Production and Activation of Matrix Metalloproteinase 7 (Matrilysin 1) in the Lungs of Patients With Idiopathic Pulmonary Fibrosis

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• ***Context.***—**Idiopathic pulmonary fibrosis (IPF) is characterized by diffuse interstitial inflammation and fibroblast proliferation with accelerated remodeling of extracellular matrix, which result in irreversible destruction of the lung's architecture.**

Objective.—**To elucidate the production levels, tissue localization, and activation of matrix metalloproteinase 7 (MMP-7) in the lungs of patients with IPF.**

Design.—**Bronchoalveolar lavage analysis was performed in 17 IPF patients and 6 healthy volunteers. Levels of MMP-7 in blood were assayed in 23 IPF patients and 20 controls. Histologic and immunohistochemical analyses were performed on paraffin sections of the lung tissues from patients with IPF, interstitial pneumonia associated with rheumatoid arthritis, or nonspecific interstitial pneumonia.**

Results.—**The proMMP-7 levels in bronchoalveolar lavage fluids from IPF patients were significantly higher than those from healthy controls, although there was no difference in the serum levels between the 2 groups. By immunohistochemistry, proMMP-7 was localized mainly to the hyperplastic alveolar and metaplastic bronchiolar epithelial cells in the lung tissues from IPF patients. Active MMP-7 was immunolocalized on alveolar macrophages and hyperplastic epithelial cells, which were also immunostained with antibody against CD151, a molecule associated with activation of proMMP-7. Immunoblot analysis indicated the overproduction of proMMP-7 together with a small amount of active MMP-7 in bronchoalveolar lavage fluids from IPF patients. The MMP-7 activity was detected in a cross-linked carboxymethylated transferrin film assay.**

Conclusions.—**proMMP-7 is excessively produced by hyperplastic alveolar and metaplastic bronchiolar epithelial cells and activated locally in the lungs of IPF, suggesting that MMP-7 may contribute to the pathology of IPF.**

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Idiopathic pulmonary fibrosis (IPF), also called usual interstitial pneumonitis on a pathologic basis, is characterized by diffuse interstitial inflammation, fibroblast proliferation, and accelerated remodeling of extracellular matrix (ECM), which result in irreversible destruction of the fine architecture of the lungs.[1,2] The disease is progressive and patients with IPF usually die (mean survival, 5 years). Although several medications such as glucocorticoids, immunosuppressants, and pirfenidone have been used with some improvement in a fraction of the patients with IPF,[3] there is no established treatment that reliably improves their prognosis. Thus, new therapies based on novel understanding of its pathogenesis are being awaited. Zuo et al[4] previously reported the overexpression of matrix metalloproteinase 7 (MMP-7), also called matrilysin 1, by oligonucleotide microarrays and immunohistochemistry in the fibrotic lung tissues of patients with IPF. MMP-7 is a member of the MMP gene family, which is currently composed of 23 members in humans. They are synthesized as inactive zymogen forms (proMMPs) and processed to active forms, which lack the NH_2-terminal propeptides. Synthesis and activation are independently regulated and thus the presence of proMMPs does not indicate their activities in situ. Accumulated lines of evidence have suggested that MMP-7 is docked to cell membranes by binding membrane proteins such as CD44 heparan sulfate proteoglycan. In addition, we have recently demonstrated that proMMP-7 binds to CD151 and this interaction leads to activation of proMMP-7.[5] MMP-7 displays strong proteolytic activity against a wide range of ECM components including collagen types IV, V, IX, and X, proteoglycans, fibronectin, laminin, gelatin, elastin, and entactin. It also cleaves various non-ECM bioactive molecules such as Fas ligand, pro–tumor necrosis factor α, E-cadherin, β4-integrin, insulin-like growth factor binding protein 3 and

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insulin-like growth factor binding protein 5, and α-defensin.[6–13] MMP-7 is produced mainly by glandular epithelial cells and macrophages in various diseased tissues[14,15] and overexpressed by carcinoma cells in human cancers with correlation to metastases.[16] In addition, tumorigenesis is suppressed in MMP-7–deficient mice.[17] Thus, MMP-7 is considered to play roles in tumorigenesis and tumor progression.[18] However, information is still limited about activation of proMMP-7 within tissues and pathobiologic roles of MMP-7 in inflammatory conditions such as IPF. In the current study, we examined the production levels and activation of proMMP-7 and tissue localization of MMP-7 in the lung tissues of IPF. The results demonstrate that proMMP-7 is overproduced in hyperplastic alveolar epithelial cells and alveolar macrophages and activated in the lung tissues of IPF. The possible functions of MMP-7 in IPF lung tissues are discussed.

MATERIALS AND METHODS

Study Population

Idiopathic pulmonary fibrosis was diagnosed according to the criteria of the American Thoracic Society/European Respiratory Society international multidisciplinary consensus classification of IPF.[19] Briefly, 23 patients satisfied the following major criteria: (1) exclusion of other known causes of interstitial lung diseases such as certain drug toxicities; no history of exposure to asbestos, silica, and any other inorganic dusts; and no evidence of arthritis, myalgia, skin eruption, or serum autoantibodies suggestive of collagen vascular diseases; (2) abnormal pulmonary function studies that include evidence of restriction; (3) bibasilar reticular abnormalities with minimal ground glass opacities on high-resolution computed tomography scans; and (4) transbronchial lung biopsy or bronchoalveolar lavage (BAL) showing no features to support an alternative diagnosis. They also had all of the minor criteria: (1) age older than 50 years; (2) insidious onset of otherwise unexplained dyspnea on exertion; (3) duration of illness more than 3 months; and (4) bibasilar, inspiratory crackles. In addition, the following patients were eliminated from the IPF group to avoid the effect of acute inflammation and drugs: patients with a possible respiratory tract infection as a complication and those with present or past history of treatment with glucocorticoid or immunosuppressive agents. Seventeen patients were enrolled for BAL analysis and 23 for blood analysis. We also collected blood from 20 healthy volunteers and performed BAL in 6 healthy volunteers.[20] Informed consent was obtained from all patients and healthy volunteers. The patients were men and older than 52 years. On high-resolution computed tomography, all patients had patchy, peripheral-dominant, reticular lesions and did not show any findings suggestive of other interstitial lung diseases. No patients showed any findings of malignancy on BAL and transbronchial lung biopsy.

Peripheral Blood and Bronchoalveolar Lavage Samples

Blood was drawn from peripheral veins into a small glass tube, and serum was obtained by centrifugation at 3000 rpm for 5 minutes. Bronchoalveolar lavage was performed according to the standard protocol as described previously.[21] Briefly, a bronchoscope was wedged in a segment of the right middle lobe or lingual under local anesthesia. Sterile normal saline at room temperature was instilled through the bronchoscope. The recovered BAL fluids (total 150 mL) were filtered through sterile gauze and the cells were counted. Cytospin preparations were made for cell differential counts. The fluids were centrifuged at 200*g* for 5 minutes and the supernatants were used for the assays.

Quantification of proMMP-7 in BAL Fluids and Serum Samples

proMMP-7 levels in the BAL fluids were measured by a sandwich enzyme immunoassay (Daiichi Fine Chemicals, Co Ltd, Takaoka, Japan) as described previously.[22] This assay system detects solely proMMP-7 and the detection limit is 0.63 ng/mL. Serum samples from the patients and healthy volunteers were also measured by the system.

Histopathologic and Immunohistochemical Examination of Human Lungs

Lung tissues of IPF (n = 6) and control normal lung tissues (n = 6), the latter of which were from patients without underling pathologic conditions in the lungs, were obtained at autopsy and fixed with 10% formalin. In addition, we obtained an autopsied lung tissue from 1 patient with interstitial pneumonia associated with rheumatoid arthritis and biopsied lung tissues from 3 patients with nonspecific interstitial pneumonia. Paraffin sections (4 μm thick) were made and stained with hematoxylin-eosin, elastica van Gieson, and silver impregnation. For immunohistochemistry, the sections were reacted with monoclonal antibodies specific to proMMP-7 (141-7B2; 4 μg/mL), active MMP-7 (176-5F12; 10 μg/mL), or CD151 (11G5a; 5 μg/mL) for approximately 18 hours at 4°C after blocking endogenous peroxidase and nonspecific binding according to our methods.[5] They were incubated with biotinylated horse immunoglobulin G (IgG) to mouse IgG (1:200; Vector Laboratories, Inc, Burlingame, California) for 30 minutes and then with an avidin-biotin peroxidase complex (Dako, Glostrup, Denmark) for 30 minutes at room temperature. Color was developed with 0.03% 3,3′-diaminobenzidine tetrahydrochloride in 50mM Tris-HCl buffer, pH 7.6 containing 0.006% H_2O_2 for Figure 2 and diaminobenzidine containing 10mM imidazole for Figure 3. Counterstaining was performed with hematoxylin. For a control, the primary antibodies were replaced with nonimmune mouse IgG (4 μg/mL) followed by the procedures described previously.

Immunoblotting

Bronchoalveolar lavage fluids were concentrated by trichloroacetic acid and then electrophoresed on 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions. Proteins in the gels were electrotransferred onto polyvinylidene difluoride membranes (ATTO Corporation, Tokyo, Japan). The membranes were reacted with monoclonal anti–MMP-7 antibody (10 μg/mL; 125-20H11), which recognizes both proMMP-7 and active MMP-7,[22] at 4°C for 12 hours after blocking nonspecific reaction with 3% bovine serum albumin, and incubated with horseradish peroxidase–labeled secondary antibody (Vector). Immunoreactive bands were detected with ECL Western blot reagents (Amersham Bioscience Co) using Lumivision HSII (Taitec Co Ltd, Tokyo, Japan).

