様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

科学研究費助成事業　　研究成果報告書

令和　４ 年　 ６ 月　 ８ 日現在

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研究課題名（和文）多発外傷時における白血球遺伝子発現プロファイル解析：T-iPS細胞療法の応用

研究課題名（英文）Evaluation of gene expression profile during multiple trauma: application of T-iPS

研究代表者

武山　直志（Takeyama, Naoshi）

愛知医科大学・医学部・名誉教授

研究者番号：００１５５０５３

交付決定額（研究期間全体）：（直接経費）　　3,400,000円

研究成果の概要（和文）：急性呼吸促迫症候群（ARDS）発症時のneutrophil extracellular traps (NETs)の関与を明らかにするために、培養血管内皮細胞（HPAEC）とphorbol myristate acetate (PMA)刺激好中球を共培養するin vitroの検討を行った。PMA刺激好中球で誘発したNETsは、HPAEC細胞間隙のアルブミン透過性を亢進した。組織学的には、サイトスケルトンのFアクチン変形と細胞間接着分子であるカドヘリンの減少を認めた。本in vitroの検討により、NETsは直接的にHPAECの細胞構造に影響を与え透過性亢進を誘発していることが明らかになった。

研究成果の学術的意義や社会的意義
ARDS病態の中心を成すと考えられていた活性化好中球に関して、これまでは、neutrophil extracellular traps (NETs)の関与は証明されていなかった。本研究によりNETsと血管透過性亢進の関係が明らかになったことにより、今後、NETs産生をコントロールすることにより、従来治療法の限られていたARDS治療が可能になると予想される。このことは、細菌感染、外傷に起因するARDSのみでなく、COVID-19をはじめとするウイルス感染によるARDSに対しても同様な効果を発揮すると考えられる。

研究成果の概要（英文）：Although activated neutrophils are thought to play a significant role in mediating acute respiratory response syndrome (ARDS), at present the contribution of neutrophil extracellular traps (NETs) to lung endothelial barrier function is unclear. To clarify their role, we co-cultured in vitro NETs induced by phorbol myristate acetate (PMA), activated neutrophils with lung endothelial cell monolayers (HPAEC) and examined the barrier function of lung endothelial cells. Co-culture with stimulated neutrophils increased the albumin permeability of HPAEC and altered cytoskeleton F-actin and vascular endothelial-cadherin in cell-cell junctions. This in vitro experiment shows that altered HPAEC barrier function and increased albumin permeability are caused by the direct effect of PMA-induced NETs and their components. NET formation may be involved in the increased vascular permeability of the lung, which is a common feature in ARDS of various etiologies.

研究分野：救急医学

キーワード：NETs　急性呼吸促迫症候群　カドヘリン　Fアクチン

様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９（共通）

１．研究開始当初の背景

Acute respiratory distress syndrome (ARDS) is an acute inflammatory lung injury characterized by hypoxemic respiratory failure as a consequence of increased permeability of the endothelial-epithelial barrier, alveolar damage, and pulmonary edema. The pathogenesis of ARDS is complex, and the syndrome has a high mortality rate in critically ill patients. Despite significant advances in mechanical ventilation aimed to better protect the lungs, ARDS remains difficult to prevent, reduce, or treat effectively. Recent research has identified a novel antibacterial strategy: neutrophil extracellular traps (NETs), which localize to and eliminate pathogens. These NETs are characterized by chromatin decorated with cytosolic and granular proteins. They immobilize or trap various pathogens, thus preventing their dissemination. However, similar to the excess production of inflammatory mediators, the excessive presence of NETs—and in particular NET-bound components—also has harmful effects. For instance, NETs contain histone, neutrophil elastase (NE), cathepsin G, and myeloperoxidase (MPO), all of which are cytotoxic to endothelial cells. Neutrophil extracellular traps are found not only at sites of infection and acute inflammation but also in the bloodstream, where they are known as circulating cell-free NETs. Researchers have hypothesized that circulating cell-free NETs interact with platelets, leucocytes, and the vascular endothelium in the lung to induce ARDS. In patients with ARDS and in murine models of lung injury, NETs develop in response to a variety of infectious stimuli and contribute to the injury.

