様式 C-19

科学研究費補助金研究成果報告書

平成２２年５月２６日現在

研究種目：若手研究(ｽﾀｰﾄｱｯﾌﾟ)
研究期間：2008　～ 2009
課題番号：20890177
研究課題名（和文）：外傷性脳損傷における軸索内輸送蛋白・炎症性サイトカインの動態に関する研究

研究課題名（英文）：Intracerebral expression pattern of various transported protein through axons and proinflammatory cytokines after fatal traumatic brain injury

研究代表者
林　敬人（HAYASHI　TAKAHITO）
鹿児島大学・大学院医歯学総合研究科・助教
研究者番号：40512497

研究成果の概要（和文）：法医実務に応用可能なより高精度のびまん性外傷性軸索損傷（TAI）の診断法，鑑別診断法及び受傷後経過時間推定法の確立を目的として，頭部外傷例の脳梁についてβ-amyloid precursor protein（APP），Neuron-specific enolase（NSE）といった軸索内輸送蛋白質及び Interleukin-8（IL-8），Tumor necrosis factor-α（TNF-α）といった炎症性サイトカインを指標として免疫組織化学的検討を行った。APP，NSE には，明らかに 2 種類の染色パターンが存在し，神経線維束の走行に沿って染色されるものは外傷性軸索損傷，神経線維束に沿わず散在性に染色されるものは低酸素血症に起因する二次性軸索損傷に相当する可能性が示唆された。また，NSE は受傷後 2 時間以上経過した例の全例で軸索損傷を示す NSE 陽性 axonal bulb が認められ，APP と比較し早期診断への有用性を期待させる結果が得られた。さらに，IL-8，TNF-αはそれぞれ受傷後 3 日，4 日より陽性 axonal bulb が認められ，受傷後経過時間推定のための新たな指標となる可能性が示唆された。

研究成果の概要（英文）：This research aimed to establish the following three methods; (1) new forensic diagnostic markers for diffuse traumatic axonal injury (TAI), (2) differential diagnosis between traumatic axonal injury and hypoxic one, (3) estimation for time interval after injury. For this purpose, we immunohistochemically examined intracerebral expressions of transported proteins through axons such as β-amyloid precursor protein(APP),Neuron-specific enolase(NSE), and expressions of proinflammatory cytokines such as Interleukin-8 (IL-8), Tumor necrosis factor-α (TNF-α) . Sections of the corpus callosum from cases of head injury were immunostained for those markers. On both APP and NSE immunostained sections, two patterns of immunoreactivity were identified in several cases of head injury. The first pattern showed that labeled axons were oriented along with white matter bundles; the second demonstrated that the axons were scattered irregularly. Our results suggested that the first and second pattern may represent traumatic axonal injury, and secondary hypoxic axonal injury, respectively. Moreover, NSE was more sensitive procedure for detecting TAI than APP, because NSE immunostaining could detect axonal injuries in cases of head injury that survived more than 2 hours (APP; more than 9 hours). Furthermore, IL-8 and TNF-α immunostaining could detect axonal injuries in cases of head injury that survived more than 3 days and 4 days, respectively. Our results suggested that immunostaining of proinflammatory cytokines such as IL-8 and TNF-α in the corpus callosum could be a new useful marker for estimating time interval after traumatic axonal injury.

交付決定額

（金額単位：円）

	直接経費	間接経費	合　計
２００５年度			
２００６年度			
２００７年度			
２００８年度	1,340,000	402,000	1,742,000
２００９年度	1,190,000	357,000	1,547,000
総　計	2,530,000	759,000	3,289,000

研究分野：法医学
科研費の分科・細目：社会医学・法医学
キーワード：びまん性軸索損傷，法医病理学的診断，軸索内輸送蛋白，炎症性サイトカイン，免疫組織化学，受傷後経過時間