Detection of MMP-7 Activity in BAL Fluids in a Cross-Linked Carboxymethylated Transferrin Film Assay

Bronchoalveolar lavage fluids were placed on cross-linked carboxymethylated transferrin (CCm-Tf) films, which were prepared by coating with cross-linked CCm-Tf solution on polyethylene terephthalate support films as described previously.[23] Immediately after placing BAL fluid specimens on the films, they were incubated at 37°C for 8 to 16 hours in a moist chamber and then stained with staining solution containing 0.3% Biebrich Scarlet. The samples were also subjected to the CCm-Tf films impregnated with a mixture of aprotinin and elastinal or a mixture of aprotinin, elastinal, and 1,10-phenanthroline.

Statistical Analysis

All data are presented as mean ± standard deviation. Unpaired Student *t* tests or 1-factor analysis of variance were used to compare mean values and χ^2 test was used to compare detection rate. *P* values less than .05 were considered significant.

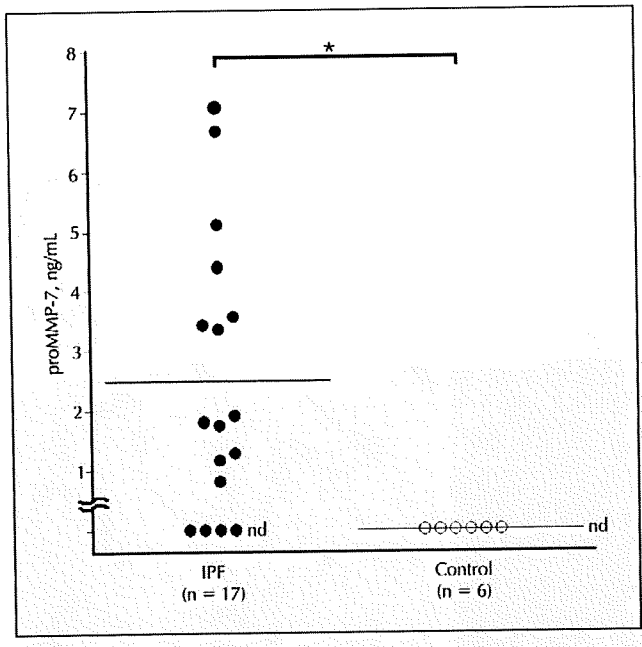

Figure 1. *Pro matrix metalloproteinase 7 (proMMP-7) levels in the bronchoalveolar lavage fluids. The bronchoalveolar lavage fluids were subjected to the enzyme immunoassay for proMMP-7 as described in "Materials and Methods." Abbreviations: IPF, idiopathic pulmonary fibrosis; nd, not detectable. *, P < .001.*

RESULTS

proMMP-7 Levels in BAL Fluids and Serum Samples of IPF Patients

proMMP-7 in BAL fluids was assayed by the enzyme immunoassay system in 17 patients with IPF. Total cell number in the BAL fluids was 5.14 ± 3.61 × 10^5/mL (mean ± standard deviation). Cell differential percentages in the BAL fluids were as follows: macrophages, 75.7 ± 19.4%; lymphocytes, 10.8 ± 8.2%; neutrophils, 11.0 ± 18.3%; and eosinophils, 6.3 ± 9.4%. As shown in Figure 1, 13 of 17 samples from the patients showed detectable levels of proMMP-7 (2.49 ± 2.29 ng/mL), but negligible levels of proMMP-7 were observed in the control samples from 6 healthy volunteers (<0.63 ng/mL). The levels were significantly higher in the patient samples than in the control samples ($P < .001$). On the other hand, although proMMP-7 was detected in the serum samples from IPF patients (3.47 ± 2.15 ng/ml) and healthy volunteers (3.77 ± 1.61 ng/mL), there was no significant difference between the 2 groups.

Immunohistochemical Localization of proMMP-7, Active MMP-7, and CD151

Histologic analysis of the lung tissues of IPF showed septal fibrosis and inflammatory cell infiltration in the alveolar walls (Figure 2, A). By immunohistochemistry, proMMP-7 was localized mainly to the hyperplastic alveolar epithelial cells in the lung tissue of IPF (Figure 2, B), whereas active form of MMP-7 was detected mainly on the cell surfaces of alveolar macrophages and epithelial cells (Figure 2, C). We observed similar findings in all 6 patients with IPF. On the other hand, in patients with nonspecific interstitial pneumonia (Figure 2, D and G) and interstitial pneumonia associated with rheumatoid arthritis (Figure 2, E and H), proMMP-7 was positive in the regenerative epithelial cells. However, no definite signal of active MMP-7 was detected (data not shown). Control normal lung tissue (n = 6) showed no staining with antibody against proMMP-7 or active MMP-7 (Figure 2, F and I, and data not shown for active MMP-7 immunostaining). Because our previous studies have demonstrated that proMMP-7 is captured and activated by CD151 expressed on the cell surfaces of lung adenocarcinoma cells[5] and osteoarthritis chondrocytes,[24] we further examined colocalization of MMP-7 and CD151 in the mirror sections of the lung tissue of IPF. As shown in Figure 3, CD151 immunostaining was prominent in hyperplastic alveolar and metaplastic bronchiolar epithelial cells and alveolar macrophages, and some of these cells also showed positive staining of MMP-7.

Immunoblotting Analysis of MMP-7

When BAL fluids were immunoblotted with anti–MMP-7 antibody, both proMMP-7 of 28 kDa and its processed forms of active MMP-7 were detected (Figure 4, lanes 1–3). In contrast, neither proMMP-7 nor active MMP-7 was observed in the control samples (Figure 4, lanes 4–6).

Detection of MMP-7 Activity in a CCm-Tf Film Assay

Bronchoalveolar lavage fluids were incubated on the CCm-Tf coated films without or with proteinase inhibitors. As shown in Figure 5, A, the digestion of CCm-Tf on the film without inhibitors was observed in 3 of 7 IPF samples (lanes 1–7), but no activity was detected in 3 samples from healthy volunteers (lanes 8–10). The CCm-Tf–degrading activity in the samples from IPF patients was not altered by incubation on the CCm-Tf film coated with a mixture of aprotinin and elastinal (serine proteinase inhibitors) (Figure 5, B), but it was completely abolished in the film coated with a mixture of the serine proteinase inhibitors and 1,10-phenanthroline (a metalloproteinase inhibitor) (Figure 5, C).

COMMENT

In the present study, we have demonstrated that significantly higher levels of proMMP-7 are detected in the BAL fluids of IPF patients. Immunohistochemistry further indicated that proMMP-7 and CD151 are localized to the hyperplastic alveolar and metaplastic bronchiolar epithelial cells. Furthermore, active form of MMP-7 was detected by immunohistochemical and immunoblotting analyses, and the activity of MMP-7 was confirmed in the CCm-Tf film assay. Based on these data, we propose that proMMP-7 is excessively produced and efficiently activated in the lung tissues of IPF probably through interaction with CD151.

Our enzyme immunoassay system has indicated significant elevation of proMMP-7 in BAL fluids from IPF patients compared with normal subjects. Because proMMP-7 was immunolocalized mainly in the hyperplastic alveolar and metaplastic bronchiolar epithelial cells and macrophages, these cells are considered to be responsible for the production. During preparation of our manuscript, other groups reported that MMP-7 levels in BAL fluids from patients with IPF or other interstitial lung diseases including nonspecific interstitial pneumonia, cryptogenic organizing pneumonia, and sarcoidosis are higher than those from normal subjects and that the levels are not different between IPF and other interstitial lung

