厚生労働科学研究費補助金
難治性疾患克服研究事業

Menkes病・occipital horn症候群の実態調査、早期診断基準確立、治療法開発に関する研究

平成23～24年度　総合研究報告書

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児玉　浩子（帝京大学医学部小児科・客員教授）

研究要旨

Menkes病およびoccipital horn症候群はATP7A遺伝子異常症であるが、発症頻度は不明で、有効な治療法がない難治性疾患である。私達は、平成22年度の研究でMenkes病の発症頻度は男子出生100万人に約8人で、女子例が２例見られた。Occipital horn症候群の発症頻度は男子出生の約68万人に１人であることを明らかにした。平成23年には詳細な二次調査を行い、神経症状発症前の特徴を明らかにし、早期診断ポイントを明らかにした。また、銅の皮下注射とノックビン経口投与の併用療法である新規治療の検討では、モデルマウスの研究で銅の代謝動態への効果を明らかにした。さらに７例のMenkes病患者、1例のoccipital horn症候群で新規治療を開始し、治療効果を評価した。また、Menkes病およびoccipital horn症候群の診断治療指針作成に当たっては日本小児神経学会の支援共同研究として承認されており（申請番号10-07）、現在、“早期診断基準および治療のガイドライン”の学会承認を準備中である。両疾患のホームページを立ち上げ、これらの成果を社会に発信している。さらに、患者家族会と定期的に交流し、患者家族の不安や悩みに対して支援している。

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A. 研究目的

Menkes 病は銅の腸管・血液脳関門などでの銅輸送障害により、銅欠乏による重篤な中枢神経障害や結合織異常を呈する疾患である。本症患児の殆どは中枢神経障害が発症する生後 3 か月以降に診断される。しかし神経障害発症後では現在の治療であるヒスチジン銅皮下注射は全く効果がなく、重

度神経障害・結合織異常が進行する。早期に診
断し治療を開始すると神経障害は予防できるが、結合織障害は予防できない。したがって早期診断と新規の有効な治療法の開発が待たれている。Occipital horn 症候群は Menkes 病の軽症型であるが、わが国での発症頻度は全く不明で、治療法もない。
本研究の目的は
①Menkes 病および occipital horn 症候群の発症頻度を明らかにする。
②遺伝子型と表現型の関係を解析する。
③神経症状発症前の特徴を明らかにし、早期診断法を確立する。
④Menkes 病モデルマウスである macular マウスで、併用療法の治療効果を判定する。
⑤両患者での新規治療の治療効果と副作用を明らかにする
⑥公開講座やホームページで、両疾患の理解および本研究の成果を世間に周知させる。
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B. 研究方法
１．早期診断のための病歴調査、広告活動
Menkes 病および occipital horn 症候群の経験の有無について、小児神経専門医 1,678 人、公立病院および大学病院の小児科 1,209 施設、重症心身障碍施設 24 施設、すでに把握している Menkes 病患者主治医 30 名（合計 3,161）に過去 10 年間での Menkes 病、occipital horn 症候群の診療経験を調査した。
患者を診療した経験のある主治医には、２次アンケートとして、詳細な病歴を調査した。　特に神経症状発症前の臨床的特徴なども詳細に調査する。その際に遺伝子診断が可能であること、Menkes 病友の会の存在なども通知する。友の会のホームページにも実態調査協力を呼びかけた。関連学会で実態調査を広報した。
さらに、全国アンケート調査の２次調査で、出生時の体重、身長、頭囲を正常児と比較した。また、小奇形の有無を調査した。
得られた知見について随時メンケス病・オクチピタールホーン症候群のホームページ等で公表した。
（http://www.pediatric-world.com/menkes/index.html）
２．遺伝子型と表現型の関連の解析
遺伝子解析を希望する当該疾患患者で、文書での同意を得た後に、ATP7A 遺伝子のゲノム解析を行い、遺伝子型と表現型の関連を解析した。
３．新規治療法（ジサルフィラム経口投与とヒスチジン銅皮下注射の併用療法）の効果の検討
①Macular マウスを用いての新期治療の効果の解析
モデルマウスを用いて、ノックビン経口投与と銅注射の併用療法を１～２か月間行い、個体としての活動性・体重増加などを評価した。さらに、解剖し、各臓器の銅濃度、銅酵素活性などを検討した。銅濃度および銅酵素活性測定はすでに習熟している。同時に各臓器の組織学的検討、肝機能や腎機能を解析し、副作用の有無を明らかにした。
②患者への新規治療の実施
ノックビンはアルコール中毒の治療薬として保険診療で認可されている薬剤である。さらに抗がん剤であるシスプラチンの副作用軽減剤、コカイン中毒の解毒薬としても用いられている。Menkes 病および occipital horn 症候群の病態および併用療法の作用

機序から考えて occipital horn 症候群に対しても唯一の治療法になると考えられる。文書で同意が得られた 7 例の Menkes 病患者および 1 例の occipital horn 症候群患者で、治療効果と副作用の有無を経時的に検討した。すでに確立している治療効果評価法（骨密度、尿 VMA/HVA、尿デオキシピリジノリン値、血液・尿カテコラミン分画、頭部 MRS など）および全身状態や神経学的所見で治療効果を明らかにするとともに副作用の有無を詳細に検討した。

（倫理面への配慮）

モデルマウスを用いたMenkes病治療法開発の研究は、帝京大学動物実験倫理委員会の承認をすでに得ている（番号；帝医動07-035）。また、実験を担当する藤澤千恵は獣医で獣医博士を取得しており、実験動物に対する動物愛護に関しても十分に配慮し、実験を行った。

Menkes病およびoccipital horn症候群患者の遺伝子解析および生化学的診断も、帝京大学医学部倫理委員会の承認をすでに得ている（2007年、帝京医倫認証済み）。Menkes病患者でノックビン経口投与と銅皮下注射の併用療法に関しても、帝京大学医学部倫理委員会の承認をすでに得ている（帝京医倫08-114）。口頭で詳細に説明し、文書で同意を得たのちに、治療を開始した。

遺伝子解析および治療を希望する患者家族には上記倫理委員会承認内容に準じて対応する。また、遺伝カウンセリング有資格者である共同研究者（黒沢健司医師）が必要に応じて遺伝カウンセリングを行った。

C. 研究結果

１．発症頻度

一次調査の回収率は小児神経専門医54.8%、病院施設45.6%、重症心身障害児施設44.3%で合計回数率は50.5%であった。その結果、62例のMenkes病（男児60例、女児2例）、occiitalhorn症候群は7例（男子6例、女子1例）であった。過去10年間の全国出生数から換算し、Menkes病は男児100万人出生当たり約8.03人、すなわち、約12.5万人当たり1人の発症率であった。Occipital horn症候群は男子出生68万人に1人の発症率であった。

２．早期診断のための方策

在胎週数に対する出生時の体重、身長、頭囲は対象児と差がなかったが、６５ 例の Menkes 病患者の 26.6%は小奇形を合併しており、これは全国の先天奇形の発症率である 1.9%より有意に高かった。主な小奇形は、高口蓋、小頭症、脳または肺のう胞、房室ブロック、眼裂狭小、停留睾丸であった。これら奇形を合併する児では本症を疑う必要があると考えられた。

広報活動：

公開シンポジウムを平成22年度から毎年２回、帝京大学および大阪市立大学で開催した。約100名の関係者が全国から参加した。Menkes病患者家族会代表の方にも講演していただき、患者家族の抱えている問題点や医療関係者への要望を聞くことが出来た。さらに患者家族と話し合う場を設け、お互いの情報交換を行った。患者家族会がホームページを充実されるのに協力した。