１．研究開始当初の背景

外傷性脳損傷は，法医解剖の中でも例数が多いものの一つであり，正確な診断が必要不可欠である．さらに，頭部への外力の作用時期が問題になることも多く，受傷後の経過時間も重要な検討項目になっている。ところが，外傷性脳損傷のうちびまん性外傷性脳損傷と呼ばれる病態は，脳挫傷などの局所性脳損傷を伴うことが少なく，特に受傷後短時間で死亡した例では診断が極めて困難である。その代表的な病態がびまん性外傷性軸索損傷 diffuse traumatic axonal injury (TAI) である。TAI は回転加速度が頭部に加わった際に白質内の軸索がびまん性に断裂して生じるもので，予後の悪い病態である。局所性脳損傷に乏しいため，その診断には組織病理学的検査が必須であるが，特異的な軸索の球状変性像 retraction ball が通常の染色で検出できるのは受傷後 15～18 時間以上生存した例に限られる。そのため，受傷後早期に死亡した例の TAI 診断法が今まで種々試みられてきた。近年，軸索内輸送蛋白β-amyloid precursor protein (APP) に対する免疫組織化学を用いた方法が注目されている。しかしながら，APP は受傷後 2 時間程度の生存例で検出できるものの，それより早期の死亡例では診断できず，また，低酸素血症に基づく軸索損傷との鑑別ができない等の問題点も指摘されてきた。そこで，TAI における様々な軸索内輸送蛋白や炎症性サイトカインの発現の動向を免疫組織学的に検討し，TAI の早期診断法，他の軸索損傷との鑑別法及び受傷後経過時間の推定法を開発する研究を企画した。本研究によりヒトにおける軸索内輸送蛋白や炎症性サイトカインの役割が明らかになれば，法医学分野のみならず，治療など臨床分野にも応用できる可能性があり，興味深いものと考える。

２．研究の目的

本研究は，TAI 剖検例における様々な軸索内輸送蛋白や炎症性サイトカインの脳内発現の動向を免疫組織学的に検討し，法医実務に応用可能な TAI の早期診断法，他の軸索損傷との鑑別法及び受傷後経過時間の推定法を開発することを目的とする。

さらに，TAI における軸索内輸送蛋白や炎症性サイトカインの役割を解明し，新たな治療法の開発へとつなげ臨床分野にも貢献していくことが本研究の最終目的である。

３．研究の方法

（１）軸索内輸送蛋白を指標とする検討：TAI 剖検例の脳梁における APP，Neuron specific enolase (NSE)の発現の動向を免疫組織化学的に検討し，その分布や経時的な動態を解析して，軸索損傷の鑑別法及び受傷後経過時間推定法の開発を試みた。

（２）炎症性サイトカインを指標とする検討：TAI 剖検例の脳梁における Tumor necrosis factor (TNF-α)，Interleukin-8 (IL-8)の発現の動向を免疫組織化学的に検討し，その分布や経時的な動態から軸索損傷の鑑別法及び受傷後経過時間の推定法に応用可能か否かを明らかにした。

４．研究成果

（１）軸索内輸送蛋白を指標とする検討

１－１）APP を指標した軸索損傷の鑑別法に関する研究(Two patterns of β-amyloid precursor protein (APP) immunoreactivity in cases of blunt head injury)

① Abstract

Immunostaining for β-amyloid precursor protein (APP) is widely recognized as an effective tool for detecting diffuse traumatic axonal injury (TAI). APP selectively labels injured axons, such as

axonal bulbs and varicose axons. However, it has been reported that axonal bulbs are detected in cases of cerebral hypoxia without head injury. Therefore, we examined whether there are differences in the morphological pattern of axonal bulbs between trauma and hypoxia. Sections of the corpus callosum from 25 cases of head injury and 23 control cases were immunostained for APP. APP staining detected axonal bulbs in 14 cases of head injury, who survived more than several hours, although it failed to label axons in control cases. In addition, two patterns of immunoreactivity were identified in several cases of head injury. The first pattern showed that labeled axons were oriented along with white matter bundles; the second demonstrated that the axons were scattered irregularly. The first pattern alone was found in 5 of 14 cases, while cases of the second pattern alone were not observed. Both patterns were detected in 5 cases and in the remaining 4 cases, clear patterns were not found. From these findings, we speculated that the first pattern may represent TAI. Further examinations are required for determining whether these two patterns are identical with patterns of trauma and hypoxic brain damage as indicated by Oehmichen *et al*. (2003) and Graham *et al*. (2004).