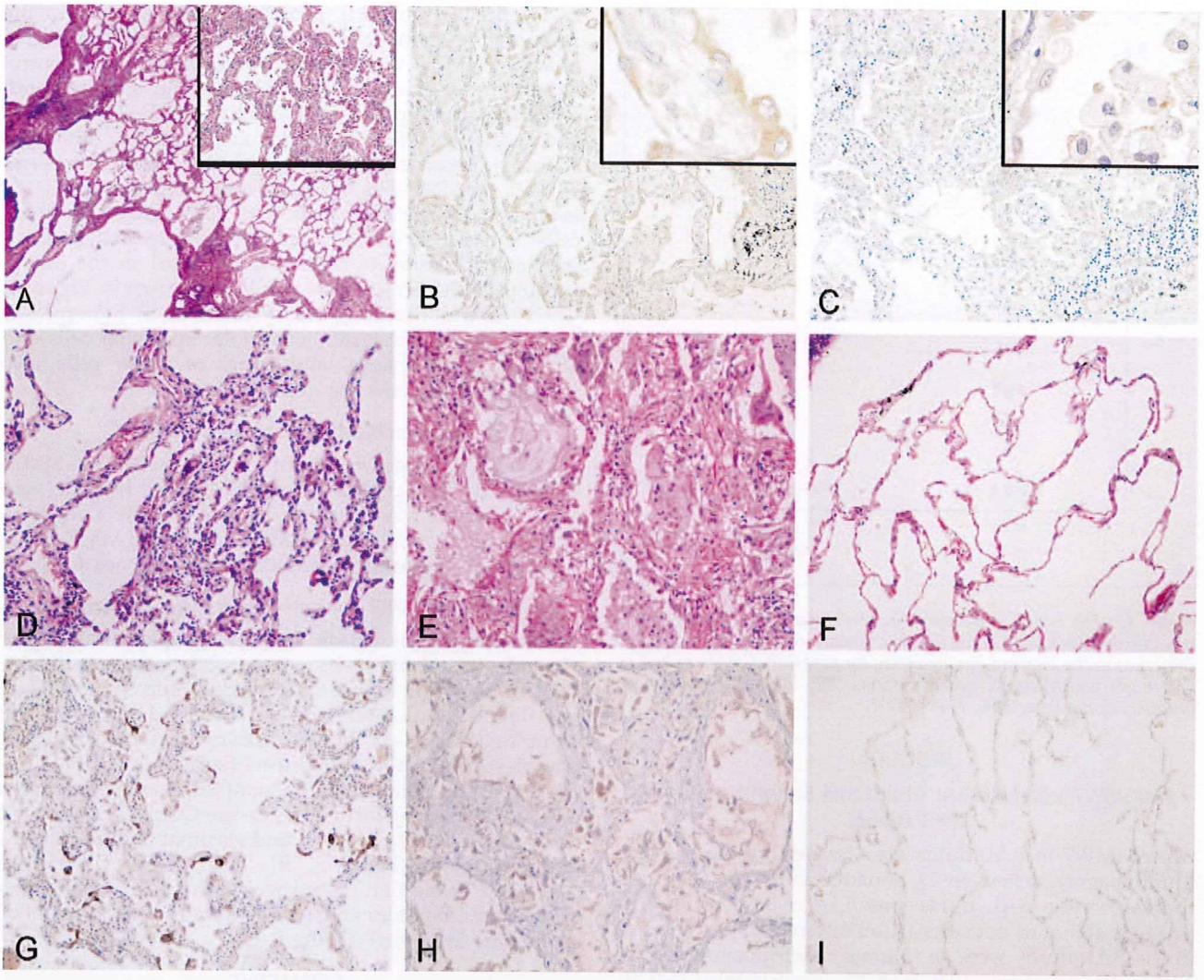

Figure 2. *Histology and immunohistochemistry for pro matrix metalloproteinase 7 (proMMP-7) and active MMP-7 in the lung tissues. A, Lung tissue of idiopathic pulmonary fibrosis (IPF) showing septal inflammation and fibrosis (hematoxylin-eosin, original magnifications ×2 [A] and ×20 [inset]). B, Immunohistochemistry for proMMP-7 in the lung tissue of interstitial pneumonia. Note that proMMP-7 is predominantly immunostained in the hyperplastic alveolar epithelial cells and alveolar macrophages. (original magnifications ×20 [B] and ×40 [inset]). C, Immunohistochemistry for MMP-7 in the lung tissue of IPF using antibody specific to active MMP-7 (original magnifications ×20 [C] and ×40 [inset]). Note that active MMP-7 is localized on alveolar macrophages and alveolar epithelial cells. D and G, Histology and immunohistochemistry, respectively, for proMMP-7 in the lung tissue of nonspecific interstitial pneumonia (original magnifications ×20). E and H, Histology and immunohistochemistry, respectively, for proMMP-7 in the lung tissue of interstitial pneumonia associated with rheumatoid arthritis (original magnifications ×20). Note that proMMP-7 is positive in the regenerative epithelial cells in nonspecific interstitial pneumonia and interstitial pneumonia associated with rheumatoid arthritis. F and I, Histology and immunohistochemistry, respectively, for proMMP-7 in the control normal lung tissue (original magnifications ×20). No positive immunostaining is observed in the sample.*

diseases.[25,26] Although we did not find differences in the serum levels of proMMP-7 between IPF and control groups, a recent article showed significantly higher levels of plasma MMP-7 in IPF[27] in an assay that detects only proMMP-7. The discrepancy may be derived from the different characteristics of the enzyme immunoassay kits between R&D Systems, Inc (Minneapolis, Minnesota) and ours.[27] Previous studies have shown that MMP-7 is preferentially secreted to the apical surface of normal mucosal and gland epithelium[14,28] and localized mainly to the luminal epithelial surface of the uterine and mammary glands.[29] Therefore, our data showing no changes in serum proMMP-7 levels may be because proMMP-7 produced by the alveolar epithelial cells is secreted mostly to the alveolar spaces but not into blood circulation. This may also be explained by the fact that the concentrations of proMMP-7 are too low to be detected by our assay system because of dilution after being drained into blood circulation. However, another plausible explanation might be that proMMP-7 is locally activated and consumed by acting as a proteinase in the extracellular milieu without drainage into blood circulation.

One of the most interesting findings in the present study is that proMMP-7 is activated within the lung tissue of IPF. This was demonstrated by immunohistochemistry using anti–MMP-7 antibody specific to active MMP-7, immunoblotting, and CCm-TF film assay. Our immunohistochemical study also showed that no definite active MMP-7 is produced in the lungs from interstitial pneumonia associated with rheumatoid arthritis and nonspecific

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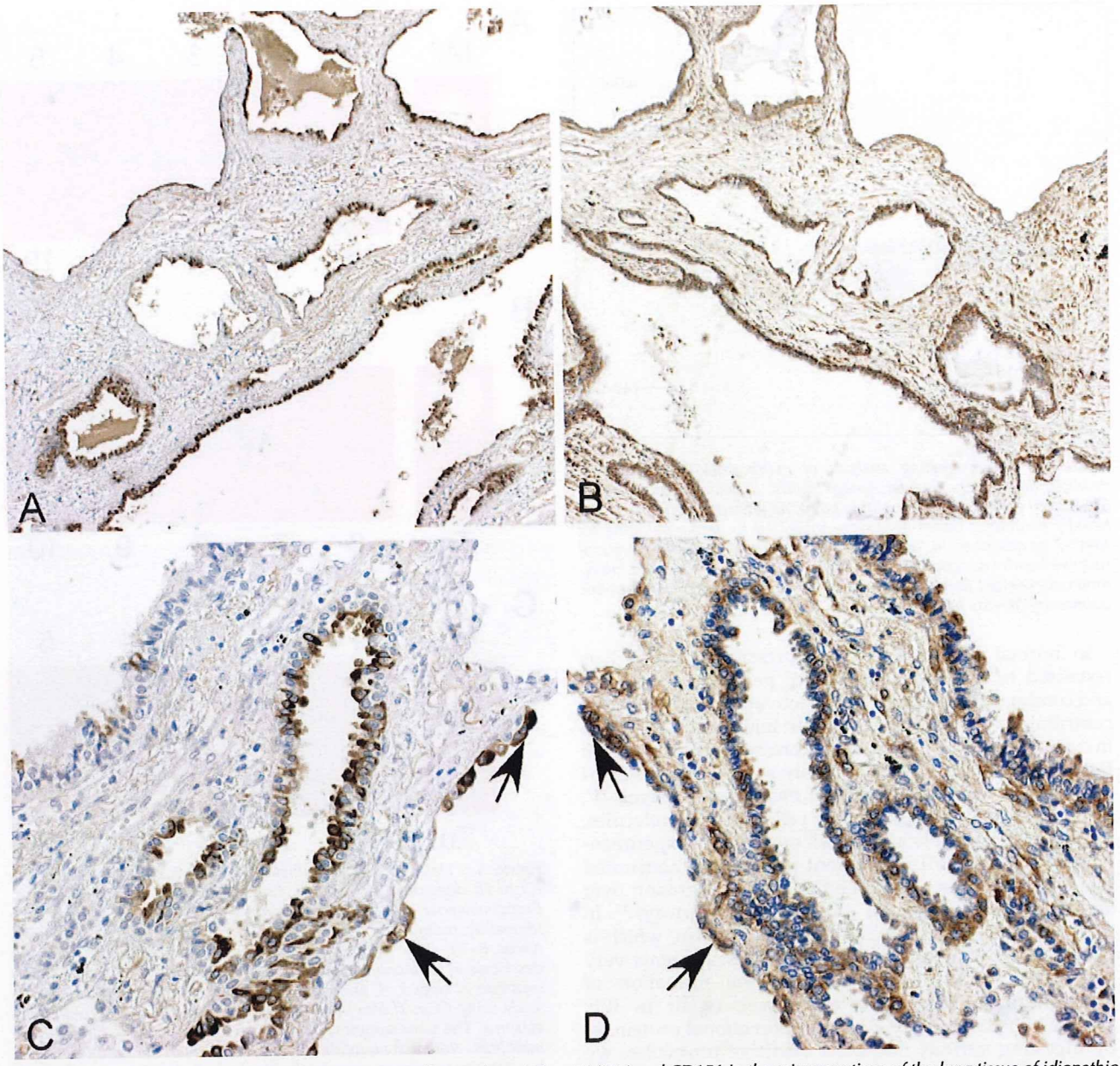

Figure 3. *Immunohistochemistry for pro matrix metalloproteinase 7 (proMMP-7) and CD151 in the mirror sections of the lung tissue of idiopathic pulmonary fibrosis. A and C, Low- and high-power views of proMMP-7 immunostaining showing that alveolar epithelial cells are positively stained (original magnifications ×20 in all). B and D, Low-power and high-power views of CD151 immunostaining. Note that proMMP-7 and CD151 are colocalized in some epithelial cells (arrows) (original magnifications ×20 in all).*

interstitial pneumonia, although proMMP-7 is expressed. Active MMP-7 has been detected in several pathophysiologic tissues including physiologic duodenal mucosa,[10] endometrial adenocarcinomas,[30] and lung adenocarcinomas,[5] but to our knowledge our observation is the first to demonstrate the proMMP-7 activation within the lung tissues of IPF. Although in vitro biochemical studies have shown that proMMP-7 is fully activated by trypsin or MMP-3, and partially by plasmin or leukocyte elastase,[31] the activation mechanism of proMMP-7 in tissues remains unknown. We have recently reported that CD151 acts as a docking molecule for proMMP-7 through binding to the propeptide of proMMP-7 on cell surfaces, leading to its activation.[5] The findings in the current study that both proMMP-7 and CD151 are localized in the hyperplastic alveolar epithelial cells and macrophages and that active MMP-7 is stained on these cells suggest activation of proMMP-7 through the interaction with CD151. We have recently shown similar CD151-associated activation of proMMP-7 in the human lung adenocarcinomas[5] and in cultured osteoarthritic chondrocytes.[24] Thus, we speculate that the activation of MMP-7 may be characteristic to IPF and contribute to the pathology of IPF. Although the expression of other MMPs (MMP-1, 2, 9, and 13 and MT1, 2, 3, 4, and 5-MMP) and TIMPs (TIMPs-1, 2, 3, 4) has been previously reported in IPF and other interstitial lung diseases, their local and specific activation in IPF has not been detected or clarified.[32–35]