３．遺伝子型―表現型の関連の解明

今までの解析も含めて６０例のMenkes病

患者および２例のoccipital horn症候群患者の遺伝子解析を行った。occipital horn症候群はsplice-site変異とmissense変異であることを明らかにした。Splice-site変異はリアルタイムRT-PCRで残存活性が存在することが確認された。遺伝子解析結果は遺伝カウンセリングを行い、患者家族に報告した。

４．新規治療法（ノックビン経口投与とヒスチジン銅皮下注射）の確立

①モデルマウスを用いた新規治療の短期的および長期的効果および副作用の解明
体重増加は併用療法群で対象群に比べて有意に増加していた（平均体重増加：併用療法群20.33±0.34g、対照群18.64±0.49g）。小脳および血清の銅濃度、小脳チトクロームCオキシダーゼ活性は併用療法群で対象群に比べて有意に改善を示した。短期併用療法群の血算、肝機能、腎機能および血液一般生化学検査に異常は認められなかったが、長期併用療法群の一部で肝機能異常が認められた。今後の課題と考えられた。

②モデルマウスでの新規治療での銅の脳への取り込みと保時の研究
マクラマウスでの 64Cu を用いた microPET の実験では、ノックビン投与マクラマウスでは、Cu の脳への取り込みが、ノックビンを投与していないコントロールマクラマウスに比べて、有意に多かった。さらに Cu が長時間脳に保持された。すなわち、ノックビン併用で、銅の脳への移行・保持が、画像検査においても証明することが出来た。

③患者への新規治療の実施

７名の Menkes 病患者でヒスチジン銅皮下注射とノックビン経口投与の併用療法を開始した。年齢は３歳～１５歳で、６例は男児、１例は女児であった。７例ともヒスチジン銅の皮下注射をすでに行っている患者であった。ヒスチジン銅は原則今までの投与量で行った。ノックビンは 30mg/日から開始し、徐々に増量し、約 10mg/kg/日で現在経過を観察している。反応は症例により異なった。血清銅およびセルロプラスミン値がノックビン併用により上昇し、ヒスチジン銅を減量できた症例も見られたが、ノックビン併用でこれらの血清値に変化がない症例もあった。しかし、活動性、神経発達、骨密度などが有意に改善した例もあり、治療効果の症例による相違の理由を明らかにすることが今後の課題と考えられた。
これらの成果は、平成 23 年 10 月の国際学会(ISTERH)でシンポジウムとして取り上げられ、諸外国の研究者に強い関心を持たれた。さらに学術英文論文として受理された。

D. 考察

本研究で、Menkes 病および occipital horn 症候群の発症頻度を初めて明らかにすることができた。また、発症前の詳細な症状が明らかになり、早期診断法を確立することができた。早期診断法のガイドラインを日本小児神経学会で承認してもらう予定である。今後は、初期に認められる症状で早期診断が可能であることを小児科医・産科医に認知してもらう必要があると思われる。
私たちが提案したヒスチジン銅皮下注射とノックビン経口投与の併用療法はモデルマ

ウスでは、体重増加、血清銅・脳内銅値の上昇、脳のチトクローム C オキシダーゼ活性の改善、カテコラミン代謝の改善が認められ、有効な治療と考えられた。これらの結果は、銅が脳血液関門を通過して神経細胞に輸送され、銅酵素であるチトクローム C オキシダーゼに利用されていることを示唆している。すなわち、当初に期待した効果が見られていると言える。
患者での新規治療の研究成果では、血清銅およびセルロプラスミン値がノックビン併用により上昇し、ヒスチジン銅を減量できた症例も見られたが、ノックビン併用でこれらの血清値に変化がない症例もあった。しかし、活動性、神経発達、骨密度などが有意に改善した例もあり、治療効果の症例による相違の理由を明らかにすることが今後の課題と考えられた。

E. 結論

Menkes 病の発症頻度は男子出生 100 万人に約 8 人で、女子例が 2 例見られた。Occipital horn 症候群の発症頻度は男子出生の約 68 万人に 1 人であることを明らかにした。
また、神経症状発症前の特徴を明らかにし、早期診断ポイントを明らかにした。
新規治療として私たちが提案した銅皮下注射とノックビン経口投与の併用療法のモデルマウスでの研究では、銅の代謝動態は改善した。7 例の Menkes 病患者、1 例の occipital horn 症候群での新規治療効果は症例により異なった。治療効果の症例による相違の理由を明らかにすることが今後の課題と考えられた。また、Menkes 病および occipital horn 症候群の診療指針を日本小児神経学会に提案し、承認される準備を行っている。詳細は、本研究事業で立ち上げた両疾患のホームページに掲載している。

F. 健康危険情報

なし

G. 研究発表

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H. 知的財産権の出願・登録状況

なし

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Original article

Congenital abnormalities in Japanese patients with Menkes disease ☆

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Abstract

Menkes disease (MNK) is an X-linked recessive disorder. Incidence of live-born infants with MNK is 2.8 per million live births in Japan. The aim of this study was to observe congenital malformations (CMs) in MNK patients. Subjects comprised 35 Japanese male patients with classical MNK who received copper histidine treatment. Patient clinical data were obtained anonymously from medical records or medical record summaries by pediatrician's retrospective review through a survey. We observed 21 different CMs in 14 patients. Eight of these had a single CM, while six had multiple CMs. The most frequent CM was higher arched palate with other CMs found in five patients. There was no relationship between CMs and mutations in the ATP7A gene. Using Mann–Whitney *U* tests, age at death was also significantly lower in MNK patients with CMs ($P < 0.05$), compared to those without CMs, even though there was no significant difference of age onset, age at diagnosis and age at start of treatment with copper histidine between both groups of patients. Sudden death occurred in three MNK patients with CMs only: two with congenital heart disease, and one with microphallus.

Keywords: Copper; Inherited metabolic disease; Menkes disease; Congenital malformations; Japanese

1. Introduction

Menkes disease (MNK, #OMIM 309400) is an X-linked recessive disorder. The combined frequency of live-born MNK patients in Denmark, France, the Netherlands, the United Kingdom, and West Germany was 1 per 298,000 live-born babies in the period 1976–1987 [1]. Incidence of live-born infants with MNK is 2.8 per million live births from 1990 to 2003 in Japan [2]. In Japan, three to four individuals with MNK are born every year [2]. Onset of MNK occurs within three months of birth in 52.2%, and within 4–9 months after birth in 47.8% of patients with MNK [2]. While most classical MNK patients die by the age of three years, some survive until their teens with continued treatment [3]. The gene responsible for MNK is *ATP7A* (GenBank L06133.1), which is located at chromosome Xq13.3 and encodes a copper-transporting ATPase (ATP7A) [4–6]. Mutations in the *ATP7A* gene or other abnormalities in the genome or chromosomes associated with the *ATP7A* gene were found in most, but not all MNK patients [7–11]. In affected cells, copper transport by ATP7A from the cytosol to the Golgi apparatus is

Abbreviations: MNK, Menkes disease; CMs, congenital malformations

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disturbed, resulting in a reduction of copper efflux, and consequent reduction of activities of copper-dependent enzymes. This can lead to neurological symptoms, hair changes and abnormalities of connective tissue including arterial tortuosity, bladder diverticula, gastrointestinal polyps and abnormalities of bone. As orally-administrated copper cannot be absorbed due to ATP7A dysfunction and accumulates in the intestine, a copper deficiency in various tissues arises. Subcutaneous injections of copper–histidine complex, the currently accepted mode of treatment, may prevent neurological degeneration in some patients when treatment is initiated soon after birth and prolong lifespan of patients [3]. However, the current treatment does not help to alleviate connective tissue disorders caused by low activity of a copper enzyme – lysyl oxidase.

