Trk was detected with an anti-phosphotyrosine antibody (α-PY; Upstate Biotechnology Inc., Waltham, MA) and Trk was detected with α-Trk. The positive bands were detected with horseradish peroxidase-conjugated secondary antibodies using enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ) (Asakura et al., 2007). The cell-free lysates were also subjected to SDS–PAGE and immunoblotted with anti-phosphospecific mitogen-activated protein kinase (p-MAPK) antibody (α-p-MAPK; New England Biolabs, Tokyo, Japan) or anti-MAPK antibody (α-MAPK; New England Biolabs). Semi-quantitative analyses of Trk autophosphorylation and MAPK phosphorylation were performed by measuring the density of each band by densitometer (Shimadzu, Kyoto, Japan). Phosphorylated Trk or MAPK at each concentration of clioquinol was adjusted with total Trk protein or MAPK protein and was compared as a ratio (Mutoh et al., 1995).

4.4. *Cell survival assay*

To detect the cell survival the cells were incubated with trypan blue (Sigma). The cells were seeded onto 24-well plates and were cultured with or without NGF (50 ng/ml) in serum-free medium. Then, the cells were cultured with various concentration of clioquinol for another 24 or 48 h. Under phase contrast microscope, the number of viable (unstained) and dead (stained) cells was counted. Each experiment was performed in triplicate and statistical analysis by Student's t-test was done.

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