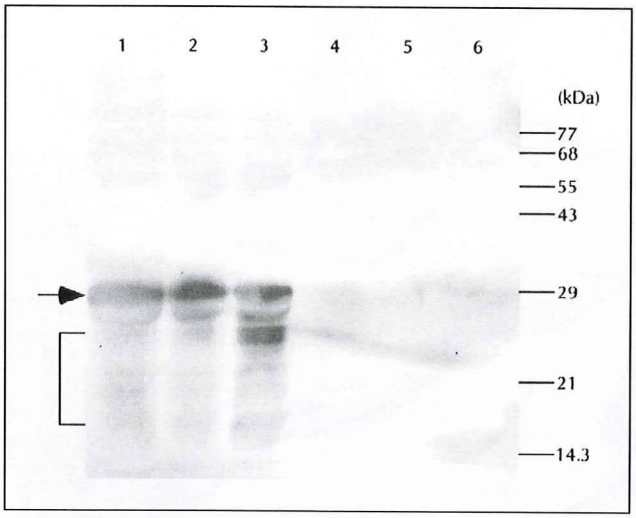

Figure 4. *Immunoblotting analysis of matrix metalloproteinase 7 (MMP-7) in bronchoalveolar lavage fluids. Bronchoalveolar lavage fluids from patients with idiopathic pulmonary fibrosis (lanes 1–3) and healthy volunteers (lanes 4–6) were subjected to immunoblotting for MMP-7 as described in "Materials and Methods." Note the immunoreactive bands corresponding to 28-kDa proMMP-7 (arrow) and lower-molecular-weight forms of active MMP-7 in the samples of idiopathic pulmonary fibrosis patients.*

In normal lung tissue, the expression of MMP-7 is restricted to the epithelial cells of peribronchial glands and conducting airways, and the activation is suspected to contribute to exocrine function.[36] In injured lungs such as in cystic fibrosis, MMP-7 is overexpressed by alveolar type II cells.[14] MMP-7 has strong activity against various ECM components including basement membrane collagen IV, elastin, proteoglycans, and other cell adhesion molecules, all of which are implicated in cell motility.[31,36] Experimental study using MMP-7–deficient mice has demonstrated that MMP-7 is essential to epithelial cell migration over and reepithelialization of the damaged airways.[14] In addition, MMP-7 is known to shed E-cadherin, which is necessary for the epithelial repair.[8] Thus, it seems very likely that MMP-7 contributes to the formation of hyperplastic epithelial foci and tissue repair in IPF. However, because MMP-7 is a multifunctional proteinase by digesting various non-ECM bioactive molecules, we can speculate other functions in addition to repair in the lung tissues of IPF: (1) MMP-7 is known to cleave Fas-L, which could trigger epithelial cell apoptosis.[11,37] Previous studies have demonstrated soluble Fas-L in the BAL fluids and apoptosis of the epithelial cells increases in IPF. Thus, MMP-7 may be involved in the epithelial cell apoptosis through cleavage of the membrane-bound Fas-L.[38,39] (2) MMP-7 could facilitate bioavailability of insulin-like growth factor binding protein 3 and insulin-like growth factor binding protein 5, which are autocrine growth factors for myofibroblasts in the colon.[6,7] Because myofibroblasts are thought to actively produce ECM molecules in IPF, it is reasonable to speculate that MMP-7 may play a role in the growth of myofibroblasts in the lungs. (3) Osteopontin is expressed in IPF[40] and particle-induced lung diseases[41] and upregulated in the bleomycin-induced lung fibrosis model.[42] Because MMP-7 is known to process osteopontin into an active form,[43] MMP-7 might act as a positive modulator for fibrosis signaling.[44] (4) We have

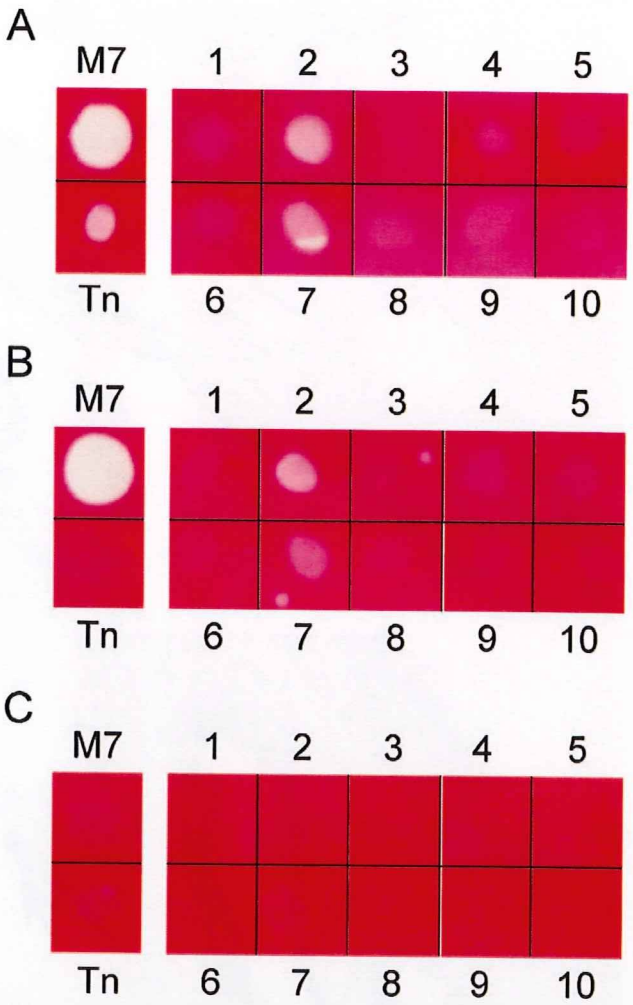

Figure 5. *Detection of cross-linked carboxymethylated transferrin (CCm-Tf)–degrading activity in bronchoalveolar lavage fluids. A, Bronchoalveolar lavage fluid specimens (3 μL) from 7 patients with idiopathic pulmonary fibrosis (lanes 1–7) and 3 healthy volunteers (lanes 8–10) were spotted and incubated on the CCm-Tf film as described in "Materials and Methods." Note the digestion of CCm-Tf substrate in lanes 2, 4, and 7 as well as positive controls. B, Inhibition study using CCm-Tf film impregnated with a mixture of aprotinin and elastinal. The same samples as in A were subjected to the film with the inhibitors. Note that no inhibition of the activity in the samples is seen, but trypsin activity is completely blocked. C, Inhibition study using CCm-Tf film impregnated with a mixture of 1,10-phenathroline, aprotinin. and elastinal. Note that activity of all the samples including positive controls is inhibited. Abbreviations: M7, positive control of matrix metalloproteinase 7; Tn, positive control of trypsin.*

previously shown that alveolar macrophages are primed for interleukin 8 production in IPF patients during its chronic phase, and high levels of interleukin 8 are detected during the phase of acute exacerbation.[21] Because MMP-7 is reported to promote neutrophil transmigration to alveolar spaces through shedding of chemokine-binding syndecan 1,[9] it might be possible to speculate that excessive MMP-7 production and activation may contribute to the induction of neutrophilic infiltration into alveolar walls in IPF, especially during the phase of acute exacerbation.[9]

In summary, our study showed that proMMP-7 is overproduced by hyperplastic epithelial cells and activated in the lung tissue of IPF. The data suggest that excessive

production and macrophages and activation of proMMP-7 may contribute to the pathology of IPF through a variety of pathways such as epithelial cell migration and apoptosis, myofibroblast autocrine growth, and neutrophil accumulation. Further studies are mandatory for establishing these pathologic roles.

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〔日本肺サーファクタント・界面医学会雑誌　第40巻　137～139（平成21年）〕

《シンポジウム2》

EBMに基づくALI/ARDS診療のUp-To-Date

慶應義塾大学医学部救急医学
藤島清太郎

急性呼吸促迫症候群(ARDS, acute respiratory distress syndrome)は病態生理学的に肺微小血管の透過性亢進，過剰な炎症反応を特徴とする症候群であるが，臨床的に$PaO_2/FIO_2 < 200$と画像上の両側浸潤影というAmerican-European Consensus Conferenceの簡便な診断基準を満たす症例には，発症機序の異なる多彩な病態が包含されている．一方，$PaO_2/FIO_2 < 300$で定義される急性肺損傷(ALI, acute lung injury)は，より軽症，初期病態を含めたさらに広い疾患概念といえる．

ALI/ARDSの病態は現在でも未解明な部分が多いが，我々はこれまで，重症傷病に罹患した患者が，経過中感染などを契機にALI/ARDSを発症し，病態が急激に重症化する現象(two hit phenomenon)をしばしば経験し，その病態解明を行ってきた(図1)．マウスの熱傷後敗血症モデルを用いた検討では，熱傷後に細菌内毒素を投与した群で，炎症性サイトカインの産生が増大かつ遷延することを認めている[1]．最近では，少量IL-18投与により，生存率が改善し，炎症性サイトカインの産生が抑制されることを見いだした(図2)[2]．

一方，臨床面では近年各領域でEBMが急速に導入されているが，救急・集中治療領域ではRCT(randomized controlled trial)の実施が困難なこともあり，エビデンスの集積が未だ不十分である．ALIやARDSの診療に関する記載のあるガイドラインとして，我が国の"日本呼吸器学会ALI/ARDS診療のためのガイドライン2005年版"，国際的には"Surviving Sepsis Champaign Guidelines 2008年改訂版"があるが，いずれも必ずしも十分なエビデンスに基づかない事項を列記せざるを得ないのが現状である[3,4]．

