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研究課題名(和文) 虐待により繰り返される損傷に基づく好中球の臓器侵襲の検討と虐待診断法の開発

研究課題名(英文) Determination of the infiltration of neutrophils into organs resulting from repetitive injuries, and development of diagnostic methods for the physical abuse

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研究成果の概要(和文)：小児・高齢者に対する身体的虐待に基づく多発外傷によって好中球が各臓器内に浸潤することを、好中球マーカー-myeloperoxidase, 接着因子 P-selectin, 遊走因子 interleukin-8 等を指標とした免疫組織化学によって証明し、身体的虐待の法医学的証明法として有用であることを明らかにした。さらに、好中球が産生する組織障害因子がすでに発現しており、被虐待者は多臓器不全の前段階ともいふべき状態にある可能性が示唆された。

研究成果の概要(英文)：We investigated neutrophil infiltration into several organs in cases of child and elder physical abuse using immunohistochemistry for myeloperoxidase, a marker for primed neutrophil, P-selectin, an adhesion molecule, and interleukin-8, a chemotactic factor, and indicated that these analyses may represent supportive diagnostic methods for the physical abuse of the child and elderly. We also indicated that infiltrating primed neutrophils released elastase, a cytotoxic mediator, and suggested that patients with physical abuse may be in a primed stage of multiple organ failure.

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## 1. 研究開始当初の背景

近年、小児・高齢者等に対する虐待が社会問題となり、各虐待防止法も施行されているが、現在でも減少しているとは言い難く、法医解剖では虐待の終末像を見せつけられている。われわれは1991年、小児の身体的虐待に基づく広範な皮下出血の出血血液から溶出した hemoglobin が循環血中に入り、腎

臓内に沈着することを明らかにした。近年では、2007年から虐待の法医病理学的証明に関する研究を集中的に行っており、虐待のストレスが高齢者の内分泌系に影響を及ぼすことを報告した。われわれの報告の他にも、1990年初頭に胸腺の退縮やストレス蛋白質 ubiquitin の出現などが報告されてきた。しかしながら、近年では小児・高齢者に対する

虐待が身体に及ぼす影響に関する報告は少ない。一方、外傷性ショックや出血性ショックをはじめ広範な皮下出血に基づいて好中球が各臓器に浸潤することが報告されてきたが、身体的虐待に基づく好中球の侵襲についての検討は現在においてもほとんどない。

## 2. 研究の目的

われわれは今まで、児童虐待に基づく腎臓内への hemoglobin 沈着に加え、頭部外傷に基づく軸索、膠細胞などの変化を免疫組織化学を用いて検討してきた。さらに、これらの免疫組織化学的研究をさらに発展させて、各虐待による広範かつ繰り返す外傷が各種の炎症性メディエーターを介して、好中球が諸臓器に浸潤・侵襲することを明らかにしてきた。今回さらに詳細に検討を加え、虐待外傷による侵襲の開始から最終的に組織障害に至るまでの好中球侵襲の全体像を明らかにすることが本研究の目的である。

得られた成果を総合して、虐待に基づく好中球侵襲の全体像に加え、今までに得られた胸腺の退縮や腎臓内 hemoglobin 沈着などの研究成果も加味して、より精度の高い虐待の法医病理学的証明法を開発したい。さらに、各々の指標の経時的変化を明らかにして、頭部外傷における軸索等の経時的変化をも指標に加え、虐待された期間のより正確な推定法を確立したい。本研究によって法医実務に貢献するのみならず、虐待が全身諸臓器の機能に悪影響を及ぼすことを社会に対して広く発信していき、ひいては虐待予防に役立てていきたい。

## 3. 研究方法

小児・高齢者に対する虐待死事例について、好中球のマーカー myeloperoxidase に対する抗体を用いた免疫組織化学によって、各虐待例の心臓、肺、肝臓、腎臓などの臓器内に浸潤した好中球の数を数えて対照例と比較した。さらに、接着因子 P-selectin、遊走因子 interleukin-8、好中球エラスターゼ等を指標とした免疫組織化学によって各臓器内の陽性細胞数や細胞周囲への広がりを検討した。得られた成績を総合的に検討して、虐待による損傷に基づく好中球侵襲の全体像を明らかにした。

## 4. 研究成果

(1) 高齢者虐待に基づく多臓器への好中球浸潤に関する免疫組織化学的研究 (The infiltration of 'primed' neutrophils into multiple organs due to physical abuse to the elderly: An immunohistochemical study.)