To date, congenital malformations (CMs) in MNK patients have not been reported details. In OMIM (http://omim.org/entry/309400) head and neck of MNK patients were described as microcephaly, brachycephaly, Wormian bone and pudgy cheeks. The present study analyzed CMs in MNK patients, which were considered to be unassociated with complications caused by reduced activity of copper enzymes, such as lysyl oxidase under current knowledge, and observed the relationship between CMs and the genetic mutation. We compared lifespan of treated MNK patients with and without CMs.

2. Methods

2.1. Subjects

A total of 35 Japanese MNK patients were investigated in the study. These patients were referred to the Department of Pediatrics of Teikyo University School of Medicine from 18 prefectures for counseling, copper histidine treatment, and biochemical and/or molecular pre- and post-natal diagnosis of MNK during 1990–2009 [7–10,12–15]. These patients were treated for classical MNK, which was diagnosed by clinical examination, measurement of the copper concentrations in cultured cells, and/or genetic mutation as previously reported [7–10,12–15]. Genetic analysis performed in the 5′-upstream region, each of the 23 exons, and the adjacent intronic sequences of the *ATP7A* genes, as described previously [7]. In Japan MNK patients were supported by medical aid programs [16], and received detail examinations including head magnetic resonance imaging and computed tomography scan.

Three pairs of patients were siblings, while the remaining patients were unrelated. Patient mortality status included dead ($n = 23$), alive (10) and unknown (2). Preparation of copper-histidine complex was according to the same protocol [3]. The dose of copper histidine parenterally administrated for one time was that could keep serum copper and ceruloplasmin within a normal range. The period of treatment with copper histidine was 26–183 (mean ± SD: 64.2 ± 39.9 months, $n = 17$) months.

2.2. Clinical data and data analysis

Clinical data for all patients were anonymously obtained from medical records or medical record summaries by retrospective review of pediatrician in charge of.

The study protocol was reviewed and approved by the Ethics Board of the Teikyo University School of Medicine. All participants provided a signed written informed consent.

To compare age differences between patients with CMs and those without CMs, the Mann–Whitney U test was performed, using the statistical package PASW® Statistics 17.0 (SPSS Inc., an IBM Company, Chicago, IL, USA).

3. Results

Among the 35 MNK patients, we observed a total of 21 different CMs in 14 MNK patients (Table 1). Eight of these (8/14, 57.1%) had a single CM, while six (6/14, 42.9%) had multiple CMs. Eight CMs (8/21, 38.1%) were minor, and the others (13/21, 61.9%) were major anomalies. The minor anomalies included higher arched palate, single transverse palmer crease, flat occiput, micrognathia, congenital microblepharia, undescended testis at birth, microphallus and accessory spleen (Table 1). Most frequent CMs were higher arched palate (five patients), single transverse palmer crease (three), micrognathis (two), flat occiput (two), and congenital microblepharia (two) (Table 1).

Age at onset, age at diagnosis, age at start of treatment with parenteral copper histidine, age at death, and age of alive patients (on August 31, 2009) are presented in Table 2. A comparison of MNK patients with CMs and those without CMs revealed no significant differences in age at onset, age at diagnosis, and age at start of treatment with parenteral copper histidine, but age at death was significantly different between these two groups ($P < 0.05$, Table 2).

Two unrelated patients with the same mutation (R986X) were both born at 38 gestational weeks weighing more than 2500 g. They both received parenteral copper histidine treatment at 6.5 months of age. One had CMs and died at 32.5 months due to a bladder hemorrhage (Patient 3 in Table 1). The other had no CMs and died at 59.0 months due to apnea. Another two patients were siblings who had a mutation of Del 2429-2430delTT. Both were born at 36 gestational weeks and weighed approximately 2300 g. The younger brother, who was diagnosed prenatally with having a

Table 1
Spectrum of congenital malformations (CMs) and mutations in the *ATP7A* gene in 14 patients with classical MNK.

Patient number	Congenital malformations	Cause of death	Age at death (Month)	Mutation in the *ATP7A* gene [References]
1	Higher arched palate, congenital microblepharia, entropion, flat occiput, and single transverse palmer crease	Unknown	17.0	IVS20+5G>A [7,9]
2	Higher arched palate, micrognathia, patent ductus arteriosus, and accessory spleen	Sudden death	34.5	2491insA (F781FS826X)
3	Higher arched palate and single transverse palmer crease	Bladder hemorrhage	32.5	3101C>T (R986X) [7–9,12]
4	Higher arched palate and undescended testis at birth	Unknown	Unknown	1730G>T (E529X) [7–9]
5	Higher arched palate, hypertrophic pyloric senosis and hydrocele	Alive	–	Not found, 46 XY [15]
6	Single transverse palmer crease, congenital microblepharia and flat occiput, hypoplastic corpus callosum, aplasia of inferior vermis	Stomach hemorrhage	57.5	IVS9+12insAATTG [9]
7	Cystic malformation in white matter of brain	Infectious disease	22.5	2429-2430delTT (S761FS770X) [9]
8	Congenital complete A–V block	Sudden death	45.0	IVS9+5G>C [8,9]
9	Microphallus	Sudden death	13.0	Not found
10	Club foot	Infectious disease	19.5	4177T>A and 4178A>T (S1344R and I1345F) [7,9]
11	Cystic malformation in lung	Infectious disease	22.5	1474delC [9]
12	Micrognathia	Infectious disease	43.0	2298-2299insAluYa5a2 [9,10]
13	Cystic malformation in arachnoid mater	Alive	–	Not detected yet
14	Cerebellar hypoplasia	Infectious disease	186.0	Not found

CM (Patient 7 in Table 1), died at 22.5 months due to sepsis and overall bad health from a tumoral lesion in his stomach. His older brother did not have any CMs, but died at 69 months of age from an infectious disease.

No relationship was found between CMs and genetic mutation (Table 1).

4. Discussion

In OMIM (http://omim.org/entry/309400) head and neck of MNK patients were described as microcephaly, brachycephaly, Wormian bone and pudgy cheeks. Horn et al. mentioned high-arched palate as one of the MNK symptoms [17]. However, to date, CMs in MNK patients have not been reported details yet. This is the first documentation of CMs in MNK patients in detail. This information can be added to what we know about MNK patients, which is that they often exhibit kinky hair, hypopigmentation, a higher arched palate, micrognathia, flat occiput and single transverse palmer crease. These minor and major anomalies presenting at birth are as clues for early diagnosis of MNK.

In this study, higher arched palate was most observed, and micrognathia and flat occiput were also observed. Along with the fact that microcephaly, brachycephaly, and pudgy cheeks described in MNK patients, it is considered that abnormal craniofacial growth and development occurred during pregnancy, although flat occiput was often considered as subjective judgment.

The main causes of death among MNK patients with or without CMs were infectious disease and abnormalities of connective tissues, such as hemorrhage and rupture of abdominal aorta (Tables 1 and 2). However, sudden death occurred in MNK patients with CMs only, especially in patients with congenital heart disease (Table 1). Moreover, MNK patients often exhibit connective tissue complications associated with lower activities of copper enzymes, such as osteoporosis, bladder diverticula, gastroesophageal reflux disease, which require surgical treatment [14,18,19]. It was reported that micrognathia made intubation and anesthesia extremely problematic [18,19], such that surgical intervention for MNK patients with CMs is avoided or difficult, although micrognathia was a minor CM. MNK is a rare disease, although we did not have enough number of patients to do statistical analysis, it was easy to understand that some CMs, such as congenital heart disease and micrognathia, lead to an inevitably shorter lifespan in these patients compared to patients without CMs. Pediatricians should pay extra attention to complications and CMs when treating MNK patients.