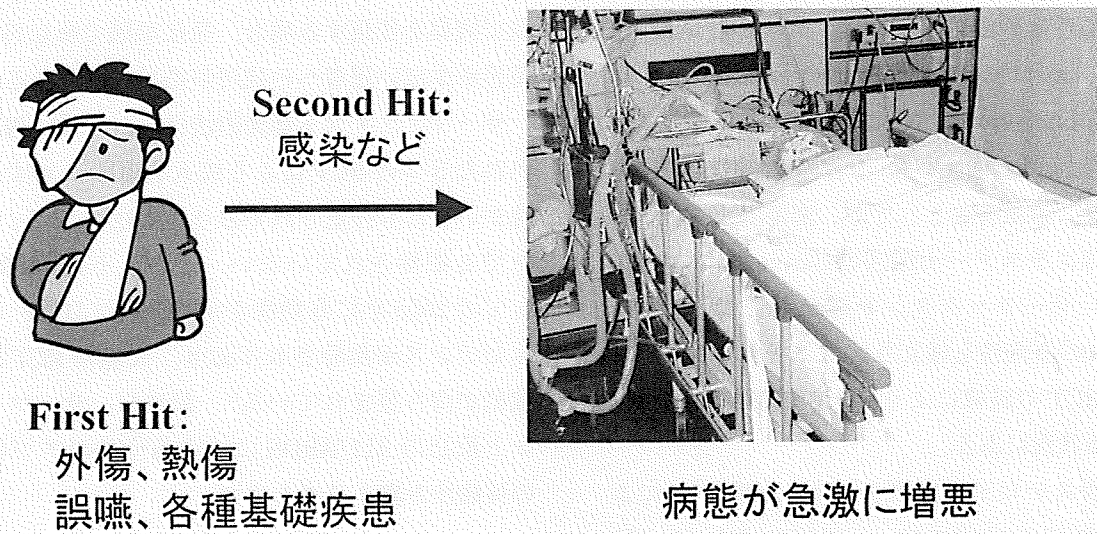

図1　Two Hit Phenomenon

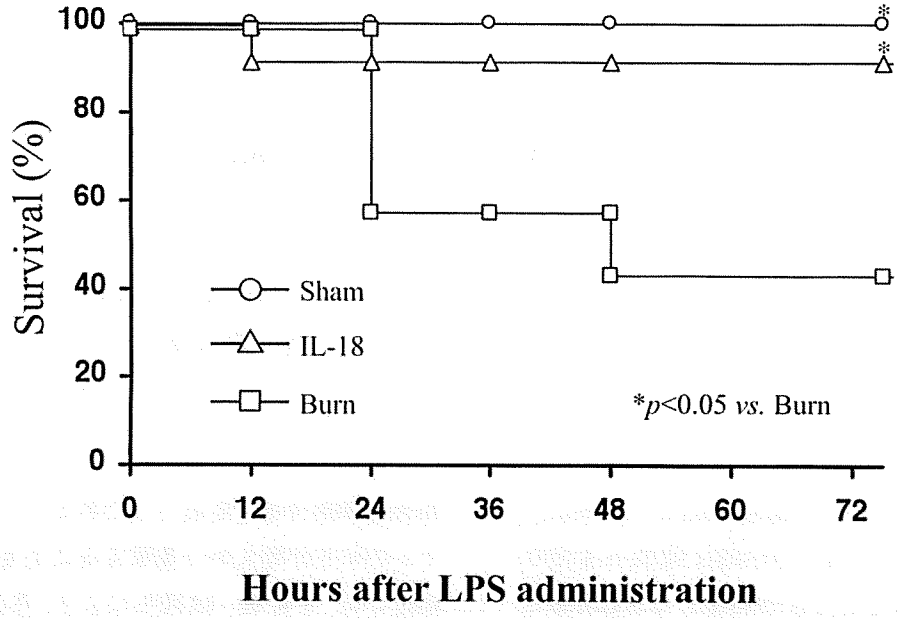

図2 Cumulative survival after LPS administration in the sham, burn and IL-18 groups
（文献2より）

表1 Surfactant療法：今後のアプローチ

1. 新たなsurfactant開発：
   リコンビナントSP-A, SP-D（親水性、生体防御機能）生体防御作用の付加
2. 対象の選定：
   直接傷害によるALI/ARDS小児・若年者
3. 投与法の改良：
   気管支鏡下instillation 一時的気管挿管
4. 投与時期：
   早期投与（second hit前）

(Lewis JF, et al. Sem Respir Crit Care Med 2006; 27(4): 377-88)
(Stevens TP, et al. Chest 2007; 131(5): 1577-82)

様々な治療法のうち，これまでにALI/ARDSの長期生存率改善が示されている治療法は，低容量換気による呼吸管理のみであり，高圧の呼気終末陽圧（PEEP, positive end-expiratory pressure）負荷，recruitment maneuver（バッグマスク換気などによる一時的な高気道内圧負荷）などの有効性は明確でない．一方，薬物療法としては，副腎皮質ステロイド，好中球エラスターゼ阻害薬（sivelestat，エラスポール®），サーファクタント補充療法などがあるが，いずれの有効性も確立されておらず，新たに報告されるRCTの成績により，その位置づけが刻々と変化する状況にある．

副腎皮質ステロイドに関しては，ARDSNetによる大規模RCTの成績では，methylprednisolone早期投与により人工呼吸器からの離脱が早まるものの，神経筋合併症が増し，さらに発症14日以降の投与では死亡率が増加したことが報告されている[5]．一方我が国における臨床治験で肺機能の改善と，人工呼吸器装着期間，ICU在室期間の短縮を認めたsivelestatは，その後の海外臨床試験では有効性が示されず，現在我が国と韓国でのみ市販されている．海外試験の対象患者の多くが重症例であり，我が国と同程度の重症度例に絞った再解析で改善傾向を認めたこと，我が国の臨床治験の層別解析で，発症後3日以内投与群での有効率が高いこと，市販後調査で臨床試験と同様の有効性が示されたことから，現状で同薬を使用する場合，発症早期で肺以外の臓器障害が少ない患者への使用が推奨されている．

サーファクタント補充療法に関しては，過去に行われた3つのRCTにより成人ARDSに対する有効性がいずれも否定されており，上述の2ガイドラインでも推奨されていない．一方，小児ARDSに対しては2005年に有効性が示唆されて以降，様々なエビデンスが集積されており，大規模RCTによる成績が待ち望まれている[6]．最後に，サーファクタント療法のALI/ARDSに対する今後のアプローチの可能性を**表1**にまとめた．

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SHORT REPORT

Distribution of *emm* genotypes among group A streptococcus isolates from patients with severe invasive streptococcal infections in Japan, 2001–2005

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SUMMARY

We surveyed *emm* genotypes of group A streptococcus (GAS) isolates from patients with severe invasive streptococcal infections during 2001–2005 and compared their prevalence with that of the preceding 5 years. Genotype *emm1* remained dominant throughout 2001 to 2005, but the frequency rate of this type decreased compared with the earlier period. Various other *emm* types have appeared in recent years indicating alterations in the prevalent strains causing severe invasive streptococcal infections. The cover of the new 26-valent GAS vaccine fell from 93·5% for genotypes of isolates from 1996–2000 to 81·8% in 2001–2005.

Group A streptococcus (GAS) is one of the most common human pathogens and causes a wide array of infections, the most frequent of which is acute pharyngitis (strep throat). Many streptococcal virulence factors have been implicated in the pathogenesis of streptococcal infection including pyrogenic exotoxins (SpeA, SpeB and SpeC) and M protein. M protein protects GAS from phagocytosis by polymorphonuclear leukocytes [1, 2]. More than 90 of M serotypes have been identified, and a molecular approach to identification of *emm* (M protein) genes has also been documented [3]. From the late 1980s, severe invasive infections such as streptococcal toxic shock-like syndrome (TSLS) and necrotizing fasciitis (NF) caused by GAS became a serious problem in both developed and developing countries. The first defined case of TSLS in Japan was reported in 1992 [4]. In almost all of the countries which have surveyed the prevalence of M serotypes, strains of *emm3* and *emm1* genotypes are dominant in causing severe invasive infections. In Japan, where periodic sampling surveys are performed on a 5-year cycle, *emm1* remained the most frequent from1992 to 2000, but *emm3* strains which predominated from 1992 to 1995 decreased during 1996–2000 [5].

In this study, we report the frequency of genotypes of isolates from patients with severe invasive

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† Other group members are listed in the Appendix.

