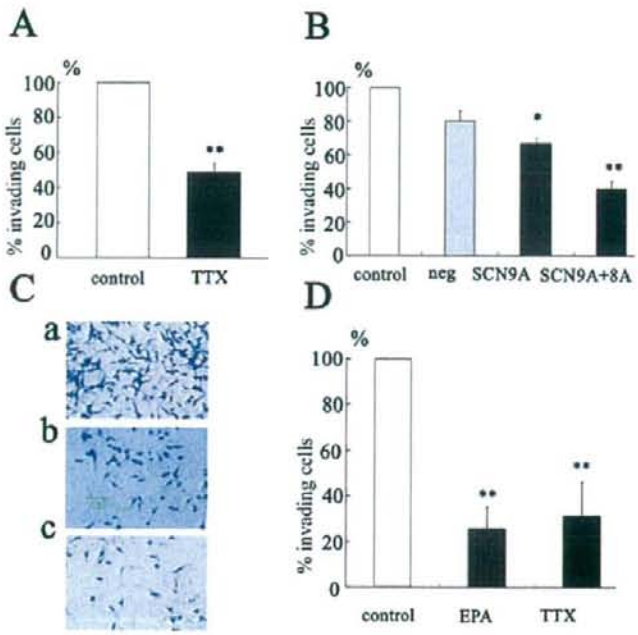

Figure 7 Effects of treatment with TTX, siRNA targeted for SCN8A or SCN9A and EPA on cell invasion in PC-3 cells. (A) Effects of TTX (10 μmol·L^{-1}) on cell invasion. (B) Effects of siRNA for SCN9A alone and in combination with siRNA for SCN8A on cell invasion. (C & D) Effects of EPA and TTX on invasion assay. In C, photographs showing typical fields of view of PC-3 cells following invasion through the Matrigel-coated chamber under control conditions (a), and in the presence of TTX (10 μmol·L^{-1}, b), and EPA (30 μmol·L^{-1}, c). In D, summary data from these assays (means ± SEM from six different experiments). *$P < 0.05$, **$P < 0.01$ vs. control. EPA, eicosapentaenoic acid; siRNA, synthetic small interfering RNA; TTX, tetrodotoxin.

7 nmol·L^{-1}. These findings indicate that I_{Na} expressed in these prostate cancer cells closely resembles TTX-sensitive I_{Na} found in human brain and skeletal muscle, but is different from the TTX-insensitive I_{Na} found in heart. By using molecular techniques, it appears that human breast cancer cells express a TTX-insensitive Na^+ channel subunit, SCN5A, while prostate cancer cells express a TTX-sensitive protein, SCN9A (Diss *et al.*, 2001; 2005). In primary cultures of human cervical cancer, TTX-sensitive I_{Na} has been reported to be carried by a number of channels, $Na_V1.2$, $Na_V1.4$, $Na_V1.6$ and $Na_V1.7$ (Diaz *et al.*, 2007). In the present studies, the transcript of $Na_V1.1$, $Na_V1.2$, $Na_V1.6$ and $Na_V1.7$ was detected in Mat-LyLu cells, as previously reported (Diss *et al.*, 2001; 2005). However, in PC-3 cells, the predominant expression was of $Na_V1.6$ and $Na_V1.7$, and there was more of SCN8A, compared with SCN9A. The presence of these Na channels was confirmed by immunohistochemical studies in these prostate cancer cells. Thus, the expression of SCN8A and SCN9A genes appears to yield TTX-sensitive I_{Na} in PC-3 cells.

The present study showed for the first time that EPA inhibited I_{Na} in prostate cancer cells. The inhibitory effect of EPA was observed at concentrations greater than 0.1 μmol·L^{-1}, and the IC_{50} of EPA was approximately 6 μmol·L^{-1}. As the plasma concentration of free EPA measured by gas chromatography was about 1–6 μmol·L^{-1} and can be increased after ingestion of cod liver oil or after treatment with EPA-ester (Okuda *et al.*, 1997), the inhibitory action of EPA on I_{Na} may play a crucial role in clinical settings. Several possible mechanisms to explain the effects of EPA on I_{Na} can be proposed. But, as shown previously (Jo *et al.*, 2005), neither indomethacin, a cyclo-oxygenase inhibitor, nor NDGA, a lipoxygenase inhibitor, prevented the effects of EPA on I_{Na} (data not shown) suggesting that the metabolites of these pathways are not involved in this action of EPA. Alternatively, EPA has been reported to inhibit $I_{Ca,L}$ in smooth muscle cells and cardiac myocytes (Xiao *et al.*, 1997). Thus, it is more likely that EPA modulates I_{Na} by an interaction with the channel protein itself or by acting at lipid sites near the channels, after partition into the lipid bilayer. The binding sites of EPA have been proposed to be on the short cytoplasmic segment of the α-subunit of Na channels, linking transmembrane repeats III and IV in cardiac myocytes (Kang and Leaf, 1996). The IC_{50} value of EPA on cardiac cells is approximately 4 μmol·L^{-1}, which is close to that in the present study using prostate cancer cells. We also reported that I_{Na} expressed in cultured human bronchial smooth muscle cells is carried by $Na_V1.7$ coded by SCN9A mRNA and inhibited by EPA (Jo *et al.*, 2004; 2005). Thus, whether EPA inhibits I_{Na} in prostate cancer cells by binding the sites similar to those in $Na_V1.5$ (Kang and Leaf, 1996) remains unclear, but I_{Na} appears to be an important target for EPA in prostate cancer cells.

Besides the acute effect of EPA, this fatty acid is incorporated into membrane phospholipids and consequently affects various membrane functions and gene expression (Asano *et al.*, 1998). Examples of this latter effect include prevention of the up-regulation of mRNA induced by mexiletine, an

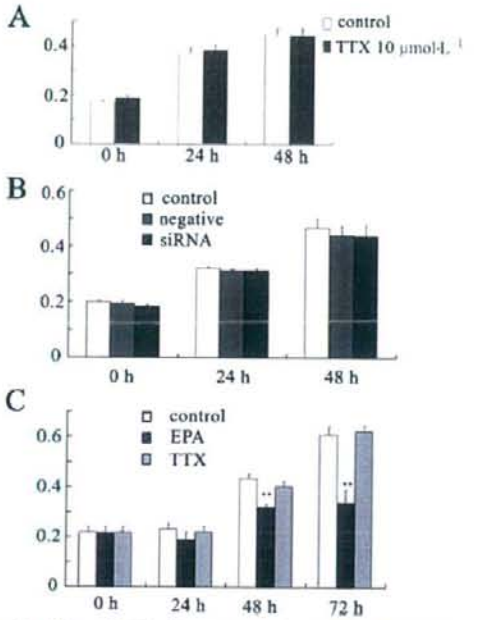

Figure 8 Effects of TTX, siRNA targeted for SCN8A and SCN9A and EPA on cell proliferation in PC-3 cells. (A) Effects of TTX on cell proliferation, assessed after cells were treated with TTX (10 μmol·L^{-1}) for 0, 24 and 48 h. (B) Effect of combined SCN8A and SCN9A siRNA on cell proliferation. Cells were transfected with siRNA targeted for both SCN8A (86) and SCN9A (91), or with non-silencing (negative control) siRNA. (C) Effects of EPA on cell proliferation after exposure to EPA (30 μmol·L^{-1}) for up to 72 h. **P < 0.01 vs. 0 h EPA, eicosapentaenoic acid; siRNA, synthetic small interfering RNA; TTX, tetrodotoxin.

anti-arrythmic agent (Kang *et al.*, 1997) in ventricular cells with EPA (20 μmol·L^{-1}) for 4 days although the basal level of mRNA for SCN5A was not altered. More recently, EPA inhibited SCN9A expression in cultured human bronchial smooth muscle cells (Jo *et al.*, 2005) and a ω-3 PUFA, DHA, inhibited SCN5A mRNA expression and I_{Na} in human breast cancer cells, where $Na_v1.5$ was predominant (Isbilen *et al.*, 2006). In our study, after chronic treatment with EPA, the EPA content of the phospholipid fraction (mol%) increased time-dependently, and that of AA decreased, increasing the ratio of EPA to AA. In our present work, EPA significantly decreased the level of both SCN8A and SCN9A mRNA in PC-3 prostate cancer cells, suggesting that EPA regulates the transcription of mRNA or its processing and stability of mRNA. Thus, it is likely that EPA may suppress the up-regulation of I_{Na} genes, by inhibiting transcription of the relevant genes in prostate cancer cells.