**1. Abstract:** The infiltration of 'primed' polymorphonuclear neutrophils into multiple organs has been reported in cases of traumatic or hemorrhagic shock. Since multiple injuries are usually observed in

cases of physical abuse of the elderly, we investigated neutrophil infiltration into the heart, lung, liver and kidney in cases of abused elderly individuals using immunohistochemistry for myeloperoxidase (MPO). In addition, we examined the expression of molecules associated with neutrophil infiltration, including P-selectin as the adhesion molecule and IL-8 as the chemotactic factor. The number of neutrophils in the physically abused elder cases was increased significantly, particularly in the lung and liver, compared with that of control cases of sharp instrument injury, single fatal blunt injury and polytrauma. In addition, P-selection expression in the endothelium and the presence of IL-8-positive cells (mainly macrophages) in the lung and liver of abuse cases were significantly greater than those in control cases. In contrast, the number of MPO-, P-selectin- and IL-8-positive cells in cases of multiple organ failure (MOF) due to various causes was significantly greater than that in abuse cases. It is known that primed neutrophils accumulation may undergo MOF by 'activation' due to secondary insults. Thus, our results suggest that MPO immunostaining can distinguish cases of elderly physical abuse from non-abuse and MOF cases. In addition, our results indicate that MPO is a potential diagnostic marker for elder physical abuse, and that P-selectin and IL-8 may be useful for a more accurate diagnosis. Finally, our results also suggest that elder cases of physical abuse may be in a primed stage of MOF, and are at risk of falling into MOF by various secondary insults including those following abuse.

**2. Introduction:** The abuse and neglect of the elderly have been recognized as widespread and growing problems. Previously, we encountered an autopsy case of a physically and financially abused elderly person, and found it difficult to diagnose the case of elder abuse. In addition, we investigated the weight and histopathological findings of the thymus and adrenal glands in abused elders in an attempt to identify a diagnostic marker for elder abuse. The aim of the present study was to develop more reliable markers for elder abuse. Evidence suggests that 'primed' polymorphonuclear neutrophils, which migrate from the bone marrow and deposit in end organs, may contribute to organ

injury in cases of extensive trauma and following traumatic or hemorrhagic shock by releasing inflammatory mediators such as proinflammatory cytokines, neutrophil elastase, and nitric oxide. In general, neutrophil infiltration is a basic biological response, and involves a multistep cascade that includes rolling, adhesion, transmigration, and chemotaxis. The initial rolling interaction between neutrophils and the endothelium is mediated by the selectin family, including P-, E-, and L-selectin. Of these 3 selectins, P-selectin mediates the rolling from the earliest phase after stimulation of the endothelial cells. The process of transendothelial migration and chemotaxis are essential for neutrophil infiltration into the tissues. Neutrophils migrate mostly to the extravascular space in response to a chemotactic gradient. TNF- $\alpha$  and CXC chemokines such as interleukin (IL)-8, have a strong chemotactic effect on neutrophils. Evidence also suggests that the primed neutrophil infiltration into organs may lead to multiple organ failure (MOF) due to various secondary insults. In physical abuse cases, multiple injuries of different ages are usually observed over the whole body. Therefore, we assumed that neutrophils may infiltrate into multiple organs during the abused state, as in the cases of traumatic or hemorrhagic shock. In this study, we investigated neutrophil infiltration into several organs in cases of elder physical abuse using immunohistochemistry for myeloperoxidase (MPO), a marker for primed neutrophils. In addition, we examined the expression of molecules associated with neutrophil infiltration, including P-selectin and IL-8, to confirm that the cascade of neutrophil infiltration was activated. We discuss whether these immunohistochemical analyses may represent supportive diagnostic methods for the physical abuse of the elderly.