As some MNK patients do not have mutations in the *ATP7A* gene (Table 1) [7–9,11], we wonder if CMs in

Table 2
Median age (range) at onset, diagnosis, start of parenteral copper histidine treatment, at death and cause of death in MNK patients who had died.

Congenital malformation	Median age (range) months					Cause of death (number of patients)
	At onset*	At diagnosis*	At start of treatment with parenteral copper histidine*	At death**	Of alive patients (as of August 31, 2009)	
Yes ($n = 13$)[a]	2.0 (0.0–9.0) ($n = 13$)	6.0 (3.0–12.0) ($n = 12$)	7.0 (3.0–15.0) ($n = 13$)	32.5 (13.0–186.0) ($n = 11$)	68.5 ($n = 1$)	Infectious disease (4); Infectious disease and hemorrhage (1); sudden death (3); bladder hemorrhage (1); stomach hemorrhage (1); unknown (2); ($n = 12$)
No ($n = 21$)	2.5 (0.0–6.0) ($n = 18$)	4.5 (0.0–33.0) ($n = 18$)	6.5 (0.5–34.0) ($n = 19$)	49.5 (38.0–97.0) ($n = 8$)	42.0 (17.0–104.0) ($n = 9$)	Infectious disease (5); central apnea (2); respiratory failure (1); rupture of abdominal aorta (1); sputum obliteration in cannula (1); unknown (1); ($n = 11$)

[a] Data from patients who received prenatal diagnoses were excluded, with the exception of data pertaining to median age at death and cause of death in patients with CMs.
* $P > 0.05$, Mann–Whitney U test.
** $P < 0.05$, Mann–Whitney U test.

MNK patients may provide some clues regarding the pathological causes of MNK. However, even if patients 1–5 had the same CM in Table 1, that is, a high arched palate, mutations in their *ATP7A* genes were not consistent. Therefore, there was no association between CMs and mutations in the *ATP7A* gene.

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Effect of copper and disulfiram combination therapy on the macular mouse, a model of Menkes disease

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ABSTRACT

Menkes disease (MD) is a genetic neurodegenerative disorder characterized by copper deficiency due to a defect in ATP7A. Standard treatment involves parenteral copper–histidine administration. However, the treatment is ineffective if initiated after two months of age, because the administered copper accumulates in the blood–brain barrier and is not transported to neurons. To resolve this issue, we investigated the effects of a combination therapy comprising copper and disulfiram, a lipophilic chelator, in the macular mouse, an animal model of MD. Seven-day-old macular mice treated subcutaneously with 50 μg of $CuCl_2$ on postnatal day 4 were used. The mice were given a subcutaneous injection of $CuCl_2$ (10 μg) with oral administration of disulfiram (0.3 mg/g body weight) twice a week until eight weeks of age, and then sacrificed. Copper concentrations in the cerebellum, liver, and serum of treated macular mice were significantly higher than those of control macular mice, which received only copper. Mice treated with the combination therapy exhibited higher cytochrome c oxidase activity in the brain. The ratios of noradrenaline and adrenaline to dopamine in the brain were also increased by the treatment, suggesting that dopamine β-hydroxylase activity was improved by the combination therapy. Liver and renal functions were almost normal, although renal copper concentration was higher in treated macular mice than in controls. These results suggest that disulfiram facilitates the passage of copper across the blood–brain barrier and that copper–disulfiram combination therapy may be an effective treatment for MD patients.

Introduction

Menkes disease (MD) is an X-linked recessive disorder caused by a defect in a copper-transporting ATPase (ATP7A). In humans, ATP7A is expressed in almost all cell types except hepatocytes. In normal cells, ATP7A is localized in the trans-Golgi network and transports copper from the cytosol into the Golgi apparatus, where copper is incorporated into secretory copper enzymes [1]. In MD-affected cells, however, copper accumulates in the cytosol and cannot be excreted. Copper accumulation in the intestines results in copper absorption failure, leading to overall copper deficiency in the body, except in the kidney. Copper also accumulates in the cells of the blood–brain barrier. Thus, copper cannot be delivered from the bloodstream to neurons after barrier maturation [2–4]. Copper concentrations in the serum, liver, and brain of MD patients are significantly lower, resulting in reduced activities of copper-dependent enzymes such as cytochrome c oxidase, dopamine β-hydroxylase, and lysyl oxidase. Currently, parenteral administration of copper–histidine is the standard treatment for MD [1,5]. However, the treatment is ineffective if initiated after two months of age, because the administered copper accumulates in the blood–brain barrier and is not transported to neurons. Therefore, copper delivery to neurons is the most important objective in the treatment of MD-associated neurological degeneration [6].

We previously reported that a combination therapy comprising copper and diethyldithiocarbonate (DEDTC) improved copper concentrations, cytochrome c oxidase activity, and catecholamine metabolism in the brain of macular mice [7]. A dimer of DEDTC, disulfiram, has been used for the treatment of alcoholism and cocaine addiction and as a modulator of cisplatin-induced toxicity [8–10]; thus, oral disulfiram is easily applicable in the clinical setting. Here, we report the effects of a combination therapy comprising copper injection and oral disulfiram on the macular mouse, an animal model of MD [11].

Materials and methods

Animals

Male hemizygous macular mice and normal littermate controls were treated with a single subcutaneous injection of cupric

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chloride solution (50 μg of $CuCl_2$) on postnatal day 4, because macular mice die without this treatment. All mice were maintained under standard conditions. Macular mice were separated into control and treated groups. The latter group was treated with a subcutaneous injection of $CuCl_2$ (10 μg) and oral administration of disulfiram (0.3 mg/g body weight) twice a week from the age of 7 days to 8 weeks, and then sacrificed. Control mice were given a subcutaneous injection of $CuCl_2$ (10 μg) as above, but disulfiram was replaced with double distilled water. Normal littermates were used as normal controls. Body weights were measured twice a week during the treatment period. The cerebrum, cerebellum, kidney, liver, and intestines were dissected. Sera and tissues were stored at −80 °C until analysis. This study was approved by Teikyo University School of Medicine Animal Ethics Committee (07-035).

Measurement of copper concentration

Tissue samples were dried at 120 °C for 12 h and wet-digested with concentrated HNO_3 by heating at 120 °C, and the resultant residues were dissolved in 2 mol/L HNO_3. Serum samples were wet-digested with concentrated HNO_3 by heating at 120 °C. Copper concentration was analyzed with a Hitachi Z-8100 atomic absorption spectrophotometer (Hitachi Industries, Japan). All glassware was washed with nitric acid to avoid metal contamination.

Assay for cytochrome c oxidase activity

Mitochondria were isolated from tissue samples immediately after sacrifice using the Mitochondria Isolation Kit for Tissue (Pierce, Rockford, IL). Cytochrome c oxidase activity in the mitochondrial solution was determined using the Cytochome c Oxidase Assay Kit (Sigma–Aldrich, St. Louis, MO). Protein concentration was determined using the Pierce BCA Protein Assay Kit (Pierce, Rockford, IL).