Na^+ current is up-regulated in several types of cancer cells, including prostate cancer, and is known to enhance other cellular functions linked to invasion, secretion, adhesion and motility (Grimes *et al.*, 1995; Laniado *et al.*, 1997; Smith *et al.*, 1998; Abdul and Hoosein, 2001; Anderson *et al.*, 2003; Fraser *et al.*, 2003; Mycielska *et al.*, 2003; Bennett *et al.*, 2004; Onganer and Djamgoz, 2005; Slade *et al.*, 2005; Fulgenzi *et al.*, 2006). The present study showed that I_{Na} was involved in cell invasion and endocytosis but not proliferation, in PC-3 cells. TTX and siRNA targeted for SCN9A or SCN8A significantly inhibited cell invasion. Interestingly, inhibition by a combination of siRNA for SCN9A and for SCN8A, was greater than that after either siRNA alone, suggesting that both subunits were contributing the I_{Na} observed. Similarly, endocytosis measured by uptake of HRP was significantly inhibited by TTX or siRNA for Na_v proteins. In the present study, we also showed that EPA significantly inhibited cell invasion and endocytosis, through a 'chronic' effect on gene expression, as

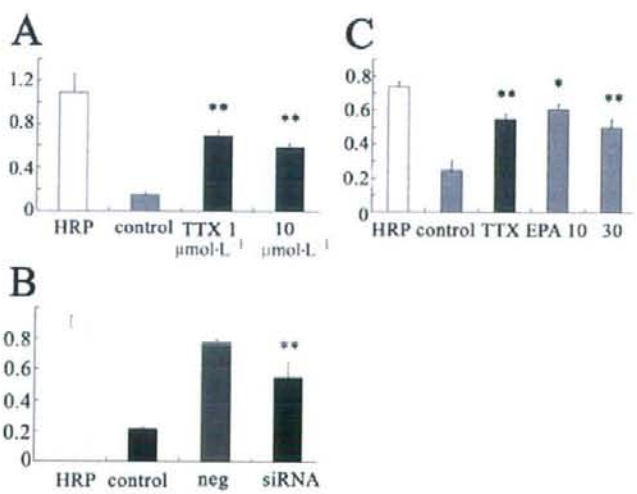

Figure 9 Effects of treatment with TTX (A), siRNA for SCN8A and SCN9A (B) and EPA (C) on endocytosis of HRP in PC-3 cells. Background (control) indicates endogenous peroxidase activity. *P < 0.05, **P < 0.01. EPA, eicosapentaenoic acid; HRP, horseradish peroxidase; siRNA, synthetic small interfering RNA; TTX, tetrodotoxin.

well as by a direct inhibition of the Na channel. The precise mechanism through which I_{Na} could regulate cellular functions is not known yet, but several possibilities can be considered. Particularly, Na^+ influx through I_{Na} may regulate cell volume (Bortner and Cidlowski, 2003), and changes in ion flux and cell volume may be integral to the invasion process (Soroceanu *et al.*, 1999). In turn, the subcellular/molecular mechanisms underlying these effects may involve the cytoskeleton (directly via β-subunit interaction and/or indirectly via local Ca^{2+} fluxes), protein kinase activity and gene expression (Fraser *et al.*, 2003; Mycielska and Djamgoz, 2004). In contrast to the effects of TTX and siRNA on cell invasion and endocytosis, cell proliferation of prostate cancer cells was not affected by TTX and siRNA, suggesting that the contribution of I_{Na} to proliferation of cancer cells was minimal, a result compatible with previous work (Fraser *et al.*, 1999; Fulgenzi *et al.*, 2006). Nevertheless, EPA did significantly suppress cell proliferation, as well as invasion and endocytosis. Thus, EPA appears to inhibit cell growth, independent of I_{Na} blockade, possibly by modulating AA metabolism and/or *Pertussis* toxin-sensitive signal transduction pathways (Sauer *et al.*, 2005). Thus, it is likely that the suppressive effects of EPA on several functions that are crucial to metastasis, such as proliferation, motility, secretion and invasion may be involved in the inhibitory effects of EPA on the metastatic activities of prostate cancer cells.

In conclusion, the present study suggests that treatment with EPA induces direct acute inhibition of I_{Na} and long-term down-regulation of expression of Na channel proteins, up-regulated in prostate cancer cells, thus inhibiting their metastatic activities. I_{Na} would appear to be one of the targets of EPA for long-term control and suppression of prostate cancer cells.

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Conflict of interests

None.

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Hyperoxia exaggerates bacterial dissemination and lethality in *Pseudomonas aeruginosa* pneumonia

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ABSTRACT

Effects of hyperoxia on lethality in mice with *Pseudomonas aeruginosa* pneumonia were defined, and protective roles of macrolides were examined both in vitro and in vivo. Sub-lethal hyperoxia accelerated lethality of mice with *P. aeruginosa* pneumonia. Bacterial number was not different in the lungs, but higher in the liver of mice in hyperoxic conditions. Filter-sterilized culture supernatants of bacteria induced loss of viability of alveolar epithelial cells, which was exaggerated in hyperoxia. Metalloprotease blocking by inhibitor or gene-disruption in bacteria resulted in partial reduction of cytotoxic activity in culture supernatants. Co-culture of bacteria with sub-inhibitory concentrations of macrolides, such as azithromycin, reduced cytotoxic activity in the culture supernatants. Azithromycin provided significant survival benefit in hyperoxia–pneumonia model, which was associated with suppression of bacterial dissemination to extra-pulmonary organs. These results suggest that hyperoxia serves as an important cofactor for bacterial dissemination and lethality of *P. aeruginosa* pneumonia. Our data identify the potential of macrolides to protect individuals with *P. aeruginosa* pneumonia in the setting of hyperoxia.

1. Introduction

Ventilator-associated pneumonia (VAP) is a life-threatening infectious disease that causes substantial morbidity and mortality in respiratory and intensive care units of hospitals. Although oxygen supplementation is a critical supportive therapy for patients with severe hypoxemia, prolonged or even transient administration of oxygen may promote cellular damage and tissue injury [1,2]. Although mechanisms of oxygen toxicity to the lungs have not been carefully defined, it is likely that apoptosis and necrosis play a certain role in hyperoxia-associated lung injury [1,2]. Previously we have reported that hyperoxia exaggerates lethality and acute lung injury due to *Legionella pneumophila* in a mouse model of pneumonia [3]. In *Legionella* pneumonia, acceleration of apoptosis in the infected lungs without affecting bacterial number was demonstrated in the setting of hyperoxia. Although *Legionella* is a major cause of serious pneumonia requiring ventilator, more ubiquitous and antibiotic resistant organisms, such as methicillin-resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa*, play a major part in etiology of VAP [4].