２．研究の目的

Direct evidence of lung hyperpermeability caused by NETs and circulating cell-free NETs is still lacking. Therefore, to examine whether NET formation is involved in lung hyperpermeability during acute inflammation, we cocultured human pulmonary endothelial cell monolayers with neutrophils after pretreatment with phorbol myristate acetate (PMA).

３．研究の方法

1) Analysis of NET formation

The NET formation of polymorphonuclear leukocytes (PMNs) after activation by PMA was assessed by the release of cf-DNA, NE-DNA, and MPO-DNA into the medium. The medium levels of all three types of DNA increased significantly after the suspension was incubated with PMA for 2 h and then without PMA for 4 h (Table 1). We also analyzed the generation of NETs by PMNs after PMA activation by immunofluorescence confocal microscopy and found that incubating the suspension with PMA for 2 h and then without PMA for 4 h caused extracellular release of MPO, NE, and DNA (Figure 1).

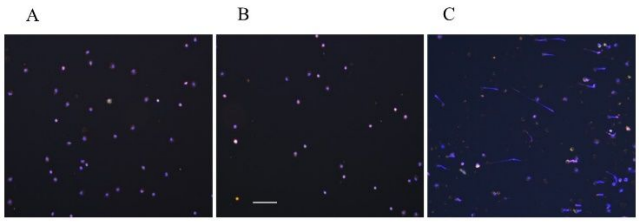
Table 1: Medium levels of cell-free DNA, NE-DNA, and MPO-DNA before and after activation of neutrophils by PMA

	Before addition of PMA, n = 5	Incubation with PMA for 2 hours before washing, n = 5	Incubation with PMA for 2 hours + incubation without PMA for 4 hours, n = 5	One-way ANOVA, P value	Tukey
Total incubation time	0 hours	2 hours	6 hours		
cf-DNA, ng/ml	82.5 ± 12.9	73.54 ± 11.08	1681 ± 326	0.008	a, b

MPO-DNA, Abs_{405}	0.29 ± 0.08	0.26 ± 0.09	5.13 ± 0.62	0.004	a, b
NE-DNA, Abs_{405}	0.049 ± 0.022	0.051 ± 0.027	0.72 ± 0.17	0.009	a, b

Figure 1: Detection of NETs by immunolabeling

Representative images showing direct immunofluorescence staining of DNA (blue), MPO, (green), and NE, (red) in neutrophils and NET structure before (A) and after addition of PMA (B and C). Neutrophils were incubated with PMA for 2 hours (B) and then washed three times to remove PMA and further incubated without PMA for 4 hours (C).

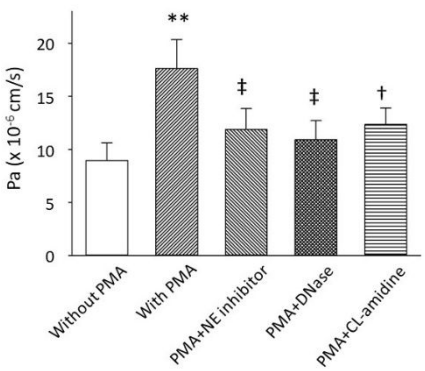


2) NETs influence permeability in HPAECs

To evaluate the effects of activated PMNs on permeability across the HPAEC monolayer, we assessed Pa in a model of HPAECs co-cultured with PMNs activated by PMA. After the addition of PMNs activated by PMA, Pa significantly increased when compared with co-culture with PMNs without PMA stimulation (Figure 2). This increase in permeability was significantly reduced by pretreatment with CL-amidine, DNase, or NE inhibitor (Figure 2).