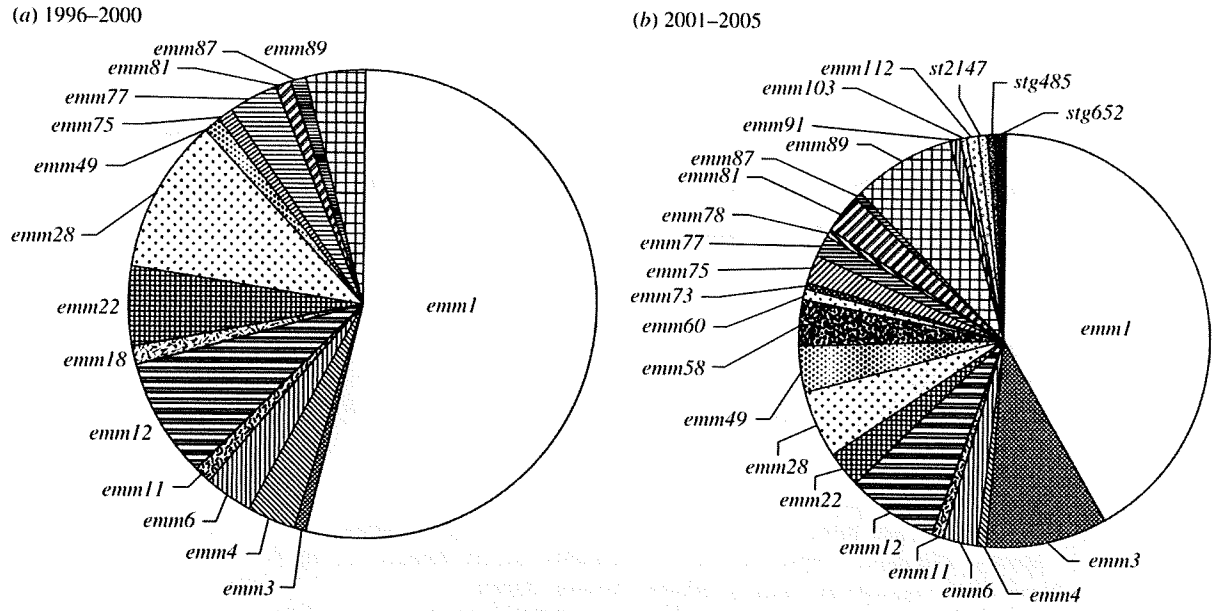

Fig. The rate of *emm* genotypes of strains isolated from patients of severe invasive streptococcal infections. (*a*) 1996–2000, (*b*) 2001–2005. (Number of each *emm* genotyped isolate during each period/number of severe invasive infections isolates during each period.)

streptococcal infections, TSLS and/or NF, from 2001 to 2005 in Japan and compare these data with the preceding sampling period from 1996 to 2000.

Information on patients with severe invasive streptococcal infections and *emm* genotypes of the isolates during the period of 2001–2005 was submitted to the National Institute of Infectious Diseases from the branch offices of reference centre and cooperative hospitals [5]. The diagnostic criteria of TSLS were based principally on those described by the Working Group on Severe Streptococcal Infections (1993) [6].

A total of 137 GAS isolates were cultured predominantly from sterile sites of patients with severe invasive infections during the period of 2001–2005. The ratio of each genotype compared between the two sampling periods is given in the Figure. Isolates of *emm1* remained dominant over the two periods, accounting for 50 out of 93 (53·8%) cases in 1996–2000 and 57 out of 137 (41·6%) cases in 2001–2005; there was a slight reduction in the ratio of *emm1* to other genotypes. On the other hand, *emm3* isolates increased dramatically from 1·08% (1/93) to 9·49% (13/137) ($P<0·01$). It is noteworthy that this genotype was already prevalent (33%) among TSLS isolates more than 10 years previously [5] and despite the marked decrease in the 1996–2000 sampling period, its prevalence is clearly increasing in more recent years. Fewer than seven *emm* genotypes were isolated each year in 1996–2000, while 8–15 *emm* genotypes were recorded in the subsequent years. A total of 15 *emm* genotypes were isolated during 1996–2000 compared with 23 *emm* genotypes in the later period; 10 genotypes (*emm58*, *emm60*, *emm73*, *emm78*, *emm91*, *emm103*, *emm112*, *st2147*, *stg485* and *stg652*) were isolated from patients in the later period but were absent in the first sampling period (Fig.). These results indicate the change and diversity of genotypes over time in Japan.

On the basis of epidemiological data demonstrating that the majority of non-invasive and invasive streptococcal infections are caused by a limited number of M-types, a multivalent vaccine containing amino-terminal fragments of M proteins from 26 different serotypes of GAS has recently been developed in the United States [7]. Based on the seroprevalence data presented here for Japanese GAS from invasive disease the vaccine would provide cover for 87 out of 93 strains (93·5%) isolated in 1996–2000 and for 112 out of 137 (81·8%) in 2001–2005. These data therefore have important implications for the formulation and composition of candidate vaccines against streptococcal infections in Japan.

APPENDIX. Working Group for Beta-haemolytic Streptococci in Japan

The following are Group members: T. Kawai (Sapporo City Institute of Public Health, Sapporo),

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DECLARATION OF INTEREST

None.

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五類感染症(全数把握)

劇症型溶血性レンサ球菌感染症

Streptococcal toxic shock syndrome

池辺忠義　　渡邉治雄

Key words：劇症型溶血性レンサ球菌感染症，*Streptococcus pyogenes*

はじめに

劇症型溶血性レンサ球菌感染症(streptococcal toxic shock syndrome)は1987年に米国で最初に報告され，その後，先進国ばかりでなく発展途上国からも報告されている．日本における最初の典型的な症例は1992年に報告されており，現在までに200人を超える患者が確認されている．そして，このうち約45%が死亡しているという極めて致死率の高い感染症である．A群レンサ球菌感染による一般的な疾病は咽頭炎であり，その多くは小児が罹患する．一方，劇症型溶血性レンサ球菌感染症は大人から子供まで広範囲の年齢層に発症するが，特に30歳以上の大人に多いのが一つの特徴である．1999年4月に施行された「感染症の予防及び感染症の患者に対する医療に関する法律(感染症法)」による集計によると，2000年には45例，2001年には43例，2002年には90例，2003年には52例，2004年には53例，2005年には60例が報告されている．2006年，劇症型溶血性レンサ球菌感染症の届出基準に変更があり，主な変更点は，今まで起因菌をA群レンサ球菌と限定していたが，今回の変更でβ溶血を示すレンサ球菌にまで広げられた．

1. 病原体と感染経路

劇症型溶血性レンサ球菌感染症は，β溶血性のレンサ球菌により引き起こされる．β溶血性レンサ球菌の中でも，特にA群溶レン菌である*Streptococcus pyogenes*による症例が最も多い．

*S. pyogenes*は，球状の菌体が種々の長さの連鎖を形成するグラム陽性の通性嫌気性菌である．鞭毛は有しておらず，芽胞も形成しない．カタラーゼ陰性である．多くの株が莢膜を形成し，β溶血を起こす．ヒツジまたはウマの脱繊維血液を5%の割合に添加した血液寒天平板培地上で*S. pyogenes*を24時間培養すると，直径0.5mm以上のコロニーを形成し，発育集落の周囲に完全に透明な溶血環が認められるβ溶血，または，β溶血に比べると，溶血環は小さく，透明度や輪郭の鮮明さが劣るα′溶血を示す．集落形態は，多様であるが，最も多く観察されるのは'glossy'型で，直径0.5–1.0mmくらいの小型，灰白色，やや不透明，湿潤，正円形を示す．そのほかには'mucoid'型という露滴状または粘液性様の辺縁のなめらかな集落がある．また，A群レンサ球菌の集落はおおむね堅く，白金線で釣菌すると1個分すべてが平板から剥がれたり，集落が割れたりする場合がある．

この*S. pyogenes*には，数多くの表層抗原因子

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が知られている．このうちM蛋白は，100以上の型が知られていることから[1]，菌の疫学マーカーとしてよく用いられ，機能的には，宿主細胞への付着や抗貪食作用をもつ病原因子の一つである．また同時に，感染防御抗原としても重要な機能を果たしている．このM蛋白は，*emm*遺伝子によりコードされており，この塩基配列を決定することにより，型別が可能となっている．劇症型溶血性レンサ球菌感染症患者から分離される*S. pyogenes*の*emm*型は，1992-95年までは，*emm3*型と*emm1*型が主であったが，1995年以降，*emm3*型は減少し，*emm1*型が主流となっている[2,3]．

Stevensら[4]の報告によると，劇症型溶血性レンサ球菌感染症の患者の約35％は皮膚(minor trauma, surgical procedures, intravenous drug abuse)，約20％は粘膜(pharynx, vagina)からの感染であり，残りの約45％は，*S. pyogenes*の正確な侵入部位が不明である．

2. 病　　態

劇症型溶血性レンサ球菌感染症は，罹患者が重篤な基礎疾患をもっていないにもかかわらず，突然発病する場合がある．初期症状としては，四肢の疼痛，腫脹，発熱，血圧低下などで，発病から病態の進行が非常に急激かつ劇的で，いったん発病すると数十時間以内には急性腎不全，成人型呼吸窮迫症候群(ARDS)，播種性血管内凝固症候群(DIC)，多臓器不全(MOF)，軟部組織壊死を引き起こし，患者をショック症状から死に至らしめる．妊産婦における症例も報告されている[5]．

Stevensら[4,6]の報告によると，劇症型溶血性レンサ球菌感染症の最も一般的な初期症状は四肢の疼痛であり，急激に始まり，重篤である．続いて，その部位の圧痛を示す．疼痛は，通常，四肢でみられる．疼痛の開始前に，20％の患者は，発熱，悪寒，筋肉痛，下痢のようなインフルエンザ様の症状を示す．臨床所見として，発熱が最も一般的な徴候である(ただし，患者の10％はショックによる低体温を示す)．錯乱状態(confusion)は患者の55％でみられ，患者によっては，昏睡や好戦的な姿勢を示すことがある．局所的な腫脹，圧痛，疼痛，紅斑のような軟部組織感染の徴候は，皮膚の傷口が存在する場合によくみられる．発熱をもつ患者において紫色の水疱が圧痛のある部位にみられると，筋炎や壊死性筋膜炎のような深部の軟部組織感染を起こしている可能性が考えられる[7]．

2006年7月31日までに衛生微生物技術協議会溶血性レンサ球菌レファレンスシステムセンター(**表1**)に集められた劇症型溶血性レンサ球菌感染症の確定診断例205例(A群177例，B群5例，C群1例，G群22例)中，腎障害は72.7％(149症例)，DICは72.7％(149症例)，肝障害は56.1％(115症例)，ARDSは28.8％(59症例)，紅斑様皮膚発赤疹は13.7％(28症例)，軟部組織壊死(壊死性筋膜炎および筋炎を含む)は74.1％(152症例)，中枢神経症状は39.0％(80症例)の頻度でみられた(**表2**)．