P. aeruginosa is an opportunistic pathogen that causes a wide range of acute and chronic infections, including sepsis, wound and pulmonary infection [5]. The recent epidemiological data demonstrated that *P. aeruginosa* is a leading cause of VAP, and pneumonia due to this pathogen is associated with extremely high rate of mortality, even when *P. aeruginosa* is isolated in relatively small numbers from the lungs [6,7]. *P. aeruginosa* is known to produce a variety of virulence factors, such as pigments, proteases and exotoxins [8]. These factors appear to contribute to injury of infected airway epithelial cells, which may cause disruption of barrier function of membrane and allow penetration of bacteria into the bloodstream [8]. However, which virulence factor is crucial for the development of VAP, or how hyperoxia modulates course and severity of VAP, remained to be elucidated.

After the first report of survival benefits of long-term macrolide treatment on chronic *P. aeruginosa* infection in diffuse

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panbronchiolitis patients [9,10], several investigators have reported efficacy of long-term macrolide therapy in cystic fibrosis and other forms of chronic pulmonary infections [11–13]. Although the exact mechanisms of macrolide efficacy are unknown, there are at least three possibilities, macrolide effects on bacteria at sub-inhibitory concentration (sub-minimum inhibitory concentration: sub-MIC), host defense systems, or both [14–16]. Several investigators including us have reported that macrolides at sub-MIC suppress major virulence factors of *P. aeruginosa*, such as pigment, exotoxin and exopolysaccharide [14,17,18].

In the present study, we examined effects of hyperoxia on lethality of mice with *P. aeruginosa* pneumonia, and dissemination of bacteria into distant organs. Also we explored protective roles of macrolides on pathogenesis of *P. aeruginosa* pneumonia in the setting of hyperoxia.

2. Materials and methods

2.1. Animal

Balb/c mice (Female, 6-week-old) were purchased from Charles River Japan. All mice were housed in the animal care facility at Toho University (Tokyo, Japan). All animal experiments were performed under the approval of animal center of Toho University (approval number: #169).

2.2. Construction of *aprA* or *lasB* deletion mutants from P. aeruginosa *PAO1*

P. aeruginosa PAO1 was used for the experiments. Elastase (*lasB*)- and alkaline protease gene (*aprA*)-deficient mutants were produced, as described previously [19,20]. Primers used for the PCR were shown in Table 1. Deficiency of these proteases was confirmed in ELISA using specific antibody [21].

2.3. Culture and inoculation of bacteria

P. aeruginosa was incubated on Mullar Hinton agar (Gibco, Grand Island, N.Y.) for overnight at 37 °C. Mice were anesthetized, and then 30 μl of bacterial suspension were administered intranasally [22].

Table 1
Primers for construction of *aprA* or *lasB* mutants.

Primer	Sequence (5′–3′)[a]	PCR step
For deletion of *aprA*		
AprA F1	CGCGCTAGCCGCTACGAGGTCGAGCAGAACCGC	1st round 5′, 2nd round 5′
AprA R1	cgcgccgtgtcagcgcgTGCAGTTATCGGCCAGATCAGGCCG	1st round 3′
AprA F2	atctggccgataactgcaCGCGCTGACACGGGCGCG	1st round 5′
AprA R2	AGCAAGCTTCGAAGGCATGATCGCCAGCGTCCG	1st round 3′, 2nd round 3′
For deletion of *lasB*		
LasB F1	GAGGCTAGCGAGGCGCTGGCGCAGCTCAAGGAG	1st round 5′, 2nd round 5′
LasB R1	cggccgggaccaccgagcCAGGCCGAAGTACTCGGCCGACGC	1st round 3′
LasB F2	gccgagtacttcggcctgGCTCGGTGGTCCCGGCCGGCAC	1st round 5′
LasB R2	CCTAAGCTTTCCTACCGGGATTTCCGCATTCGCCGCG	1st round 3′, 2nd round 3′

[a] Uniderlined sequences and lower-case letters indicate the endonuclease restriction sites added for cloning into the plasmid pMT5059 and the sequences added for overlap-extention PCR, respectively.

2.4. Protease inhibitors used

The following protease inhibitors were purchased from Sigma-Aldrich: Pepstatin A, a potent inhibitor of acid proteases; AEBSF [4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride], an irreversible serine protease inhibitor; E-64 [trans-epoxysuccinyl-L-leucylamino(4-guanidino)butane], an effective irreversible inhibitor of cystein protease; EDTA (Ethylenediaminetetraacetic acid), a potent inhibitor of metallopoteases.

2.5. Oxygen exposure to mice

Two hours after intranasal administration of bacteria, one group of mice was kept in hyperoxia for 60 h in an airtight chamber, whereas another group was placed in room air [3]. For hyperoxic exposure, the oxygen concentration in the chamber was kept at 90% by a regulated flow of oxygen, which was monitored with an in-line oxygen analyzer (model D2; Beckman, Fullerton, CA).

2.6. Lungs, blood and liver harvesting for analysis

After CO_2 asphyxia, the blood was collected by heart puncture, and 10 μl of a serial dilution of blood were inoculated on agar for determination of bacterial count. The lungs and liver were homogenized in 2 ml saline using a tissue homogenizer. Homogenates (10 μl) were inoculated on agar after serial 1:10 dilutions. The remaining homogenates were centrifuged at 2500 rpm for 10 min. The supernatants were passed through a 0.45 μm filter and stored at −20 °C until used.

2.7. Determination of caspase-3 and histone-associated DNA fragments in the lungs of mice infected with P. aeruginosa

To evaluate induction of apoptosis, levels of histone-associated DNA fragments and caspase-3 activity were determined in lung homogenates. DNA fragmentation was quantified by measuring histone-associated DNA fragments using an ELISA kit (Cell Death Detection ELISA^{plus}, Roche Diagnostics GmbH, Germany). Caspase-3 activity was determined by a colorimetric assay (R&D systems), in which caspase-specific peptide conjugated to the color reporter molecule *p*-nitroanilide was used. The data are expressed as a fold increase, comparing to those of control mice ($n = 5$).

2.8. Co-culture of bacteria with sub-MICs of macrolide

Bacteria were incubated in Muller Hinton broth at 37 °C for 48 h with or without sub-MICs of macrolides. The supernatants were prepared by centrifugation and filter-sterilization (0.2 μm), and then used for cell viability assay. The following macrolides were used in this study; azithromycin (AZM), erythromycin (EM), clarithromycin (CAM), oleandomycin (OL), josamycin (JM), rokitamycin (RKM), midecamycin (MDM), telithromycin (TEL). Previously, we have reported that effects of sub-MICs of macrolide on virulence factors and quorum-sensing molecules of *P. aeruginosa* PAO1 [23,24]. During these experiments, we have confirmed that 2 μg/ml of macrolides did not affect the bacterial number in test tube, although higher concentrations may suppress the growth of *P. aeruginosa* PAO1.