Measurement of catecholamine and enzyme concentrations

Cerebrum and cerebellum were homogenized in 0.4 N perchloric acid and centrifuged at 4 °C (12,000 × *g*, 5 min). Catecholamines, including dopamine, noradrenaline, and adrenaline, in the supernatant were analyzed using a catecholamine autoanalyzer (HLC-8030; Toso Ind., Tokyo, Japan). Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), and creatinine were analyzed by Oriental Yeast Co., Ltd. (Shiga, Japan).

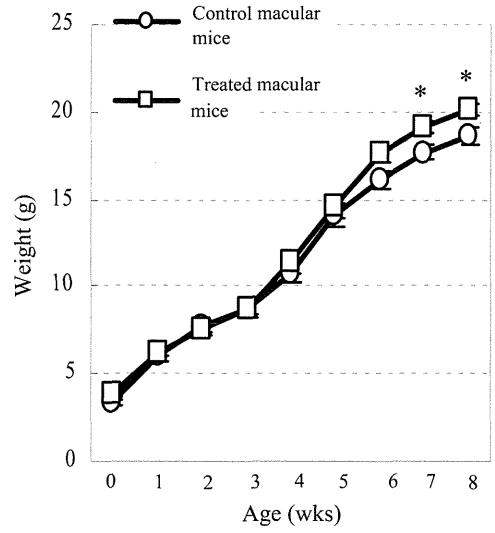

Fig. 1. Body weight changes in control and treated macular mice from birth to 8 weeks of age. Weights are presented as mean (SE) (control macular mice: $n = 19$; treated macular mice: $n = 24$). *$p < 0.05$.

Statistical analysis

Data are presented as mean ± standard error (SE). The SE is computed from known sample statistics, and it provides an unbiased estimate of the standard deviation of the statistic. Differences in body weight changes between the two groups were analyzed using two-way repeated measure ANOVA. Differences in copper concentration, CCO activity and catecholamine between the two groups were analyzed using one-way repeated measure ANOVA (Tukey post hoc test); $p < 0.05$ was considered significant.

Results and discussion

As shown in Fig. 1, the weight gain in treated macular mice was significantly higher than that of control macular mice after week 7. Fig. 2 shows the copper concentration in tissues and serum.

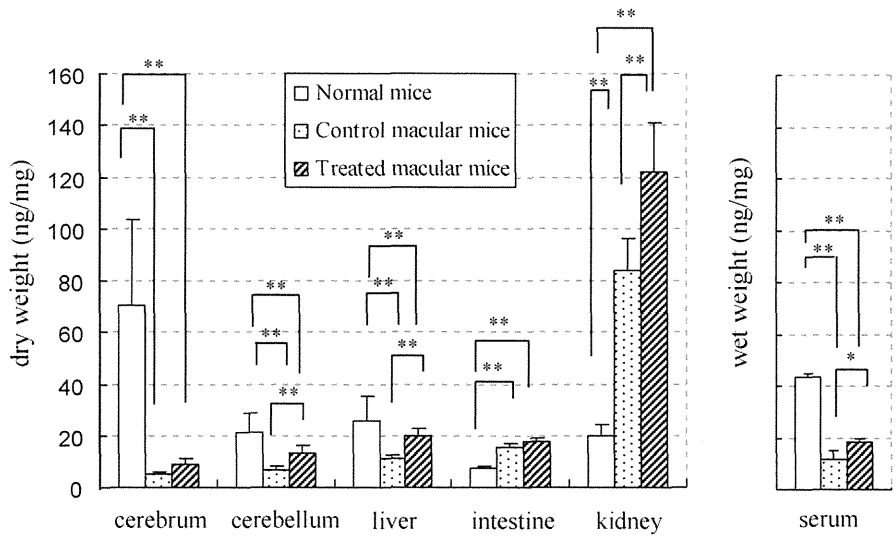

Fig. 2. Copper concentrations in the cerebrum, cerebellum, liver, intestines, kidney, and serum of normal mice ($n = 5$), control macular mice ($n = 19$), and treated macular mice ($n = 24$). *$p < 0.05$; **$p < 0.01$.

Consistent with a previous report [12], control macular mice exhibited low copper concentrations in serum, liver, and brain, and high copper concentrations in the intestine and kidney. Copper concentrations in the cerebellum, liver, and serum of treated macular mice were significantly higher than those of control macular mice, suggesting that disulfiram improved intestinal copper absorption. The activity of cytochrome c oxidase, a copper-dependent enzyme, is reduced in the brain of macular mice [12]. Combination therapy improved cytochrome c oxidase activity (Fig. 3).

Dopamine β-hydroxylase is also a copper-dependent enzyme. Copper is incorporated into dopamine β-hydroxylase in the Golgi apparatus in normal cells. Dopamine β-hydroxylase activity is decreased in the brain of the brindled mouse, an animal model of MD [13]. Furthermore, even in patients with MD, the activity of this enzyme is reduced and cannot be improved by parenteral copper administration [14]. These findings indicate that copper cannot be incorporated into dopamine β-hydroxylase in MD-affected cells due to ATP7A defects. Given that dopamine β-hydroxylase converts dopamine to noradrenaline, which is subsequently metabolized to adrenaline, the ratios of noradrenaline and adrenaline to dopamine serve as indicators of dopamine β-hydroxylase activity. As shown in Fig. 4, the ratios of noradrenaline and adrenaline to dopamine were higher in treated macular mice, suggesting that dopamine

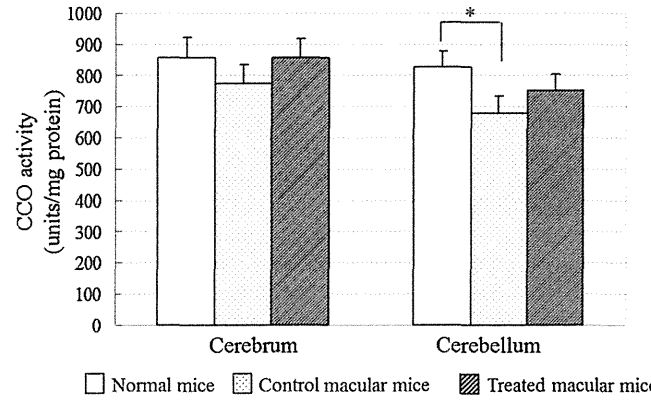

Fig. 3. Cytochrome c oxidase (CCO) activity in the cerebrum and cerebellum of normal mice ($n=4$), control macular mice ($n=3$), and treated macular mice ($n=4$). *$p<0.05$.

β-hydroxylase activity was improved by the therapy. The results of our study suggest that disulfiram facilitates copper transport into the Golgi apparatus of affected cells, including cells in the intestines and blood–brain barrier, thereby making it available to copper-dependent enzymes.