3. 診　　断

特に，四肢や体幹の激しい疼痛，発熱およびショック症状を伴う患者を診た場合には，劇症型溶血性レンサ球菌感染症を鑑別診断の一つに考える必要がある．

劇症型溶血性レンサ球菌感染症の場合には，通常無菌的である部位(血液，脳脊髄液，胸水，腹水，生検組織，手術創など)からβ溶血性レンサ球菌が検出される．特に，顕著な菌血症を示すので，血液のグラム染色標本を検鏡するとレンサ球菌を直接観察できる．分離培地には血液寒天培地を用い，A群レンサ球菌はこの培地上でβ溶血またはα′溶血を示す直径0.5mm以上のコロニーを形成する．C，G群レンサ球菌は，血液寒天培地上で24時間培養後，鮮明なβ溶血を示す．血清群別凝集試験，糖分解試験などの生化学的性状試験や同定用検査キットにより溶血レンサ球菌の血清群および菌種を同定できる．

劇症型溶血性レンサ球菌感染症は，病態の進行が急激であるため，早期の診断が重要である．先ほど述べたように，β溶血性レンサ球菌が無菌部位から検出されることが確定診断の一つの

表1 衛生微生物技術協議会溶血性レンサ球菌レファレンスシステムセンター窓口

A群レンサ球菌のT，M型別試験，および劇症型A群レンサ球菌感染症に関する情報についての窓口は以下の機関になっておりますので，お問い合わせをお願いいたします．

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表2 A群，B群およびG群劇症型溶血性レンサ球菌感染症

	A群	B群	G群
症例数	177	5	22
症　状			
腎障害	71.8%	80.0%	77.3%
DIC	71.2%	100%	77.3%
肝障害	65.0%	80.0%	63.6%
ARDS	29.4%	40.0%	18.2%
紅斑様皮膚発赤疹	14.7%	0%	9.1%
軟部組織壊死	75.1%	60.0%	68.2%
中枢神経症状	36.7%	60.0%	50.0%

項目となっている．血液培養の結果を待っていると，手遅れになる場合がある．近年，亜硝酸により菌の多糖体を抽出し，A群レンサ球菌を迅速に検出するキットが市販されている．患者の血清や膿を検体として用いたとき，市販のA群レンサ球菌迅速診断キットで陽性反応を示すことから，この迅速診断キットがA群溶血性レンサ球菌による劇症型溶血性レンサ球菌感染症の診断に有用であることが報告されている[8]．また，末梢血塗抹標本または壊死軟部組織の検鏡によるレンサ球菌の確認も有用である．

4. 治療・予防

抗菌薬としてはペニシリン系薬が第一選択薬である．また，組織内の菌密度が上昇すると菌の発育が抑制され，β-ラクタム系薬の効果が低下する現象が知られており，劇症型溶血性レンサ球菌感染症のように極端な敗血症病態を示す場合では細胞内移行性の高いクリンダマイシン(clindamycin)を推奨する意見もある[9]．診断が確定したらアンピシリン12g/日を6時間ご

ととクリンダマイシン1,200mg/日，分4の投与を速やかに開始することが推奨されている[8]．更に免疫グロブリン製剤の効果も報告されている[10]．

血圧維持には大量の輸液が必要であるが，輸液の許容範囲が狭いため，肺動脈圧の経時的観察が必要である．壊死に陥った軟部組織は菌の生息部位であること，および，筋壊死により腎不全および代謝性アシドーシスが増悪されるので，可及的に壊死部位を含む広範囲な病巣を切除することが必要である．

A群レンサ球菌による感染の拡散は，特に，咳やくしゃみをした後，および食事を準備する前や食べる前に，手をよく洗うことにより減らすことができる．傷口はすべて消毒して清潔に処置しておくべきであり，傷部位に赤色，腫脹，膿瘍および疼痛のような，感染の徴候がある場合には適切な医療処置を行う必要がある．

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Incompetence of Neutrophils to Invasive Group A *streptococcus* Is Attributed to Induction of Plural Virulence Factors by Dysfunction of a Regulator

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Abstract

Group A *streptococcus* (GAS) causes variety of diseases ranging from common pharyngitis to life-threatening severe invasive diseases, including necrotizing fasciitis and streptococcal toxic shock-like syndrome. The characteristic of invasive GAS infections has been thought to attribute to genetic changes in bacteria, however, no clear evidence has shown due to lack of an intriguingly study using serotype-matched isolates from clinical severe invasive GAS infections. In addition, rare outbreaks of invasive infections and their distinctive pathology in which infectious foci without neutrophil infiltration hypothesized us invasive GAS could evade host defense, especially neutrophil functions. Herein we report that a panel of serotype-matched GAS, which were clinically isolated from severe invasive but not from non-invaive infections, could abrogate functions of human polymorphnuclear neutrophils (PMN) in at least two independent ways; due to inducing necrosis to PMN by enhanced production of a pore-forming toxin streptolysin O (SLO) and due to impairment of PMN migration via digesting interleukin-8, a PMN attracting chemokine, by increased production of a serine protease ScpC. Expression of genes was upregulated by a loss of repressive function with the mutation of *csrS* gene in the all *emm49* severe invasive GAS isolates. The *csrS* mutants from clinical severe invasive GAS isolates exhibited high mortality and disseminated infection with paucity of neutrophils, a characteristic pathology seen in human invasive GAS infection, in a mouse model. However, GAS which lack either SLO or ScpC exhibit much less mortality than the *csrS*-mutated parent invasive GAS isolate to the infected mice. These results suggest that the abilities of GAS to abrogate PMN functions can determine the onset and severity of invasive GAS infection.

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Introduction

Streptococcus pyogenes (group A *streptococcus*; GAS) is one of the most common human pathogens. It causes a wide variety of infections ranging from uncomplicated pharyngitis and skin infections to severe and even life-threatening manifestations, such as necrotizing fasciitis (NF) and streptococcal toxic shock-like syndrome (STSS) [1,2], with high mortality rates ranging from 20% to 60% [3]. Several streptococcal virulence factors, including streptolysin, and M protein, have been reported to be involved in these diseases, by genetic studies or animal-passaged models [1,2,4–6]. However, which of factors are involved in pathogenesis mediated by clinically isolated severe invasive GAS remains obscure.

The strains of *emm1* genotype, among more than 100 *emm* genes encoding the serotype-determinant M protein, are the predominant cause of severe GAS infections in Japan [7]. Recently, GAS with diverse *emm* genotypes, especially, *emm49*-genotype, have been isolated from patients of severe invasive GAS infections since 2000; however, these genotypes were not isolated before 1999 in Japan [8]. Therefore, *emm49* GAS isolated from invasive infections seems to acquire the novel or altered virulence factors by mutations, genomic additions, or deletions.

Epidemiological and pathological findings, including sporadic incidents of severe invasive GAS infections [9], high frequency of severe invasive infections in immunocompromised host [9], and aggregation of bacteria and a paucity of polymorphnuclear neutrophils (PMN) in foci of invasive GAS infection [10] suggest that host defense factors play an important role in the onset of invasive infections. These findings led us to postulate that invasive GAS infections hampered host innate immune defense, especially on PMN, providing the front-line defense against GAS infection by quick recruitment to infection site and clearance of bacteria following phagocytosis [11,12]. So far, using animal-passaged GAS mutants, gene-manipulated GAS, many virulence-associated molecules are pointed out to play some roles in the bacterial evasion from phagocytic ingestion by neutrophils [13]. However, restricted availability of clinical isolates with the same serotypes fail to elucidate direct relationship between definitive genetic changes in clinically isolated severe invasive GAS and the lack of PMN at the site of bacterial growth.

In the present study, we aimed to explore the crucial factors in the pathogenesis of severe invasive GAS infections in the context of PMN-GAS relationship, using a panel of *emm49* clinical isolates from patients with or without severe invasive infection, and their

gene-manipulated mutants. We now show a direct and previously unrecognized link between functional loss of a factor CsrS of two-component sensor/regulator system (CsrS/CsrR: also known as CovS/CovR) and escape from killing by PMN via inducing necrosis to them and digesting IL-8, a PMN-attracting chemokine. We further determined CsrS mutations in the severe invasive GAS was essential to control the expression of various virulence genes and contributed to the *in vivo* virulence and disease-specific pathophysiology in a mouse model. These data may participate in prediction of GAS potential for future invasive infection as well as risk assessment of patients by measuring PMN function.

Results

Group A streptococcus isolates from severe invasive infections is resistant to killing by human PMN

To examine whether *emm49* GAS isolated from severe invasive infection might alter human PMN function, we performed phagocytosis assay *in vitro*. As non-opsonized GAS was resistant to the phagocytosis by PMN [14], we opsonized GAS with human plasma in advance to the assay. As shown in Figure 1A, there was no significant difference between GAS that were isolated from non-invasive and severe invasive infections in phagocytosis by PMN ($p = 0.5556$). However, as shown in Figure 1B, *in vitro* killing assay revealed that PMN killed non-invasive GAS, resulting in 15–42% of initial number of bacteria, but not invasive GAS ($p = 0.019$). The similar results were obtained when opsonized with either FCS or human serum regardless of complements immobilization (data not shown). These results were common among all PMN donors. These data indicated that clinically isolated severe invasive GAS were phagocytosed, but escaped from killing by human PMN.