2.9. Cell culture, oxygen exposure and cell viability assay

The human lung alveolar epithelial cell line A549 was obtained from ATCC, and the cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum (FBS). A549 cells were seeded to wells ($2–3 \times 10^4$ cells per well) in a 96-well plate. The cells were

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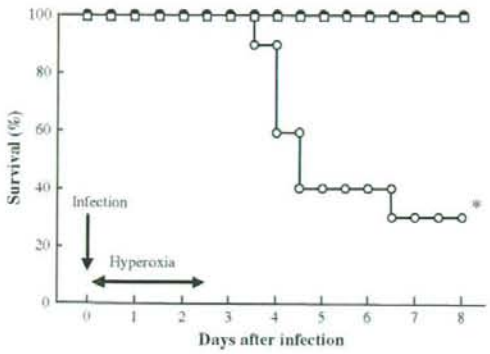

Fig. 1. Lethal sensitivity of mice with *P. aeruginosa* pneumonia in the setting of hyperoxia. Balb/c mice were intranasally inoculated with *P. aeruginosa* (6×10^6 CFU/mouse). One group (●) was kept in room air, whereas another group (○) was placed under hyperoxic conditions for 60 h, and then survival was observed in room air 8 days after bacterial challenge ($n = 10$). No death of mice was observed in hyperoxia alone without infection (□). *$P < 0.01$, compared with room air control group.

incubated in chamber of 5% CO_2 or 5% CO_2 plus 90% O_2 (ASTEC, Ltd., ACL-165D/ACM-165D, Japan) at 37 °C for 12–24 h with or without bacterial culture supernatants at 5% of final concentration. Cell viability was examined using the MTT assay using TetraColor ONE (Seikagaku Kogyo, Tokyo, Japan).

2.10. Statistical analysis

Statistical significance was determined using the unpaired, two-tailed alternate student's *t*-test. Survival curves were constructed by the Kaplan–Meier method, and were analyzed by logrank test. A significant difference was considered to be $P < 0.05$.

3. Results

3.1. Lethal sensitivity of mice with P. aeruginosa pneumonia in the setting of hyperoxia

As shown in Fig. 1, hyperoxia significantly exaggerated lethality of mice with *P. aeruginosa* pneumonia ($P < 0.01$). By 7 days after infection, approximately 70% of mice died under hyperoxia, whereas no death was observed in either infection in normoxic conditions or hyperoxia alone without infection. These data demonstrated that sub-lethal hyperoxia may potentiate lethality in *P. aeruginosa* pneumonia.

3.2. Bacterial number in the lungs and liver of infected mice under hyperoxia

We next examined bacterial number in the infected lungs and liver on day 1. As shown in Fig. 2, no difference in bacterial burden was observed in the lungs of mice exposed to hyperoxia as compared to animals maintained in room-air conditions. In contrast, drastic difference of bacterial number was observed in the liver. *P. aeruginosa* CFU in the liver of infected mice kept in room air was below the detection limit, whereas the liver of the hyperoxia group contained bacteria at a concentration of $>10^3$ CFU ($P < 0.01$). In blood, 2 of 7 mice (28.6%) were positive in *P. aeruginosa* in room-air group (mean bacterial number: 100 cfu/ml), whereas 3 of 5 mice (60.0%) were complicated with bacteremia in hyperoxia group (mean bacterial number: 133 cfu/ml). These data suggest that hyperoxia exaggerates dissemination of bacteria to extra-pulmonary organ liver, probably through bloodstream.

3.3. Caspase-3 and histone-associated DNA fragments in the lungs of mice infected with P. aeruginosa

After the induction of *P. aeruginosa* pneumonia, mice were kept in room air or hyperoxic condition for 1 day, and then caspase-3 activity and histone-associated DNA fragments in the lungs of mice were examined. As shown in Table 2, *P. aeruginosa* infection induced increase of caspase-3 activity in the lungs of mice kept in room air to approximately 180% of the control. Importantly, caspase-3 activity of the infected lungs of mice kept in hyperoxia did not differ from those of mice kept in room air. Although slightly higher values were observed in hyperoxia group in histone-associated DNA fragments, it was not statistically significant. These data demonstrated that hyperoxia is not likely to exaggerate apoptosis in the lungs of mice infected with *P. aeruginosa*, judging from apoptosis makers, such as caspase-3 and histone-associated DNA fragments.

3.4. Effects of filter-sterilized culture supernatants on epithelial cells

Effects of bacterial culture supernatants on epithelial cells were examined in an in vitro cell culture system. The

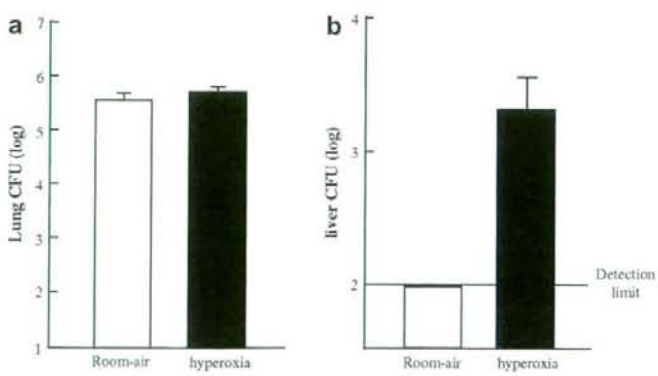

Fig. 2. Bacterial number in the lungs and liver of mice with *P. aeruginosa* pneumonia under hyperoxia. Balb/c mice were intranasally inoculated with *P. aeruginosa*. Then, one group was kept in room air (open column), whereas another group was placed under hyperoxic condition (closed column). The bacterial burden in the lungs and liver was assessed on day 1 post-infection ($n = 5$). *$P < 0.01$, compared with room air control group.

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Table 2
Caspase-3 and histone-associated DNA fragments in the lungs of infected mice kept in room air or hyperoxia.

Factors examined	Condition kept	Mean ± SD
Caspase-3	Room air	1.79 ± 0.44
	Hyperoxia	1.50 ± 0.40
Histone-DNA	Room air	64.6 ± 7.1
	Hyperoxia	73.5 ± 14.2

Mice were infected with *P. aeruginosa* and then kept in room air or hyperoxia. On day 1 after infection, caspase-3 and histone-associated DNA fragments in the lungs were examined ($n = 5$), and the results were expressed as fold increase of the control (no infection).

supernatants were prepared by centrifugation and filter-sterilization of culture supernatants of *P. aeruginosa*. Human alveolar epithelial cells (A549) were incubated with culture supernatants for 8 h, and change of morphology was examined. As shown in Fig. 3a, drastic alterations of cellular morphology, such as rounding, aggregation and detachment, were observed in a concentration-dependent manner. Next, we examined changes of cell viability in the presence of various concentrations of culture supernatants for 48 h in the setting of normoxia (21% O_2) or hyperoxia (90% O_2). Addition of culture supernatants decreased cell viability in a concentration-dependent manner under normoxia and hyperoxia. Especially, in the setting of hyperoxia, significantly higher reduction of cell viability was observed in 0.62–2.5% of culture supernatants, comparing to those in normoxia (Fig. 3b, $P < 0.01$). These results suggested that a high concentration of oxygen exaggerates epithelial cell damage by culture supernatants of *P. aeruginosa*.

3.5. Effect of protease inhibitors on cell viability in the presence of culture supernatants

Morphological changes of cells, such as rounding and detachment, prompted us to examine the contribution of bacterial proteases in culture supernatants, as it is known that *P. aeruginosa* produces several proteases. We examined effects of protease inhibitors Pepstatin A (acid proteases inhibitor), AEBSF (serine proteases inhibitor), E-64 (cysteine proteases inhibitor), EDTA (metalloproteases inhibitor) on cell viability in the presence of culture supernatant (5%) for 48 h under hyperoxia (Fig. 4). No protective effects of Pepstatin A, AEBSF or E-64 were observed in concentrations examined. On the other hand, the metalloprotease inhibitor EDTA significantly reduced loss of viability of epithelial cells at concentrations of 250 and 500 μM ($P < 0.05$).