Figure 2: The apparent permeability coefficients of albumin (Pa) in HPAEC monolayer induced by co-culture with PMA-activated neutrophils FITC-labelled albumin in the lower chamber with permeation through the HPAEC monolayer co-cultured with and without PMA-treated neutrophils in the presence and absence of DNase, a neutrophil elastase (NE) inhibitor, and a CL-amidine. Values are means ± SD; n = 4. ***P* < 0.01 vs without PMA. †*P* < 0.05; ††*P* < 0.01 vs with PMA.

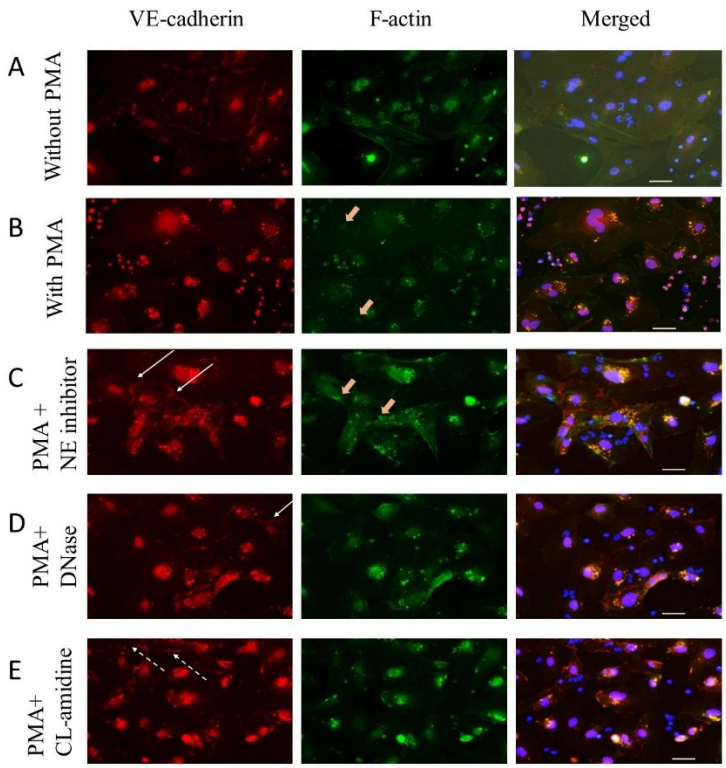
Figure 2



3) NETs affect HPAEC cytoskeletal and VE-cadherin

Endothelial adherens junctions play a key role in maintaining the integrity of cell-cell junction structures. We examined the adherens junction component VE-cadherin under co-culture conditions. The distribution of VE-cadherin in HPAECs co-cultured with stimulated PMNs was significantly altered compared with HPAECs co-cultured with unstimulated PMNs: the latter showed a straight, linear distribution of VE-cadherin between the cells (Figure 3A); however, almost all VE-cadherin expression disappeared in HPAECs co-cultured with stimulated PMNs (Figure 3B and F). When HPAECs were pretreated with DNase (Figure 3D), a PAD4 inhibitor (Figure 3E), or an NE inhibitor (Figure 3C), an interrupted (Figure 3E, dashed arrows) and a zig-zag (Figure 3C and D, white arrows) redistribution patterns appeared in the HPAECs. Many cytoplasmic bodies visualized by double-immunofluorescence staining were observed in cells co-cultured with PMA-activated neutrophils (Figure 3B to E). Cellular actin filament alignment was assessed by F-actin staining. HPAECs co-cultured with stimulated PMNs caused the remodeling of the endothelial actin cytoskeleton, i.e., actin fibers moved from the cortical rim and were collected and condensed as intracellular stress fiber (Figure 3B and C). The alteration in F-actin in HPAECs co-cultured with stimulated PMNs was partially prevented when HPAECs were pretreated with DNase, PAD4 inhibitor, or NE inhibitor (Figure 3C-E).