Severe invasive GAS rapidly induce necrosis to human PMN

In an acute bacterial infection, PMN were quickly recruited at the site of infectious foci according to the gradient of chemoattractants. Therefore, we examined whether GAS clinically isolated from severe invasive infections could affect the migration ability of PMN in response to chemokines. As a model of local infection of the initial phase, we utilized a transwell system and added IL-8 and GAS in culture medium within the lower wells. PMN were applied in the upper wells and subsequently incubated for 90 min. As shown in Figure 2A and 2B, a substantial number of PMN, consisted largely of viable cells, was detected in the lower wells consisted of IL-8 and non-invasive GAS, as a control. Contrarily, number of PMN was significantly low in the presence of severe invasive GAS ($p = 0.016$) compared to that of control culture. Flow cytometry analysis suggested that although PMN was detected in the lower well consisted of IL-8 and severe invasive GAS, but most of them were dead as defined by propidium iodine staining ($p = 0.016$) (Figure 2A and 2C) demonstrating that severe invasive GAS affected survival of PMN and its migration activity in a transwell system.

PMN were killed by streptolysin O (SLO) from severe invasive GAS

PMN death was induced shortly after encounter with severe invasive GAS, and PMN were not in apoptotic death because of low frequency of cells positive for annexin V (data not shown), which was seen in the case of cytolysin-dependent cell injury [15]. GAS produce two cytolysins that may contribute to pathogenesis. Streptolysin S (SLS) is an oxygen-stable β-hemolysin and Streptolysin O (SLO) is a pore-forming cholesterol-binding toxin [16]. Therefore, to know the mechanism underlying GAS-mediated killing of PMN, we investigated whether SLO produced by invasive GAS affect survival of PMN in an *in vitro* migration assay system.

Figure 3A shows that PMN killing by invasive GAS was blocked by anti-SLO Ab in culture medium within lower wells of a transwell system ($p = 0.018$ compared with control Ig), at similar extent by adding free cholesterol in the medium (data not shown). Furthermore, an SLO deficient mutant from a STSS isolate NIH230 (NIH230*slo*) lost the killing activity for PMN (Figure 3A), thereby, strongly suggesting that SLO is a key element for PMN killing mediated by invasive GAS. Contrarily, SLS-deficient mutant from NIH230 strain (NIH230*sagA*) killed PMN, as efficiently as did parent strain, indicating that SLS is dispensable for killing of PMN mediated by invasive GAS. SLO and SLS double mutant GAS from NIH230 strain (NIH230*slosagA*) displayed the killing activity indistinguishable from that of NIH230*slo*, confirming the primarily role of SLO for GAS-mediated PMN killing. As shown in Figure 3B, incubation of PMN with supernatant from co-culture of IL-8 and invasive or non-invasive GAS did not affect PMN viability, suggesting that severe invasive GAS causes PMN killing following encounter with bacteria in a contact-dependent manner.

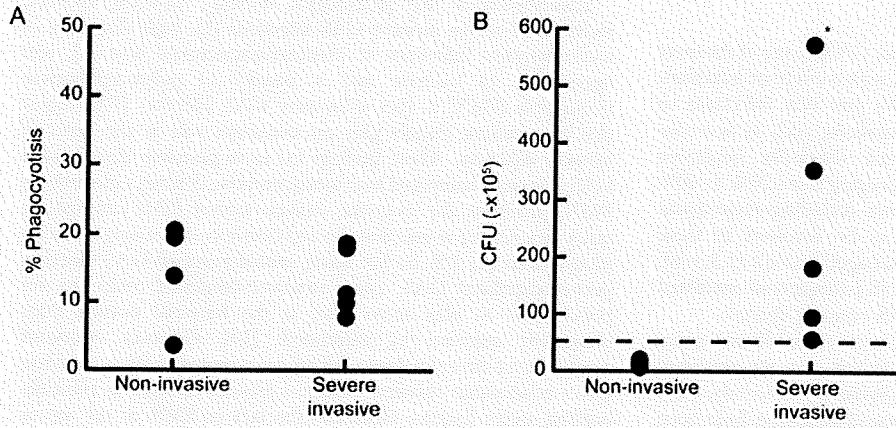

Figure 1. Severe invasive GAS evade killing activity of PMN. (A) Phagocytosis activity of PMN to *emm49* GAS. Four non-invasive strains and 5 severe invasive strains labeled with Alexa-488, followed by incubation with PMN at MOI 10 for 60 min. The proportion of GAS-phagocytosed PMN was then analyzed using flow cytometry. (B) Severe invasive or non-invasive strains were incubated with PMN at MOI 10. After 2 hours, the killing capacity of PMN was estimated by counting the number of bacteria colonies. The dotted line indicates the number of bacteria applied to the culture.
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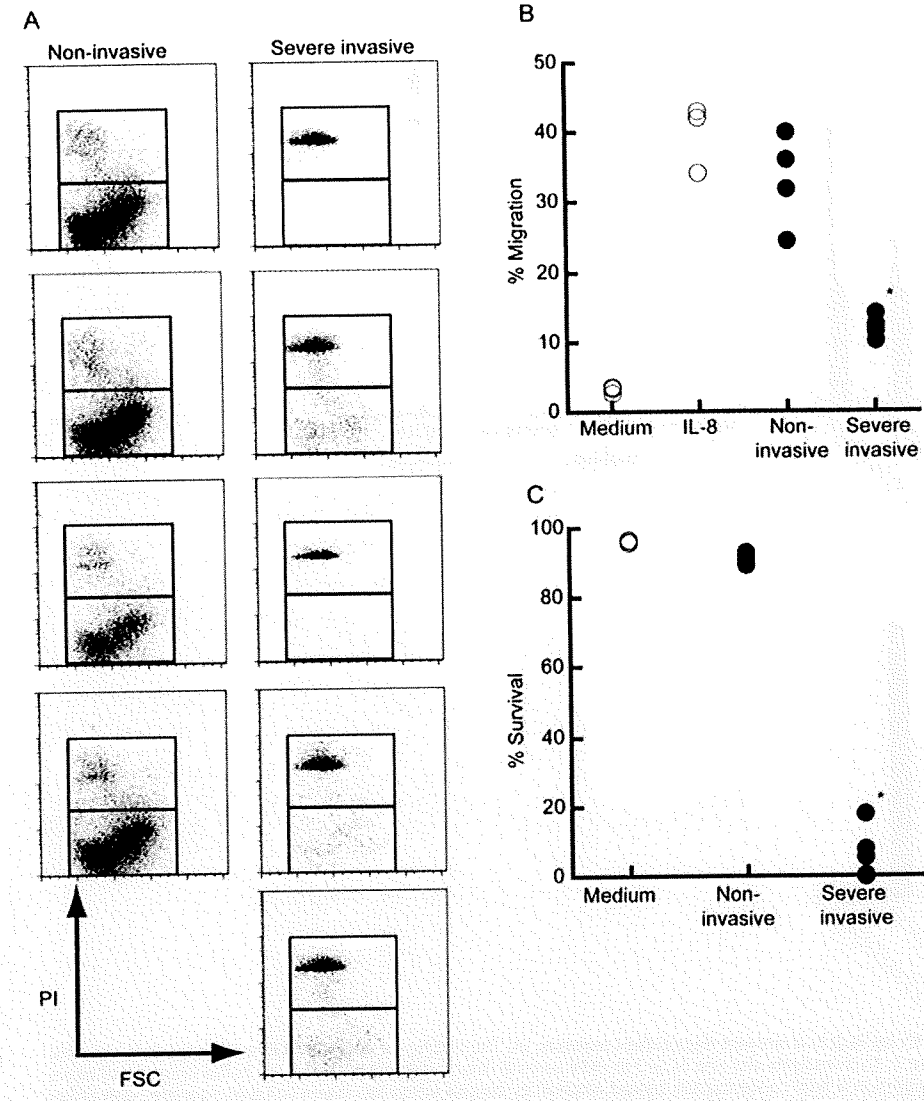

Figure 2. Severe invasive GAS kill human PMN and impair migration ability in response to IL-8. (A) Flow cytometry profiles of migrated PMN in response to 100 nM IL-8 plus GAS. PMN were applied into the upper well (5×10^5 cells) and those migrated into the lower well in a transwell system in response to IL-8 in the presence of GAS (5×10^6 CFU) were stained with propidium iodine and were analyzed using flow cytometry. Each panel represents migrated PMN encountered with individual clinically isolated GAS strain. The representative data are shown. (B) The proportion of PMN that migrated into lower wells in response to IL-8, in the presence (closed circles) or absence (open circles) of invasive or non-invasive GAS strains. Total cell numbers consisted of both viable and dead cells were estimated 60 minutes after incubation. (C) The proportion of live PMN that migrated into lower wells in response to IL-8 alone (open circles) or IL-8 in the presence of severe invasive or non-invasive GAS strains (closed circles). Viable cell numbers were analyzed at 60 minutes incubation. *$p<0.05$ estimated by Mann-Whitney's U test.
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To confirm that SLO activity is increased in invasive GAS strain, we measured SLO hemolytic activity of GAS strains used in this study. As shown in Figure 3C, SLO activity of severe invasive isolate NIH230 is increased as twice as that of non-invasive strain 1566 ($p = 0.017$).

Impaired migration of PMN is due to degradation of IL-8 by serine proteinase ScpC

Although NIH230*slo* lost the killing activity for PMN, migration of PMN in response to IL-8 in a transwell system was not restored in the presence of this mutant (Figure 4A), thereby, suggesting that severe invasive GAS blocks PMN migration by influence on IL-8 activity. Therefore, we quantified the amount of IL-8 in culture before and after co-culture with clinically isolated GAS or its mutants. Figure 4B shows that the amount of IL-8 was significantly reduced by 60-min co-culture with NIH230, as well as NIH230*slo*, but not with non-invasive GAS 1556. As previous reports suggested that the GAS envelope peptidase ScpC (also known as SpyCEP) degrades the CXC chemokines, such as human IL-8, Groα, murine KC and MIP-2 [17–19], we established a NIH230 mutant deficient with ScpC (NIH230*scpC*) and analyzed the property in a PMN migration assay. The results showed that NIH230*scpC* neither digested IL-8, like 1566 strain, (Figure 4B) nor abrogated the migration of PMN in response to IL-8,