② Introduction

APP is a transmembrane glycoprotein synthesized within neuronal cytoplasm. It is carried along axons to the synapse by fast anterograde transport, and is normally undetectable. However, APP may accumulate in axonal bulbs, and reach detectable levels when axonal transport is disturbed. These APP-positive bulbs are now used as a marker of diffuse traumatic axonal injury (TAI). A survival period of 15-18 hours following head injury is necessary before the axonal bulbs can be detected using conventional hematoxylin and eosin (H-E) or silver staining. However, immunostaining for APP can detect axonal bulbs as early as 2-3 hours after head injury.

Although diffuse axonal injury was regarded as the only consequence of head injury until recently, several investigators have stressed that axonal bulb formation may occur in the absence of a head injury, usually in the presence of both intracranial and systemic pathology. Hypoxia, ischemic brain damage, post-traumatic edema, and cerebral and cerebellar herniation are often secondary complication of head injury, and all may have a role in the formation of axonal bulbs. Accordingly, it is necessary to distinguish traumatic axonal injuries from other axonal injuries since APP immunoreactivity is observed in both cases.

In order to determine whether there are differences in the morphological pattern of axonal bulbs between trauma and hypoxia, we compared APP immunostained sections of the corpus callosum from head injury cases and control cases showing hypoxia/ischemia without head injury.

③ Materials and methods

Twenty-five cases of blunt head injury and 23 control cases showing hypoxia/ischemia without head injury were collected from our department. From each case, paraffin-embedded section of corpus callosum was immunostained for APP. Briefly, immunohistochemistry for APP was carried out on 4.5 μm serial sections of corpus callosum. After deparaffinization, the sections were immersed in 0.3% H_2O_2-phosphate-buffered saline (PBS; pH 7.2) for 30 minutes to block endogenous peroxidase activity. All sections were then rinsed in PBS and incubated with mouse anti-APP monoclonal antibody (clone 22C11, Boehringer, Mannheim, Germany) diluted in PBS containing 1% normal goat serum and 5% bovine serum albumin at 4° C, overnight. Thereafter, sections were incubated with Envision$^+$ (Dako, Kyoto, Japan) for mouse immunoglobulin at room temperature for 30 minutes, and positive signals were visualized with 3, 3'-diaminobentizine. The sections were then dehydrated and mounted without counter-staining.

Semiquantitation of axonal bulbs was performed under ×200 magnification using a Nikon Optiphot microscope (field area calculated as 0.64 mm^2). Axonal bulbs were counted as an average of 10 fields in each case.

④ Results

In all control cases without head injury, APP immunoactivity was not detected regardless of the post mortem periods (9-48 hours). On the other hand, in 14 out of 25 cases of head injury with more than 9

hours survival period, at least 1 axonal bulb per ×200 microscopic field was labeled by APP. Injured axons, such as varicose axons and waving axons, were also detected by APP in head injury cases and usually associated with axonal bulbs. Furthermore, two patterns of positive staining for APP could be identified in several cases of head injury. The first pattern revealed that labeled injured axons were regularly oriented along the white matter bundles (**Fig. 1**). The second pattern demonstrated that the injured axons were scattered irregularly in the white matter. The first pattern alone was found in 5 out of 14 APP-positive cases, while cases of the second pattern alone were not observed. Both patterns were detected in 5 cases, in which more than 5 APP-labeled axonal bulbs per ×200 microscopic field were observed. In the remaining 4 out of 14 cases, clear patterns were not observed.