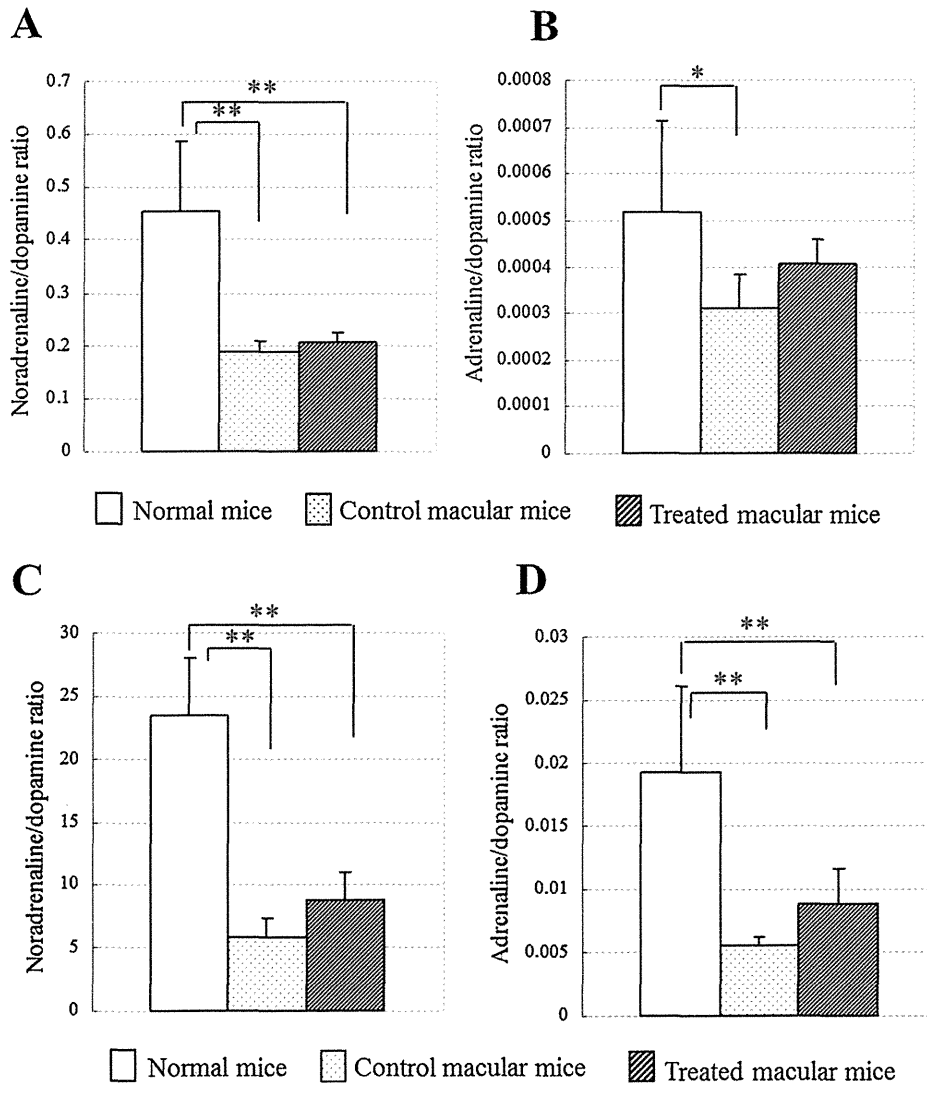

Fig. 4. Ratios of noradrenaline/dopamine and adrenaline/dopamine in the cerebrum (A and B) and cerebellum (C and D) of normal mice ($n=7$), control macular mice ($n=7$), and treated macular mice ($n=7$). *$p<0.05$; **$p<0.01$.

MD patients and macular mice exhibit high renal copper concentrations [1]. Indeed, control macular mice exhibited a high concentration of copper in the kidney. The renal copper concentration of treated macular mice was higher than that of control macular mice. Serum creatinine levels were normal (normal, 0.08–0.24 mg/dL) in 23 treated mice; one treated mouse exhibited a slightly higher serum creatinine level (0.3 mg/dL). Serum BUN, AST, and ALT levels were normal in all treated macular mice. To apply the combination therapy to patients with MD, the effects of long-term combination treatment must be investigated in mouse models of MD.

Conclusions

A combination therapy comprising copper injection and oral disulfiram improved copper concentrations in serum, liver, and brain, and enhanced cytochrome c oxidase activity and catecholamine metabolism in the brain of the macular mouse, a model of MD. These results suggest that the lipophilic copper–disulfiram complex can penetrate cellular membranes, including the blood–brain barrier and Golgi membranes, in MD-affected cells, thereby making copper available to copper-dependent enzymes. Connective tissue disorders, including arterial abnormalities, bladder diverticula, and osteoporosis, are also serious problems in patients with MD. These disorders are caused by reduced activity of lysyl oxidase, a secretory copper-dependent enzyme. Thus, the copper–disulfiram combination therapy may also be effective in treating connective tissue disorders associated with MD.

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Effects of disulfiram treatment in patients with Menkes disease and occipital horn syndrome

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Disulfiram

ABSTRACT

The clinical and biochemical effects of disulfiram were evaluated in three boys with the disorders characterized by copper deficiency due to the defect of ATP7A. Two suffered from Menkes disease (MD) and one from occipital horn syndrome. Disulfiram was orally given, in addition to a parenteral administration of copper-histidine in the case of MD patients. Serum levels of copper and ceruloplasmin slightly increased in one MD patient, and he showed favorable emotional expression and behavior more often than before according to his caretakers. However, no obvious changes were observed in the other two patients. Serum ratios of noradrenaline to dopamine, and adrenaline to dopamine, which are thought to be the indicators of dopamine β-hydroxylase activity, one of the copper requiring enzymes, were unaltered after disulfiram treatment. No adverse effects were recognized during the treatment period in all patients. Although the major improvement was not observed clinically or biochemically by disulfiram treatment so far, the trial will be continued to see the possible effects in these disorders with copper transport defect.

Introduction

A clinical phenotype of Menkes disease (MD) is characterized by copper deficiency due to the functional loss of ATP7A, the copper transporter, and occipital horn syndrome (OHS) is a milder form of this defect. The current standard therapy for copper deficiency is a parenteral administration of copper-histidine, however, the treatment is not effective for neurologic symptoms or connective tissue abnormalities, because administered copper is not transported across to the neurons nor to the trans Golgi network [1]. Only if the treatment is started at the very early neonatal period when the blood–brain barrier is still immature, and also in those with residual function of ATP7A, it may modify the disease progression [2].

Diethyldithiocarbamate (DEDTC), a lipophilic chelator, has shown beneficial effects in macular mice, an animal model of MD, on copper metabolism in the brain [3,4]. Therefore, DEDTC may act to transport copper to neurons and to the Golgi apparatus in the brains of the animals. Disulfiram, a dimer of DEDTC, is a drug used for treatment of chronic alcoholism, and is immediately converted to DEDTC in vivo, including in the human body. In the macular mouse, disulfiram showed similar effects to DEDTC, such as increase in copper concentrations in the brain (unpublished data). The purpose of this study is to evaluate clinical and biochemical effects of disulfiram in patients with MD and OHS.

Patients and methods

Two patients with MD and a patient with OHS, aged between 10 and 19 years old, were enrolled in this study as shown in Table 1. Two MD patients were clinically diagnosed during infancy, and copper-histidine treatment was initiated immediately after the diagnosis, but both are now bed-ridden, fed through nasogastric tube, with seizures despite of several anti-epileptic medications. Their diagnosis was later confirmed by genetic study. In patient 1, deletion of two base pairs in exon 3 of the ATP7A gene caused frame shift, resulting in premature termination of transcription. In patient 2, duplication of exon 3 to exon 5 reduced the estimated normal protein production to 4% of normal [5]. The OHS patient was diagnosed at 4 years of age. His uncle also suffered from OHS, and died 36 years old due to respiratory failure. They shared the same splice site mutation causing skipping of exon 6, which has been found also in other OHS patients [6,7]. He is usually on a wheelchair, but can walk slowly. He can communicate with others, and goes to work at a place for handicapped people. He has had frequent episodes of urinary tract infection due to the diverticulum of the bladder, and needs self-catheterization. Their body weights at study initi-

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Table 1
Patients' profiles.