3.6. Effects of deletion of *lasB*- or *aprA*-gene in P. aeruginosa on cytotoxic activity in the setting of hyperoxia

Next, we examined production of cytotoxic virulence factors in culture supernatants of *lasB*- or *aprA*-deficient isogenic mutants (Fig. 5). The culture supernatants (5%) of *P. aeruginosa* PAO1 parent strain induced reduction of viability of epithelial cells to approximately 50% of the control (no treatment). Although the restoration of viability was partial and modest, the deletion of *lasB*- or *aprA*-gene in *P. aeruginosa* reduced cytotoxic activity in culture supernatants from these strains. The data also suggested that alkaline metalloprotease (*aprA*) play more important role in cytotoxic activity than elastase (*lasB*) in *P. aeruginosa* PAO1 in the setting of hyperoxia.

3.7. Effects of co-culture with macrolide antibiotics on production of cytotoxic virulence factors by P. aeruginosa

P. aeruginosa was grown in the presence or absence of sub-MICs of macrolides, and then the culture supernatants were examined for their cytotoxic activities. A549 cells were incubated with 5% of culture supernatants for 48 h in the setting of hyperoxia, and then

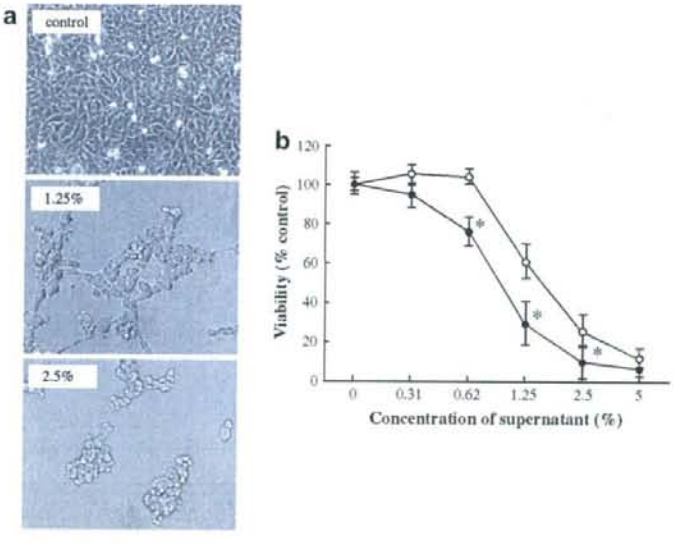

Fig. 3. Change of morphology and viability of epithelial cells in the presence of bacterial supernatants. a. A549 cells were incubated with filter-sterilized *P. aeruginosa* culture supernatant (0%, 1.25%, 2.5%) for 8 h in 5% CO_2 plus 90% O_2, and then the cell morphology was examined. b. A549 cells were incubated with various concentrations of culture supernatants for 48 h in 5% CO_2 plus 20% O_2 (ambient air) (○) or 5% CO_2 plus 90% O_2 (●), and then the cell viability was examined by MTT assay, as described in materials and methods. *$P < 0.01$, compared with 5% CO_2 group.

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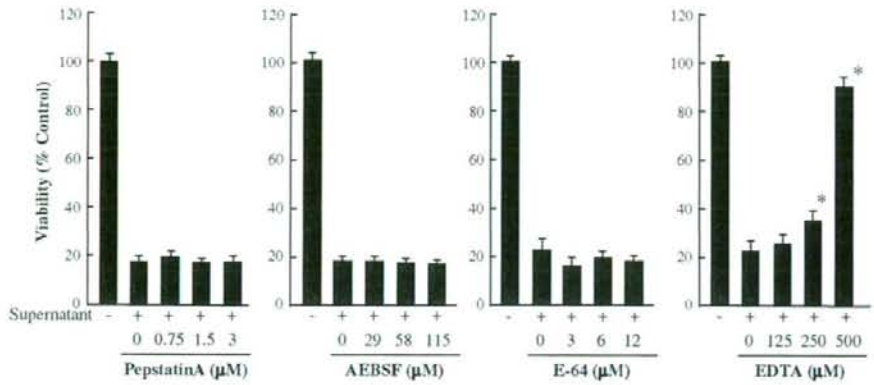

Fig. 4. Effect of protease inhibitors on induction of loss of cell viability by culture supernatants of *P. aeruginosa*. A549 cells were incubated with 5% of *P. aeruginosa* culture supernatant for 24 h in the presence of various concentrations of protease inhibitors under 5% CO_2 plus 90% O_2, and then the viability of cells was compared. *$P < 0.05$, compared with no protease inhibitor.

cell viability was compared. As shown in Fig. 6, AZM, TEL, CAM and EM suppressed production of cytotoxic virulence factors in a concentration-dependent manner. In contrast, JM, RKM, MDM and OL exhibited only subtle effects, even at a concentration of 10 μg/ml. From these experiments, we could not exclude effects of macrolide carried over on cell viability assay, although it seems unlikely because the expected final concentration of macrolides carry over was 0.125–0.5 μg/ml (20 times dilution of the supernatant).

3.8. Effects of AZM treatment on survival of mice with P. aeruginosa pneumonia in the setting of hyperoxia

AZM (30 mg/kg, PO) or control solvent was administered starting 2 days before infection for a total of 5 days, and *P. aeruginosa* was intranasally inoculated on day 0. The infected mice with or without AZM treatment were kept in 90% O_2 for 60 h, and then survival was monitored in room air for 10 days after infection (Fig. 7a). Infected mice exposed to hyperoxia started to die from day 4, with a final survival rate being approximately 50% by day 10. In contrast, no death of mice was observed in AZM treatment group ($P < 0.01$). These data suggest that AZM treatment is beneficial for *P. aeruginosa* pneumonia in the setting of hyperoxia.

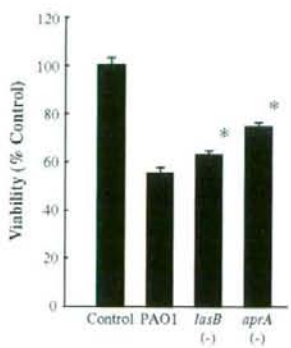

Fig. 5. Cytotoxic activities in culture supernatants of *lasB*- or *aprA*-deficient mutants. Cytotoxic activities in culture supernatants (5%) of *lasB*- or *aprA*-deficient mutants were compared to that of parent strain *P. aeruginosa* PAO1 in the setting of hyperoxia. *$P < 0.05$, compared with *P. aeruginosa* PAO1.

3.9. Effects of AZM treatment on bacterial number in the lungs and liver

We examined bacterial number in the lungs and liver of mice 1 day after the infection. Administration of AZM and treatment of hyperoxia were performed as described in Fig. 7a. In the lungs, bacterial numbers were 4.5×10^5 and 2.5×10^4 cfu/lung for the control and AZM treatment group, respectively (Fig. 7b). Interestingly, bacteria were not detected in the liver of mice with AZM treatment group, whereas approximately 10^4 cfu/liver of *P. aeruginosa* was present in the control mice. These data demonstrated that

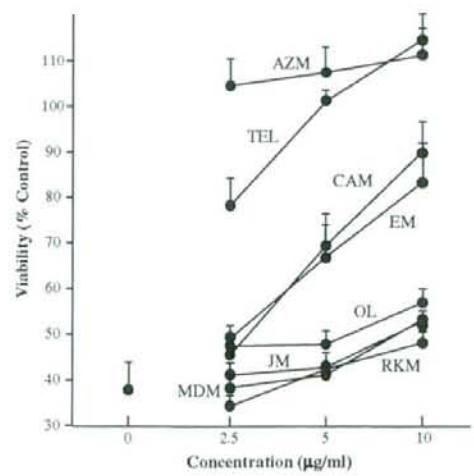

Fig. 6. Effects of co-culture with macrolide antibiotics on production of cytotoxic virulence factors of *P. aeruginosa*. *P. aeruginosa* PAO1 was incubated with 2.5, 5, 10 μg/ml of macrolide antibiotics at 37 °C for 24 h, and then the culture supernatants were prepared by centrifugation and filter-sterilization. A549 cells were incubated with 5% of the culture supernatants for 48 h in the setting of 5% CO_2 plus 90% O_2, and then the cell viability was compared.