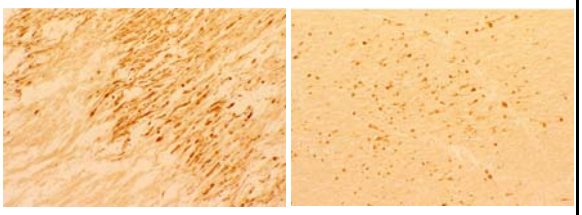

Fig. 1 Immunostaining for APP in a head injury case. APP-positive axons are oriented along with white matter bundles (left; Pattern 1). APP-positive axons are scattered irregularly in white matter (right; Pattern 2). Magnification, ×50.

⑤ Discussion

There are several studies that have examined the different pattern of APP immunoreactivity between trauma and hypoxia. Graham et al. suggested that an irregular or often 'Z' shaped pattern indicates the boundary of an infarct and, therefore, result from the vascular complications. Oehmichen et al. suggested that a wave-like pattern should be produced by the mechanical impact, and the irregularly aggregated type should result from hypoxic insult. In our cases, the 'Z' shaped pattern was seldom observed. The results were thought to be appropriate because H-E stained sections showed no histological findings of infarction in any case. In the wave-like pattern, the injured axons were scattered, but confined to individual white matter bundles, so that the first pattern found in our study may correspond to the wave-like pattern. In addition, the irregularly aggregated type was thought to resemble the second pattern observed in our results. However, it was difficult to distinguish the two patterns in 4 out of 25 cases. Therefore, further studies are needed to determine whether those patterns indicate traumatic and hypoxic axonal injury.

No APP-positive staining was observed in any of the control cases with hypoxia/ischemia in the absence of head injury examined in this study. Kaur et al. demonstrated that 12 out of 25 (43%) cases of hypoxia without head injury showed positive staining for APP in the corpus callosum, although the specific APP immunoreactive pattern was not mentioned in their study. In their cases showing positive staining for APP, the survival time ranged from a few hours to 68 days. In 7 of 23 control cases in the present study, the survival time ranged from 1 hour to 9 days. Although no APP-positive staining was observed in these control cases in which the survival time was longer, further investigation of the cases of hypoxia alone with longer survival time should elucidate the APP immunoreactive pattern of hypoxic axonal injury.

In this study, we describe different two patterns of APP immunoreactivity in head injury cases with several different survival times. The first pattern alone was detected in 5 APP-positive head injury cases, while cases of second pattern alone were not found. These results suggest that the first pattern may represent traumatic axonal injury. However, further investigations are required for determining whether these two patterns are identical with patterns of trauma and hypoxic/ischemic brain damage.
(*Legal Medicine* 11:171-173, 2009)

１－２）NSE を指標した検討

APP を指標とした検討と同じく頭部外傷例 25 例，対照例 23 例の脳梁を試料として NSE に対する免疫染色を行った。その結果，対照例は全例で NSE 陽性所見は見られなかったが，頭部外傷例では，25 例のうち受傷後 2 時間以上生存した 21 例全例で NSE 陽性の injured axon あるいは axonal bulb が認められた。APP は受傷後 9 時間以上経過例で認められたことを考えると，NSE はより早期診断に有用である可能性が示唆された。また，染色パタ

ーンについては，APP 同様，明らかに 2 種類の染色パターンが存在し，神経線維束の走行に沿って染色される Pattern 1 と神経線維束に沿わず散在性に染色される Pattern 2 がみられた。Pattern 1 単独例は 6 例，Pattern 2 単独例はなく，Pattern 1 と 2 の混在例が 5 例認められた。APP 同様に Pattern 1 は外傷性軸索損傷，Pattern 2 は低酸素血症に起因する二次性軸索損傷に相当する可能性が示唆された。