### **3. Materials and methods:**

**3-1. Autopsy samples:** Eleven autopsy cases of elder physical abuse (65 years and over of age; average age 77.5 y.o.) were collected. The abuse cases were defined based on the autopsy findings and police reports. The degrees of physical abuse were determined by estimating the whole area of injured skin area on the entire body surface based on the Lund-Browder chart, and by calculating the Injury Severity

Score (ISS). In addition, the severity of the final fatal injury was also estimated by ISS. Eighteen age-matched elder control cases (average age 76.0 y.o.) without any history of abuse were also collected. The control cases were divided into the following three groups according to the cause of death: sharp instrument injury (I-S, n=6), single fatal blunt injury (I-B, n=6) and polytrauma (Poly, n=6). Cases in which a primary focus of infectious disease was detected and/ or serum C-reactive protein levels were elevated pathologically were excluded. Further, although age was not matched with abuse cases, middle to elder cases (average age 64.0 y.o.) of fatalities due to MOF (n=6) were added. From each case, heart, lung, liver, and kidney samples were taken and were fixed in formalin, embedded in paraffin, and sectioned.

**3-2. Immunohistochemical analyses:** Immunohistochemistry for MPO, P-selectin, and IL-8 was performed on the obtained sections of the heart, lung, liver, and kidney using rabbit anti-MPO pAbs, mouse anti-P-selectin mAb, or mouse anti-IL-8 mAb. MPO or IL-8-positive cells were counted on 10 randomly chosen visual fields, and the average positive cells were calculated. As for P-selectin, the intensity of the immunohistological staining reaction was graded as absent (0), weak (1), moderate (2) or strong (3) and the percentage of blood vessels involved per visual field was graded as absent (0), <10% (1), 10–50% (2) or >50% (3). The total score was achieved by adding both variables, and the average score was calculated. The relationships of the duration of abuse and the ISS of the whole injury or final fatal injury to the immunoreactivity of MPO, P-selectin, or IL-8 were investigated.

**3-3. Double-color immunofluorescence analysis:** In order to determine the types of IL-8-expressing cells in the lungs of abuse cases, double-color immunofluorescence analysis was performed using anti-IL-8 mAb anti-MPO pAbs, anti-human macrophage Ab, and fluorochrome-conjugated secondary Abs.

**3-4. Statistical analyses:** Statistical analyses were performed using one-factor analysis of variance to determine whether differences existed among the group means, followed by Scheffe test to identify significantly different means. Regression equation analysis was used to study the

relationships between pairs of parameters. A value of  $p < 0.05$  was considered to indicate a significant difference.

#### 4. Results

**4-1. Immunohistochemical analysis of MPO:** There was no obvious difference in the number of MPO-positive neutrophils in each organ between the three control groups. By contrast, in abuse cases, a large number of neutrophils were detected in the lung, liver and kidney. Semiquantitatively, the number of neutrophils in the abuse cases was increased significantly in the lung and liver, compared with that of the three control groups (Fig. 1).

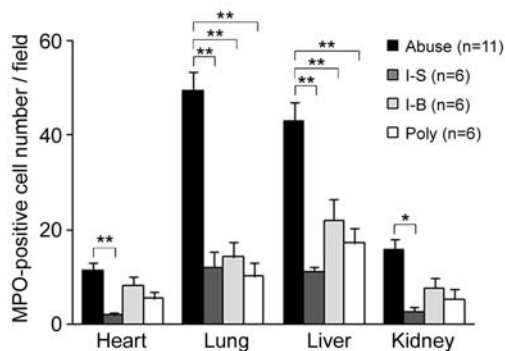


Fig. 1. Semiquantitation of MPO immunoreactivity. \*\* $p < 0.01$ ; \* $p < 0.05$  vs. abuse.