Patient	1	2	3
Sex	Male	Male	Male
Diagnosis	MD	MD	OHS
Birth date	10/Jan./2001	11/Dec./1996	18/Jul./1992
Age at			
Diagnosis	10 m	8 m	4 y 2 m
Copper histidine start (present dosage)	10 m (900 μg/w)	8 m (6750 μg/w)	-
Disulfiram start	9 y 5 m	13 y 8 m	18 y 3 m
ATP7A mutation	Del 608AA	Dup Ex3-5	del TAAG (IVS6, DS)

ation ranged 10–20 kg in the MD patients, and 35 kg in the OHS patient.

Disulfiram was given orally once daily, starting with lower dosages such as 30–60 mg per day, and then increased to the maintenance dosage of 100 mg per day. The dosage approved in Japan for adults is between 100 and 500 mg per day. In the MD patients, the dosage of copper-histidine administration and the amount of copper in the formula diet (1.2–1.6 mg daily) were unchanged during the study period.

Serum copper (Cu) and ceruloplasmin (Cp), catecholamines such as noradrenalin (NAD), adrenaline (AD) and dopamine (DA), and urinary vanillylmandelic acid (VMA) and homovanillic acid (HVA) were measured regularly. If the Cu and Cp levels increase, it is assumed that copper is transported through enterocytes. Catecholamines were measured to see ratios of NAD to DA, and AD to DA, and also to see a ratio of VMA to HVA. These ratios are thought to be indicators of activity of dopamine β-hydroxylase, which is one of secreting copper enzymes. If these ratios increase, it is assumed that copper is transported into trans-Golgi network and corporated into this copper requiring enzyme. Other measurements included bone mineral density (BMD) by dual energy X-ray absorptiometry to see probable connective tissue improvement. In addition, as renal dysfunction is a concern as an adverse effect by the treatment due to the accumulation of copper, serum urea nitrogen and creatinine, and urinary β-2 microglobulin were measured.

Informed consent was taken from their parents, and the study was approved by the Ethical Committee of Teikyo University Hospital (No. 08-114).

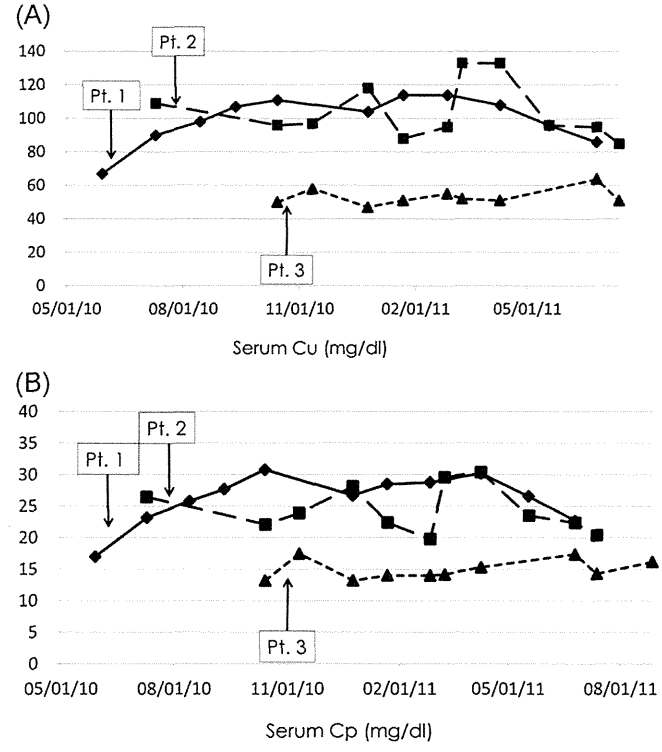

Fig. 1. Serum copper (A) and ceruloplasmin (B) levels in the three patients. Arrows indicate the start of disulfiram treatment. The normal values range between 80 and 130 mg/dl for copper, and between 20 and 35 mg/dl for ceruloplasmin.

Results and discussion

Serum levels of Cu and Cp are shown in Fig. 1. In patient 1, both Cu and Cp were below the normal limits at the onset of disulfiram, in spite of the copper-histidine treatment. The values then increased after disulfiram treatment into the normal range, and Cu has almost remained over 100 ng/ml without changing the dose of copper-histidine. In patient 2, serum Cu and Cp were within normal ranges with copper-histidine administration, with no apparent change after the addition of disulfiram. In patient 3 with OHS, both serum Cu and Cp were below the normal range before treatment, and these values did not change after disulfiram administration.

According to the parents of patient 1, he showed favorable emotional expression and behavior, such as smiling, laughing, pulling his nasogastric tube off, and so on, more often than before, shortly after starting disulfiram, however, further clinical improvement was not observed later on. In the other two patients, any beneficial change was not recognized clinically.

The indices of dopamine β-hydroxylase activity, such as NAD to DA, AD to DA, and VMA to HVA, were low before disulfiram treatment. Since dopamine β-hydroxylase catalyzes DA to NAD conversion, the NAD to DA ratio is low when the activity of this enzyme is disturbed as in MD. However, these ratios did not change after the treatment (NAD to DA ratio is shown in Fig. 2).

Lumbar BMDs before disulfiram in the two MD patients were 0.245 and 0.541 g/cm^2, which were around 50% of the mean values for their age, and 0.704 g/cm^2 in the OHS patient, which was

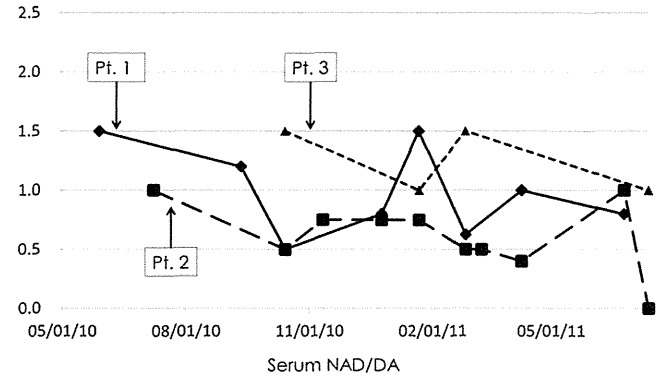

Fig. 2. Serum ratio of noradrenaline to dopamine in the three patients. Arrows indicate the start of disulfiram treatment.

about 70% of the mean. Low BMD is indicative of connective tissue impairment, however, it is greatly affected by mobility. Therefore, the very low values in the bed-ridden MD patients may be due to immobilization. BMD slightly increased in patients 1 and 3, whereas slightly decreased in patient 2 after the treatment.

Urinary β-2 microglobulin, an index of renal tubular function, which was quite high in the two MD patients with copper-histidine treatment, and normal in the OHS patient, did not change after addition of disulfiram, and serum urea nitrogen and creatinine levels remained normal during the period. No other adverse effects related to disulfiram have been recognized.

Thus, disulfiram treatment appeared marginally effective with regard to serum Cu and Cp in patient 1, but not in two other patients, and showed no beneficial changes in markers for dopamine β-hydroxylase. Reasons for failure of a favorable outcome by disulfiram treatment in the patients, in contrast to the previous animal studies with DEDTC, might be the differences in blood–brain barrier between humans and rodents, the observed rather small effects in the previous studies, and the much smaller disulfiram dosage used in this study than that of the animal studies. As blood–brain barrier is known to be immature in the mouse, the effect of DEDTC might have been modified by the immatureness. Increase in the copper concentrations by addition of DEDTC in the mouse brain was rather small, less than 50%, and the concentration was still below half of the normal control [4], therefore, such small effect may not cause clinical and biochemical improvement in humans. The dose of DEDTC (or disulfiram) used in the animal studies was 200 mg/kg body weight, which was more than 20-fold larger than that used in this study. Future studies will be required in younger patients with larger doses to clarify the effects of disulfiram.