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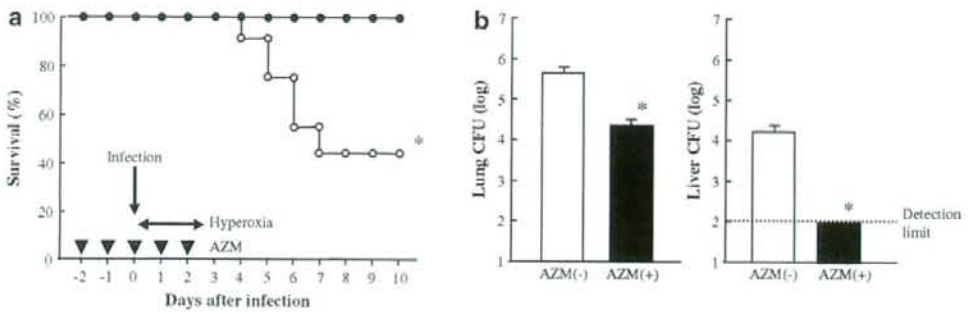

Fig. 7. AZM effects on bacterial number and survival of mice with *P. aeruginosa* pneumonia in the setting of hyperoxia. a. AZM (30 mg/kg, PO) (●) or control solvent (○) was administered from 2 days before infection for 5 days, and *P. aeruginosa* PAO1 was intranasally inoculated on day 0. The infected mice with or without AZM treatment were kept in 90% O_2 for 60 h, and then survival was monitored in room air for twice a day for 10 days after infection. b. AZM (30 mg/kg, PO) or control solvent was administered from 2 days before infection for 3 days, and *P. aeruginosa* PAO1 was intranasally inoculated on day 0. The infected mice with (closed column) or without AZM treatment (open column) were kept in 90% O_2 for 24 h, and then the bacterial number in the lungs and liver was examined ($n = 5$). $^*P < 0.01$, compared with no treatment group.

AZM treatment reduced bacterial burden in the lungs and dissemination of bacteria to the liver in the setting of hyperoxia.

4. Discussion

The present study demonstrates that sub-lethal treatment of oxygen exaggerates *P. aeruginosa* pulmonary infection. Our data showed significantly higher bacterial burden in the liver, but not in the lungs, indicating development of bacterial dissemination in the setting of hyperoxia. The data obtained from protease blocking experiments and specific gene-disrupted mutants suggested involvement of metalloproteases, especially alkaline protease, for epithelial cell damage in the setting of hyperoxia. Importantly, this cytotoxic activity observed in culture supernatants was substantially abrogated when the bacteria were co-cultured with sub-MICs of AZM, TEL, EM and CAM. Consistent with these data, AZM treatment provided survival benefit in hyperoxia–pneumonia model. Collectively, these data suggest that hyperoxia may be an important cofactor for pathogenesis of *P. aeruginosa* pneumonia, and certain macrolides may be a therapeutic option for these individuals.

Previously, we have reported that hyperoxia lethally sensitized mice to *L. pneumophila* in a mouse model of pneumonia [3]. In the *Legionella* experiment, the mechanisms of exaggerated death of mice in the setting of hyperoxia were speculated to be an accelerated apoptosis in pulmonary cells and acute lung injury. In addition, no changes of bacterial burden were observed not only in the lungs, but also in the distant organs, liver and spleen. In the present study, pulmonary apoptosis markers, such as caspase-3 and histone-associated DNA fragments, were not different in *P. aeruginosa* pneumonia between the room air and hyperoxia groups, while significantly higher bacterial burden was observed in the liver of infected mice under hyperoxia. Although a clear mechanism to explain the difference between *Legionella* and *P. aeruginosa* infection is still unknown, these data suggested a minimum contribution of apoptosis in the lungs for hyperoxia-mediated high lethality in *P. aeruginosa* pneumonia model.

Alkaline protease and elastase are major extracellular virulence factors of *P. aeruginosa* [8,25]. These metalloproteases exhibit strong proteolytic activities for cells and tissues, including fibrin and elastin. It has been demonstrated that these enzymes induce rupture of tight-junction of epithelium and increase of permeability, which may be associated with tissue invasion and spreading of bacteria to distant organs [26,27]. Although the present data indicated a role of metalloproteases in cytotoxic activity in *P. aeruginosa*, those contributions appear to be partial. Conversely, these data suggested an involvement of other and/or unknown virulence factors for cytotoxic activity in culture supernatants of *P. aeruginosa*. In this regard, Marquart and associates recently found that *P. aeruginosa* produces a novel secreted protease (*P. aeruginosa* small protease; PASP), which exerts strong proteolytic activity in cornea [28]. Also, it is possible that multiple virulence factors may function additively or synergistically, as Azghani and colleagues have reported complementary actions of elastase and exotoxin A in disruption of epithelial barrier function and bacterial translocation [29].

Although hyperoxia promoted the *P. aeruginosa* culture supernatant-induced cell death of A549 cell, the difference between 5% CO_2 and 5% CO_2 plus 90% O_2 seems to be slight. It looks like that the results obtained from A549 cells could not explain the whole mechanisms of exaggerated lethality under hyperoxia. In vivo pneumonia model may include multiple factors, such as inflammatory cells recruited and production of oxygen-scavenger substances.

Efficacy of certain macrolides against chronic *P. aeruginosa* infection has been confirmed in several clinical settings, including cystic fibrosis and diffuse panbronchiolitis, although the exact mechanisms of actions are still unknown. Several investigators have reported macrolide effects on host immunological responses, bacterial virulence factor expression, or both [14–16]. We have previously reported sub-MIC macrolide effects on *P. aeruginosa*, such as suppression of virulence factors (pigment, protease and extotoxin A) [23], quorum-sensing systems [24], alterations in cell surface structures and exposure time-dependent bactericidal activity [18]. The present results are substantially consistent with previous reports, and further demonstrated protective roles of macrolides in epithelial cell damages by culture supernatants. Also, we observed that AZM treatment reduced bacterial burden in the lungs and liver, and provided survival benefits in *P. aeruginosa* pneumonia in the setting of hyperoxia. Although the exact mechanisms are unknown in *P. aeruginosa* clearance by AZM, it may be possible that AZM-exposed bacteria became less virulent and readily cleared. To this end, Giamarellos-Bourboulis and associates have reported efficacy of CAM in sepsis and VAP patients, in which acceleration of resolution of VAP and weaning from mechanical ventilation were demonstrated, although the mortality rate at day 28 was not altered [30]. Further research regarding the mechanisms of macrolide actions on bacteria, particularly metalloprotease-suppressing activity, in addition to clinical usefulness of

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macrolides, is warranted to prevent and treat a life-threatening *P. aeruginosa* infection, including VAP.