**4-2. Immunohistochemical analysis of P-selectin:** In the abuse cases, P-selectin showed a significant increase in the intensity of the immunoreactivity and a significantly higher number of immunoreactive blood vessels in the lung and liver, compared with the control groups.

**4-3. Immunohistochemical analysis of IL-8:** In the abuse cases, the number of IL-8-positive cells was increased significantly in the lung and liver, compared with that of the control groups.

**4-4. Double-color immunofluorescence analysis of IL-8-expressing cells:** IL-8 was detected in a small proportion of MPO-positive neutrophils (Fig. 2a) in the lungs of abuse cases. However, IL-8 immunoreactivity was detected mainly in macrophages (Fig. 2b).

**4-5. The relationship of inflammatory cell infiltration with the duration of abuse and the severity of physical abuse:** There was no significant correlation between the duration of abuse and the number of infiltrating neutrophils in the lung and liver. The number of neutrophils in the lung and liver of abuse cases was not correlated with the injured skin area. By

contrast, these values were correlated positively with the ISS of the whole injury (lung,  $r=0.715$ ,  $p=0.0112$ ; liver,  $r=0.766$ ,  $p=0.0043$ ). There was a similar correlation between the ISS of the whole injury and P-selectin expression in the lung ( $r=0.602$ ,  $p=0.0488$ ), and IL-8 immunoreactivity in the lung ( $r=0.690$ ,  $p=0.0165$ ) and liver ( $r=0.665$ ,  $p=0.0233$ ). However, neutrophil infiltration was not correlated with the ISS of fatal injury due to a final insult. In addition, the ISS of fatal injury was correlated negatively with survival time after the final insult ( $r=-0.633$ ,  $p=0.0485$ ).

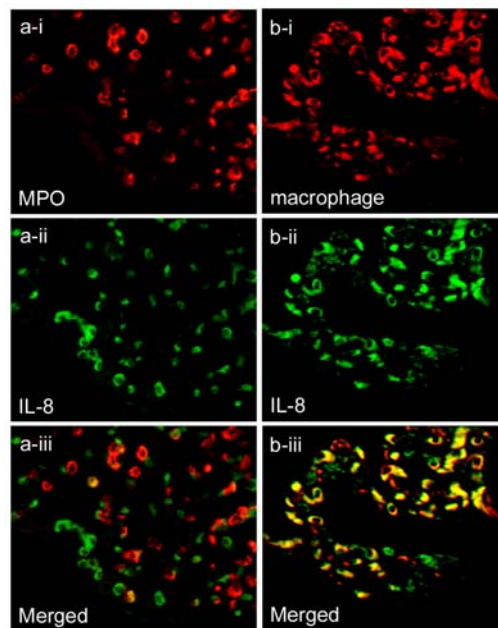


Fig. 2. The lung samples of abuse cases were double-color immunostained. Signals in i and ii were digitally merged in iii.

**4-6. Comparison of inflammatory reactions between cases of abuse and cases of fatalities due to MOF:** The number of neutrophils in MOF cases in all organs was significantly greater than that of abuse cases. P-selectin expression and IL-8-positive cells were also significantly greater in all organs of these MOF cases than in the abuse cases.

**5. Discussion:** The results of the present study indicate that a massive MPO-positive neutrophil infiltration occurred in the lung and liver of elder physical abuse cases, and that the number of neutrophils in the abuse cases was increased significantly compared with that in the three control groups. In accordance with the massive neutrophil infiltration into the lung and liver, the endothelial expression of P-selectin and the number of IL-8-

positive cells also increased significantly in those organs of abuse cases compared with that of the control groups.