Conclusions

Although major improvement was not observed clinically or biochemically by disulfiram treatment so far, the trial will be continued to see the possible effects in these disorders with copper transport defect.

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Review article

Pathology, clinical features and treatments of congenital copper metabolic disorders – Focus on neurologic aspects

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Abstract

Genetic disorders of copper metabolism, including Menkes kinky hair disease (MD), occipital horn syndrome (OHS) and Wilson's disease (WD) are reviewed with a focus on the neurological aspects. MD and OHS are X-linked recessive disorders characterized by a copper deficiency. Typical features of MD, such as neurologic disturbances, connective tissue disorders and hair abnormalities, can be explained by the abnormally low activity of copper-dependent enzymes. The current standard-of-care for treatment of MD is parenteral administration of copper–histidine. When the treatment is initiated in newborn babies, neurologic degeneration can be prevented, but delayed treatment is considerably less effective. Moreover, copper–histidine treatment does not improve connective tissue disorders. Novel treatments targeting neurologic and connective tissue disorders need to be developed. OHS is the mildest form of MD and is characterized by connective tissue abnormalities. Although formal trials have not been conducted for OHS, OHS patients are typically treated in a similar manner to MD. WD is an autosomal recessive disorder characterized by the toxic effects of chronic exposure to high levels of copper. Although the hepatic and nervous systems are typically most severely affected, initial symptoms are variable, making an early diagnosis difficult. Because early treatments are often critical, especially in patients with neurologic disorders, medical education efforts for an early diagnosis should target primary care physicians. Chelating agents and zinc are effective for the treatment of WD, but neurologic symptoms become temporarily worse just after treatment with chelating agents. Neurologic worsening in patients treated with tetrathiomolybdate has been reported to be lower than rates of neurologic worsening when treating with other chelating agents.

Keywords: Copper; Menkes disease; Wilson's disease; Occipital horn syndrome; ATP7A; ATP7B; Neurologic diseases

1. Introduction

Copper is an essential trace element for all living organisms and functions as an integral component of cuproenzymes, which include cytochrome C oxidase, lysyl oxidase, dopamine-β-hydroxylase, superoxide dismutase, tyrosinase, ascorbic acid oxidase and ceruloplasmin. When present in excess amounts, however, its oxidative potential induces reactive free radical production that results in cellular damage. Thus, the tight regulation of copper homeostasis, which is maintained by mechanisms including uptake, transport, storage and excretion of copper, is required. Disruptions to normal copper homeostasis are evident in three human genetic disorders: Menkes disease (MD), occipital horn syndrome (OHS) and Wilson's disease (WD) [1]. Each disease is caused by the absence or dysfunction of homologous copper-transporting ATPases. The responsible gene for MD and OHS is the *ATP7A* gene, and the *ATP7B* gene is responsible for WD [1]. These three diseases exhibit neurologic disorders. However, the pathology of MD and OHS is completely different from that of WD; that is, MD and OHS are characterized by a copper deficiency while WD is characterized by toxicity due

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to an excess of copper. The reason for this difference is related to the particular cell types in which the ATP7A and ATP7B proteins are expressed. ATP7A is expressed in almost all cells with the exception of hepatocytes, whereas ATP7B is primarily expressed in hepatocytes. Here we review MD, OHS and WD with a focus on neurologic aspects.

2. Copper homeostasis

The average daily intake of copper in healthy adults is about 2 mg. Ingested copper is absorbed from the intestine into the blood, and the majority is then transported into the liver. The majority of copper in the liver is excreted via the bile, but a small amount is excreted in the urine. Fig. 1 shows the molecular mechanism of copper metabolism in cells. Ctr1 is a high-affinity copper transporter located on the plasma membrane of the cells and mediates copper uptake. Copper in the cytosol is delivered to Cu/Zn superoxide dismutase in the cytosol, to the Golgi apparatus, and to mitochondria by Ccs2, HAH1 (ATOX1) and Cox 17, respectively, and are generically named copper chaperones [1]. ATP7A and ATP7B are localized to the trans-Golgi membrane and transport copper from the cytosol into the Golgi apparatus within cells. Copper transported into the Golgi apparatus is excreted from cells as a part of copper enzymes. ATP7A is expressed in almost all cells other than hepatocytes, including those of the intestine, kidney and components of the blood brain barrier; ATP7B is mainly expressed in hepatocytes and acts to excrete copper into the bile and blood [2,3].

Genetic disorders of copper metabolism in humans manifest in the form of MD, OHS and WD (Table 1).

3. Menkes disease (MD) and occipital horn syndrome (OHS)

3.1. Genetics

The phenotypic features of ATP7A mutations can be divided in at least three categories; Classical MD with death in the early childhood (generally called as MD), mild MD with long survival, and OHS (the mildest features) [4]. Inheritance of MD and OHS is X-linked recessive; patients are typically male, and their mothers are heterozygous carriers of the disease. The incidence of MD in Japan is estimated to be 1/140,000 live male births [5]. A small number of females with X-linked chromosomal abnormalities have also been reported to be affected by MD [1]. Patients with MD exhibit a large variety of mutations in the *ATP7A* gene [1,6,7]. Moller described that they had identified about 357 different mutations [4].

OHS and mild MD are extremely rare. Major mutations in the *ATP7A* gene in OHS and mild MD are splice-site or missense mutations [4], and residual activity of ATP7A still exists [6,7].

3.2. Pathology

In MD-affected cells, copper accumulates in the cytosol and cannot be excreted. Copper accumulation in the intestine results in a failure of copper absorption, which leads to copper deficiency in the body and reduces the activity of copper-dependent enzymes. Copper also accumulates in the components of the blood–brain barrier and cannot be transported from the blood vessels to neurons. The characteristic features of MD can be explained by a decrease in the activity of copper-dependent enzymes (Table 2).

Neurologic degeneration in MD is mainly caused by decreased activity of cytochrome C oxidase in neurons. In addition, subdural hemorrhage often occurs secondary to disorders of brain arteries due to decreased activity of lysyl oxidase, resulting in neurologic damage. Hypotonia may be caused by reduced activity of cytochrome C oxidase in the muscle [8].

Characteristics of OHS include connective tissue disorders caused by a decrease in lysyl oxidase activity.

3.3. Clinical features

3.3.1. Neurologic manifestations

Characteristic clinical features including seizures, delayed development, marked muscular hypotonia and abnormal hair, become prominent between the ages of 2 and 4 months when copper deficiency becomes advanced. Because clinical abnormalities are absent or subtle in affected newborns, the diagnosis is difficult prior to 2 months of age. As the disease progresses, patients are bedridden and never smile. Most patients with MD die by the age of 3 years, although some patients survive into their teenage years [1,7].

Epilepsy is observed in most patients with MD. Bahi-Buisson et al. reported the characteristics of epilepsy as divided into three periods [9]. Focal clonic status epilepticus and intractable infantile spasms are observed in the early stage (median age: 3 months) and intermediate stage (median age: 10 months), respectively. Multifocal seizures, tonic spasms and myoclonus are observed in the late stage (median age: 25 months). Ozawa et al. also reported that infantile spasms with EEGs containing hypsarrhythmic patterns are observed in 50% of epileptic patients with MD [10]. Brain MRIs of the patient shows brain atrophy and delayed myelination or demyelination. Subdural hemorrhage/effusion is often observed (Fig. 2). Magnetic resonance angiography shows a tortuosity of intracranial and cervical blood vessels [1,7]. Additionally, ^{1}H-magnetic resonance spectroscopy (^{1}H-MRS) shows a lactate peak and a decrease in