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JOURNAL OF CLINICAL ONCOLOGY

ORIGINAL REPORT

First-Line Gefitinib for Patients With Advanced Non–Small-Cell Lung Cancer Harboring Epidermal Growth Factor Receptor Mutations Without Indication for Chemotherapy

Akira Inoue, Kunihiko Kobayashi, Kazuhiro Usui, Makoto Maemondo, Soji Okinaga, Iwao Mikami, Masahiro Ando, Koichi Yamazaki, Yasuo Saijo, Akihiko Gemma, Hitoshi Miyazawa, Tomoaki Tanaka, Kenji Ikebuchi, Toshihiro Nukiwa, Satoshi Morita, and Koichi Hagiwara

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Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

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ABSTRACT

Purpose

This multicenter phase II study was undertaken to investigate the efficacy and feasibility of gefitinib for patients with advanced non–small-cell lung cancer (NSCLC) harboring epidermal growth factor receptor (*EGFR*) mutations without indication for chemotherapy as a result of poor performance status (PS).

Patients and Methods

Chemotherapy-naïve patients with poor PS (patients 20 to 74 years of age with Eastern Cooperative Oncology Group PS 3 to 4, 75 to 79 years of age with PS 2 to 4, and ≥ 80 years of age with PS 1 to 4) who had *EGFR* mutations examined by the peptide nucleic acid-locked nucleic acid polymerase chain reaction clamp method were enrolled and received gefitinib (250 mg/d) alone.

Results

Between February 2006 and May 2007, 30 patients with NSCLC and poor PS, including 22 patients with PS 3 to 4, were enrolled. The overall response rate was 66% (90% CI, 51% to 80%), and the disease control rate was 90%. PS improvement rate was 79% ($P < .00005$); in particular, 68% of the 22 patients improved from ≥ PS 3 at baseline to ≤ PS 1. The median progression-free survival, median survival time, and 1-year survival rate were 6.5 months, 17.8 months, and 63%, respectively. No treatment-related deaths were observed.

Conclusion

This is the first report indicating that *EGFR* mutation-positive patients with extremely poor PS benefit from first-line gefitinib. Because there previously has been no standard treatment for these patients with short life expectancy other than best supportive care, examination of *EGFR* mutations as a biomarker is recommended in this patient population.

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INTRODUCTION

Lung cancer is the leading cause of cancer death worldwide, and approximately 80% of cases are non–small-cell lung cancer (NSCLC). Many patients with NSCLC have advanced disease at diagnosis and a poor prognosis. For patients with advanced NSCLC who are young and have a good performance status (PS), systemic chemotherapy, such as cisplatin plus gemcitabine or carboplatin plus paclitaxel, is administered as a standard treatment and has been shown to prolong their overall survival.[1,2] However, for patients with poor PS (generally ≥ PS 3), there is no standard treatment except best supportive care (BSC), and most die within 3 to 4 months.[2]

Gefitinib (Iressa; AstraZeneca, Wilmington, DE), an orally active, epidermal growth factor receptor (*EGFR*) tyrosine kinase inhibitor (TKI), has shown novel antitumor activity in patients with advanced NSCLC.[3,4] A recent large randomized trial proved noninferiority of gefitinib to docetaxel in the second- or third-line treatment of NSCLC,[5] although, when used in combination with standard chemotherapy, gefitinib failed to demonstrate efficacy in chemotherapy-naïve patients with advanced NSCLC.[6,7] Because the toxicity of gefitinib is less than that of cytotoxic agents, its utility as first-line treatment for patients with NSCLC having poor PS was studied. The previous study conducted in Japan suggested that gefitinib should not be used in unselected patients with poor PS due to low efficacy and

high toxicity, including interstitial lung disease.[8] However, this study was done at the time when the close relationship between some specific *EGFR* somatic mutations and the sensitivity to EGFR-TKI was unknown.

Two pivotal studies have revealed that somatic mutations of the *EGFR* gene in exons 18 to 21, which encode for the regions close to the adenosine triphosphate-binding pocket of the kinase domain, are associated with the response in patients treated with gefitinib.[9,10] Most of these mutations were observed in two hotspots: in-frame deletions, including amino acids at codons 746 to 750 (E746-A750) in exon 19, and an amino acid substitution at codon 858 (L858R) in exon 21, which is more frequent in patients with NSCLC in Asian countries but is also detected in those in Western countries.[11-13] In a prospective phase II trial of first-line gefitinib for patients with advanced NSCLC with such *EGFR* mutations, we have demonstrated a high response rate (75%) and long progression-free survival (PFS; 9.7 months).[14] Several other studies have supported this result, indicating that an EGFR-TKI provides a clinical benefit in selected patients with NSCLC,[15-19] although these studies enrolled only patients with PS 0 to 2. Some reports have described patients with NSCLC and poor PS who dramatically improved with gefitinib treatment,[20,21] but these reports were case studies.

We have developed the peptide nucleic acid (PNA)-locked nucleic acid (LNA) polymerase chain reaction (PCR) clamp method, a new technology to detect sensitive and insensitive *EGFR* mutations from both cytologic and tissue specimens[22] that has become available in clinical practice in Japan. This technique enabled us to assess the *EGFR* mutation status in patients with poor PS using specimens isolated by noninvasive procedures.[17,22,23] This led us to test our hypothesis that, for selected patients with NSCLC patients having *EGFR* mutations, gefitinib may be a salvage therapeutic option, even for patients in a poor condition who would not be candidates for standard chemotherapy.

In this context, we conducted this prospective phase II study to evaluate the efficacy and feasibility of first-line gefitinib for patients with NSCLC having extremely poor PS and harboring *EGFR* mutations.

PATIENTS AND METHODS

Patient Selection

This study was performed in accordance with the Helsinki Declaration (1964, amended in 2000) of the World Medical Association, and the protocol was approved by the institutional review board of each participating institution. The main eligibility criteria was to select chemotherapy-naïve patients with NSCLC with both sensitive *EGFR* mutations and no indication for chemotherapy because of poor PS. Namely, patients having exon 19 deletions, L858R, L861Q, G719A, or G719S were included, but those with a resistant T790M mutation were excluded. Patients 20 to 74 years of age with Eastern Cooperative Oncology Group PS 3 to 4, those 75 to 79 years of age with PS 2 to 4, and those ≥ 80 years of age with PS 1 to 4 were eligible. Patients were also required to have estimated life expectancy of less than 4 months by BSC alone. Other eligibility requirements were stage IIIB to IV or postoperative recurrent NSCLC, presence of a measurable lesion according to the Response Evaluation Criteria in Solid Tumors criteria, adequate liver function (AST and ALT ≤ 100 U/L, total bilirubin < 2.0 mg/dL), and written informed consent. Oxygen therapy was permitted in cases of tumor progression.

EGFR Mutation Analysis

Cytologic specimens such as those obtained from sputum, bronchial washing, pleural effusion, and needle aspiration biopsy were divided into two samples. The first sample was used to confirm pathologically that cancer cells were present in the specimen. The second sample was subjected to an *EGFR* mutation test based on the PNA-LNA PCR clamp method. Briefly, genomic DNA fragments surrounding mutation hot spots of the *EGFR* gene are amplified by PCR in the presence of a clamp primer synthesized from PNA and with a wild-type sequence. This leads to the preferential amplification of the mutant sequence, which is detected by a fluorescent primer that incorporates LNA to increase the specificity. As a result, a mutant *EGFR* sequence is detected in the presence of a 100- to 1,000-fold wild-type sequence.[17,22,23] Thus by the PNA-LNA PCR clamp, a small number of *EGFR* mutation-positive cancer cells are detected within 3 hours. The sensitivity and specificity of the PNA-LNA PCR clamp were 97% and 100%, respectively.[23]

Four institutions routinely examined *EGFR* mutations by the PNA-LNA PCR clamp in our laboratory in Saitama Medical University as part of their clinical practice. Another nine institutions, where *EGFR* mutation testing was not routine, sent materials to the same laboratory after obtaining the informed consent for both testing *EGFR* mutations and the possibility to enter this study in the event of a mutation-positive result.