These findings suggest that elder physical abuse induces massive primed neutrophil infiltration into the vital organs, as in cases of traumatic or hemorrhagic shock, and that this infiltration occurs particularly in the lung and liver with activation of a multistep cascade mediated by P-selectin and IL-8. Neutrophils predominate for the first 6–24 h in most forms of acute inflammation. Therefore, it is probable that the primed neutrophil infiltration into the organs was not induced by final fatal injury, since the survival time of many of the control cases was less than 6 h and since the infiltration was also observed in abuse cases with a short period of survival time after the final insult. Moreover, the ISS of final fatal injury was negatively correlated with survival time after the final insult, and was not correlated with degree of the infiltration. In contrast to final fatal injury, the severity of physical abuse estimated by ISS of the whole injury was positively correlated with neutrophil infiltration. Accordingly, these observations suggest that primed neutrophil infiltration may correspond to the degree of accumulation of less fatal injuries caused by repetitive physical abuse. In addition, the infiltration was not correlated with the duration of abuse. It is possible that the number of neutrophils fails to increase with a low frequency of abuse because of the short cellular survival time (less than 24–48 h) of neutrophils, even if the duration of abuse was long.

Our results indicate the occurrence of a massive neutrophil infiltration in the liver as well as in the lung, although the lung and kidney are known to be sensitive to various types of shock. Abundant resident macrophages such as alveolar macrophages and Kupffer cells are present in the lung and liver, respectively. Recent studies have demonstrated that alveolar macrophages and Kupffer cells are the primary abundant source of proinflammatory cytokines including IL-8 when activated by antigens or inflammatory stimuli. Our results also show that the number of IL-8-positive cells increased significantly in the lung and liver of abuse cases, and that IL-8 was produced mainly by macrophages in the lung (**Fig. 2**).

Recently, it is becoming increasingly clear that infiltrating primed neutrophils not only release cytotoxic mediators such as elastase and superoxide, but also modulate the immune system by the release of both proinflammatory and counterinflammatory cytokines. Thus, infiltrating neutrophils are involved in the development of post-traumatic MOF, which remains the leading cause of late post-traumatic mortality. Numerous neutrophils were detected in multiple organs of MOF caused by various pathophysiologies including severe trauma in our study. The number of neutrophils in MOF cases was significantly greater than that of abuse cases. Primed neutrophil accumulation into several organs does not cause MOF immediately after the insult, but is prone to undergo MOF by secondary insults such as trauma, infection, hypotension, circulation failure, and disseminated intravascular coagulation (DIC). This mechanism is termed the “two-hit” model of MOF. According to the model, priming and activation of the inflammatory response by the above insults are considered to play a pivotal role in the pathogenesis of MOF. Thus, the abused elderly may be in a “primed stage of MOF” and possibly fall into MOF by ‘activation’ due to secondary insults including following physical abuse, even if fatal trauma did not occur. Consequently, our results suggest that primed neutrophil infiltration induced by extensive trauma of different ages due to elder physical abuse may cause MOF and late post-traumatic mortality. Further investigations are needed to determine whether MOF occurs in elder abuse.

Elderly people usually have more or less chronic diseases on the background, in which inflammatory cell infiltration into organs may be observed. Although mononuclear cells, but not polymorphonuclear neutrophils, predominate in the chronic inflammation, neutrophil infiltration due to chronic infection must be excluded when neutrophil infiltration is evaluated for the diagnosis of elder physical abuse.

In conclusion, massive primed neutrophil infiltration particularly into the lung and liver was observed in elder abuse cases. The demonstration of neutrophil infiltration in the lung and liver by immuno-

histochemistry for MPO may be a new diagnostic marker for elder physical abuse when chronic infectious diseases are excluded. The additional demonstration of P-selectin and IL-8 may further support a diagnosis of abuse. Furthermore, elder patients with physical abuse may be in a “primed stage of MOF” and may fall into MOF by various secondary insults. Our results emphasize the importance of prevention against elder abuse.

#### 5. 主な発表論文等

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#### 6. 研究組織

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