（2）炎症性サイトカインを指標とする検討

頭部外傷例 25 例，対照例 23 例の脳梁を試料として TNF-α，IL-8 に対する免疫染色を行った。その結果，対照例では全例で TNF-α，IL-8 はいずれも陽性所見は見られなかった。頭部外傷例では，TNF-αは受傷後 3 日以内の 14 例では陽性所見は見られず，4 日以上生存した 8 例全例に TNF-α陽性の axonal bulb がみられた。一方，IL-8 は，受傷後 2 日以内の 10 例では陽性所見は見られず，3 日以上生存した 10 例中 8 例に IL-8 陽性の axonal bulb がみられた（図 2）。以上の結果から，炎症性サイトカイン TNF-α，IL-8 は，TAI の受傷後経過時間推定のための新たな指標となる可能性が示唆された。

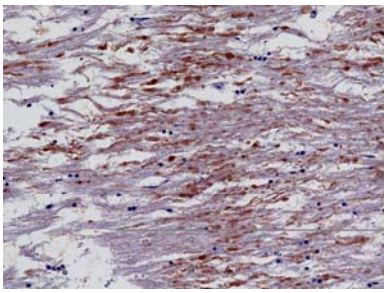

図 2．IL-8 陽性の頭部外傷例（受傷後 5 日）

５．主な発表論文等

（研究代表者、研究分担者及び連携研究者には下線）

〔雑誌論文〕（計 8 件）

① Hayashi T, Ishida Y, Mizunuma S, Kimura A, Kondo T. Differential diagnosis between freshwater drowning and saltwater drowning based on intrapulmonary aquaporin-5 expression. Int J Legal Med 123: 7-13, 2009, 査読有.

② Ishibe T, Kimura A, Ishida Y, Takayasu T, Hayashi T, Tsuneyama K, Matsushima K, Sakata I, Mukaida N, Kondo T. Reduced acetaminophen-induced liver injury in mice by genetic disruption of IL-1 receptor antagonist. Lab Invest 89: 67-79, 2009, 査読有.

③ Hayashi T, Ago K, Ago M, Ogata M. Two patterns of beta-amyloid precursor protein (APP) immunoreactivity in cases of blunt head injury. Legal Med 11: S171-173, 2009, 査読有.

④ Ogata M, Ago K, Ago M, Nakashima H, Hayashi T. Food poisoning due to Salmonella Enteritidis - A case report. Legal Med 11: S533-534, 2009, 査読有.

⑤ Nosaka M, Ishida Y, Tanaka A, Hayashi T, Miyashita T, Kaminaka C, Eisenmenger W, Furukawa F, Kimura A. Aberrant expression of histo-blood group A type 3 antigens in vascular endothelial cells in inflammatory sites. J Histochem Cytochem 56: 223-231, 2008, 査読有.

⑥ Fukube S, Hayashi T, Ishida Y, Kamon H, Kawaguchi M, Kimura A, Kondo T. Retrospective study on suicidal cases by sharp force injuries. J Forensic Legal Med 15: 163-167, 2008, 査読有.

⑦ Takayasu T, Hayashi T, Ishida Y, Nosaka M, Mizunuma S, Miyashita T, Kawaguchi M, Kimura A, Kondo T. A fatal intoxication from ingestion of 2-methyl-4-chlorophenoxyacetic acid (MCPA). J Anal Toxicol 32: 187-191, 2008, 査読有.

⑧ Ishida Y, Hayashi T, Goto T, Kimura A, Akimoto S, Mukaida N, Kondo T. Essential involvement of CX3CR1-mediated signals in the bactericidal host defense during septic peritonitis. J Immunol 181: 4208-4218, 2008, 査読有.