Figure 3: Intercellular junctions were evaluated by detecting the adherens junction protein VE-cadherin (red). The cytoskeleton was evaluated by detecting F-actin (green). DNA was stained using Hoechst 33248 (blue). Brown arrows indicate stress fibers. White arrows indicate a zig-zag pattern; dashed arrows indicate an interrupted pattern. NET inhibition was performed by DNase, a CL-amidine, and NE inhibitor.



4．研究成果

This *in vitro* study showed that co-culture with stimulated PMNs increases the permeability of the HPAEC monolayer and causes cytoskeleton remodeling and alterations of VE-cadherin at the cell-cell junction. These effects were prevented by pretreating HPAECs with DNase, a PAD4 inhibitor, and an NE

inhibitor, suggesting that formation of both NETs and NET components affects HPAEC barrier function. This study shows that both NETs and NET components are factors that probably account for the lung endothelial barrier dysfunction caused by activated neutrophils. Our results are in line with the data recently published by Lv et al., although that group used a cell line of different origin. With the direct visualization xCELLigence system, they showed that lipopolysaccharide-activated neutrophils (referred to as NETing neutrophils) caused homologous lung epithelial injuries in a time- and number-/concentration-dependent manner. Together, they showed that both pulmonary endothelial and epithelial cells are injured directly by NETs, a process that might be involved in the pathogenesis of ARDS.

Previous studies demonstrated that plasma levels of circulating cell-free NETs were higher in patients with bacterial and viral pneumonia-associated ARDS, blood transfusion–associated ARDS, and remote lung injuries than in individuals without ARDS. In addition, the levels of circulating cell-free NETs correlated to the severity of ARDS, indicating that NETs were accelerated in ARDS and may play a central role in the pathogenesis of ARDS. NET-targeted therapies, such as those inhibiting *de novo* NET synthesis or accelerating the degradation of preformed NETs, are potential therapeutic avenues to be explored in further investigations.

This *in vitro* study indicates that lung endothelial barrier functions are altered by NET formation and the extracellular release of NET components. Hyperpermeability, cytoskeleton remodeling, and alteration of VE-cadherin in cell-cell junctions are prevented by inhibition of NET formation and increased degradation of NET structure. NETs would be involved in the increased vascular permeability of the lung, which seems to be a common feature in ARDS of various etiologies. Thus, this study provides insights that may help generate novel approaches for medical interventions.

5．主な発表論文等

〔雑誌論文〕　計2件（うち査読付論文　2件／うち国際共著　0件／うちオープンアクセス　1件）

1．著者名 Hisatake Mori, Muhammad Aminul Huq, Md. Monirul Islam, Naoshi Takeyama	4．巻 19
2．論文標題 Neutrophil extracellular traps are associated with altered human pulmonary artery endothelial barrier function	5．発行年 2021年
3．雑誌名 European Journal of Inflammation	6．最初と最後の頁 1，10
掲載論文のDOI（デジタルオブジェクト識別子） 10.1177/20587392211062386	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 －

1．著者名 Hisatake Mori, Muhammad Aminul Huq, Md. Monirul Islam, Naoshi Takeyama	4．巻 19
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オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 －

〔学会発表〕　計1件（うち招待講演　0件／うち国際学会　1件）

1．発表者名 Mori H, Huq MA, Kajita Y, Islam MM, Takenaka N, Gocho T, Maruchi Y, Tsuda M, Takeyama N.
2．発表標題 Influence of Neutrophil Extracellular Traps on Vascular Permability.
3．学会等名 41st Annual conference on Shock（国際学会）
4．発表年 2018年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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6．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考
研究分担者	森　久剛 (Mori Hisatake) (10620867)	愛知医科大学・医学部・助教 (33920)	

６．研究組織（つづき）

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考
研究分担者	富野　敦稔 (Tomino Atsutoshi) (70440980)	愛知医科大学・医学部・講師 (33920)	

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関