〔学会発表〕（計 15 件）

① 林　敬人，吾郷一利，吾郷美保子，小片守．小児虐待死例の諸臓器における好中球浸潤の検討と虐待診断への応用（第 2 報）－Interleukin-8 の動態を中心として－．第 15 回日本子ども虐待防止学会学術集会埼玉大会，埼玉，2009 年 11 月．

② 林　敬人，池松和哉，安倍優樹，吾郷一利，吾郷美保子，中園一郎，小片　守．拘束ストレスによる副腎皮質内分泌系の動態に関する実験的検討と虐待解剖例への応用．第 59 回日本法医学会九州地方会，佐賀，2009 年 10 月．

③ 林　敬人，吾郷一利，吾郷美保子，中島弘志，小片　守．傍食道型食道裂孔ヘルニアに基づく左胸腔内胃破裂の一剖検例．第 59 回日本法医学会九州地方会，佐賀，2009 年 10 月．

④ 林　敬人，吾郷一利，吾郷美保子，中島弘志，小片　守．Head-down position で発見され体位性窒息，頚部圧迫による窒息との鑑別を要した糖尿病性ケトアシドーシスの一剖検例．法医病理研究会第 16 回夏期セミ

ナー，箱根，2009 年 7 月.
⑤ 林　敬人，吉田愛知，吾郷一利，吾郷美保子，中島弘志，小片　守. 全身を打撲され 3 ヵ月後に死亡した例に認められた膵臓のほぼ完全な脂肪化. 第 17 回法医病理勉強会，大阪，2009 年 5 月.
⑥ 林　敬人，吾郷一利，吾郷美保子，小片　守. P-selectin と Interleukin-8 を指標とした高齢者虐待例臓器への好中球浸潤の検討. 第 93 次日本法医学会学術全国集会，大阪，2009 年 5 月.
⑦ 吾郷美保子，林　敬人，吾郷一利，中島弘志，小片　守. クロロホルム吸引による夫妻同時死亡例の中毒学的・組織病理学的分析. 第 93 次日本法医学会学術全国集会，大阪，2009 年 5 月.
⑧ 林　敬人，小片　守. 小児虐待例の諸臓器における好中球浸潤の検討と虐待診断への応用. 第 14 回日本子ども虐待防止学会学術集会ひろしま大会，広島，2008 年 12 月.
⑨ 林　敬人，吾郷一利，吾郷美保子，林園典子，小片　守. 小児・高齢者虐待例の諸臓器における好中球浸潤の検討と虐待診断への応用.第 58 回日本法医学会九州地方会，大分，2008 年 10 月.
⑩ 林　敬人，吾郷一利，吾郷美保子，中島弘志，小片　守. Head-down position で発見された糖尿病性ケトアシドーシスの一剖検例. 第 58 回日本法医学会九州地方会，大分，2008 年 10 月.
⑪ 吾郷一利，林　敬人，吾郷美保子，林園典子，小片　守. ヒト D13S317 プライマーによるニホンザルの PCR 産物について. 第 58 回日本法医学会九州地方会，大分，2008 年 10 月.
⑫ 林園典子，吾郷一利，林　敬人，吾郷美保子，小片　守. イヌ ZEBECA4 プライマーにより増幅されるニホンタヌキの PCR 産物について. 第 58 回日本法医学会九州地方会，大分，2008 年 10 月.
⑬ 吾郷美保子，林　敬人，吾郷一利，中島弘志，小片　守. 無理心中により夫婦ともに死亡したクロロホルム中毒例. 第 58 回日本法医学会九州地方会，大分，2008 年 10 月.
⑭ Hayashi T, Ago K, Ago M, Ogata M. Two patterns of b-amyloid precursor protein (APP) immunohistochemistry in cases of blunt head injury. Seventh International Symposium on Advances in Legal Medicine (ISALM), Osaka, 2008. 9.
⑮ Ogata M, Ago K, Ago M, Nakashima H, Hayashi T. Food poisoning due to *Salmonella* Enteritidis - A case report. Seventh International Symposium on Advances in Legal Medicine (ISALM), Osaka, 2008. 9.

〔その他〕
ホームページ等
http://www.kufm.kagoshima-u.ac.jp/~legalmed/

６．研究組織
(1)研究代表者
林　敬人（HAYASHI　TAKAHITO）
鹿児島大学・大学院医歯学総合研究科・助教
研究者番号：40512497