Drug Administration

Gefitinib (250 mg/d) was orally administered once daily. Patients continued uninterrupted treatment until they experienced disease progression or appearance of intolerable toxicity or until they withdrew consent. For patients with severe toxicity, the gefitinib dosing schedule could be modified to every second day. Second-line chemotherapy or other treatments after the termination of gefitinib therapy were permitted.

Treatment Assessment

We evaluated objective tumor responses as complete response, partial response, stable disease, or progressive disease in accordance with the Response Evaluation Criteria in Solid Tumors. Disease control was defined as the best response out of complete response, partial response, or stable disease, which was confirmed and sustained for 4 weeks or longer. Baseline assessments were performed within 28 days of treatment commencement. During treatment, assessments were performed every 4 weeks for the first 4 months and then every 8 weeks until disease progression. All adverse events were reported, and severity was graded by the National Cancer Institute Common Toxicity Criteria (version 3.0). Data were also collected when gefitinib treatment was interrupted or withdrawn due to adverse events. Routine clinical and laboratory assessments were performed at least every 4 weeks. PS was assessed according to Eastern Cooperative Oncology Group criteria.

Statistical Analyses

There has been little information available on an active treatment for patients with NSCLC having extremely poor PS. From our clinical experience, it seemed that gefitinib was effective in *EGFR* mutation-positive patients, even if their PS was poor. Therefore, we hypothesized that at least half of the poor PS patients would benefit from gefitinib if their tumor was positive for *EGFR* mutation. As the primary end point of this study, we used an overall response rate (ORR), defined as the proportion of patients in whom best response was a complete or partial response among all per-protocol patients. We assumed that an ORR of 75% in eligible patients would indicate potential clinical usefulness, whereas an ORR of 50% would be the lower limit of clinical usefulness, taking into account the retrospective studies of EGFR-TKI for patients with good PS.[24] On the basis of this assumption, our study was designed to have 95% power and had a .10 level of significance. The most informative secondary end point to clinicians was PS improvement rate, which was defined as the proportion of per-protocol patients whose PS during gefitinib treatment was improved from baseline. We also assumed that a PS improvement rate of 50% in eligible patients would be the lower limit of clinical usefulness. The other secondary end points were toxicity, PFS, and overall survival (OS). PFS was defined as the interval between the start of the treatment and the date of the first observation of disease progression or death from any cause. Patients who were alive without disease progression at the data cutoff point (April 30, 2008) were censored at the last point that the patients were assessed to be progression-free. PFS and OS were estimated by the Kaplan-Meier method, and the differences between subgroups were analyzed by log rank. *P* values less than .05 was considered to be significant.

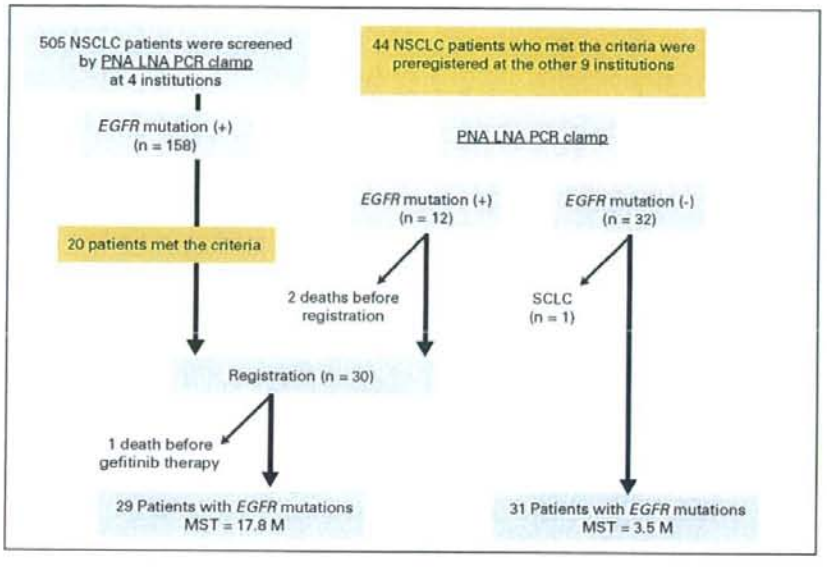

Fig 1. Flowchart of patient accumulation. Epidermal growth factor receptor (*EGFR*) mutations were routinely examined by the peptide nucleic acid-locked nucleic acid polymerase chain reaction (PNA-LNA PCR) clamp in four institutions, and the other nine institutions sent materials to the same laboratory after informed consent was obtained. From the four screening institutions, 20 patients with *EGFR* mutations were entered onto the study. From the other institutions, 12 of 44 patients tested were found to have *EGFR* mutations; of these 12 patients, 10 were enrolled and two died before they could be enrolled. Of the 30 eligible patients, 29 patients, except for one patient who died as a result of rapid disease progression, received gefitinib. NSCLC, non–small-cell lung cancer; pts, patients; SCLC, small-cell lung cancer; MST, median survival time.

RESULTS

Patient Characteristics

Two groups of institutions participated in this study. The first group included four institutions where *EGFR* mutation was tested routinely by the PNA-LNA PCR clamp test, and information on *EGFR* mutation was available at the time of diagnosis. During the study period, the four institutions screened 505 patients with NSCLC, and 158 patients (31%) were found to have sensitive *EGFR* mutations, regardless of their PS. Among them, 20 patients with poor PS entered this study (Fig 1). The second group included nine institutions where *EGFR* mutation testing was not routine. Informed consent for both testing *EGFR* mutations and for the possibility to enter this study was obtained from 44 patients in this second group. *EGFR* mutation was positive in 12 of the 44 patients, and 10 patients entered onto this study (two patients died before enrollment). In contrast, except for one patient whose diagnosis was changed to be small-cell lung cancer, 31 patients with NSCLC and poor PS and without *EGFR* mutation from these institutions were considered to be free from selection bias (Fig 1). Among these 31 patients (21 men and 10 women, with average age of 72.6 years), eight patients received a carboplatin-based doublet, two patients received mono-chemotherapy, three patients received palliative irradiation, and 18 patients were treated with BSC alone.

Between February 2006 and May 2007, a total of 30 patients with NSCLC and *EGFR* mutations were enrolled: 20 from group 1 and 10 from group 2 (Table 1). Of the 30 eligible patients, one patient did not receive gefitinib as a result of bacterial pneumonia and rapid disease progression after registration. Most patients had stage IV disease, and 16 patients had multiple sites of distant metastasis. Twenty-two patients had PS 3 or 4 because of various cancer-related conditions, as follows: respiratory failure owing to multiple pulmonary metastasis, carcinomatous lymphangiosis, malignant pleural effusion, superior vena cava stenosis owing to mediastinal lymph node invasion, carcinomatous pericarditis, symptomatic brain metastasis, carcinomatous meningitis, symptomatic vertebral metastasis, uncontrollable pain because of bone metastasis, and other conditions. Eight patients were oxygen dependent. Three patients with PS 1 and four patients with PS 2 were all ≥ 80 years of age. Because smoking status and adenocarcinoma histology were not included in the selection criteria, heavy smokers (defined as > 10 pack-years) and patients with nonadenocarcinomas were also enrolled, although we found the majority of patients were nonsmokers or light smokers and had adenocarcinomas. The mutations were mainly exon 19 deletions or L858R, which is similar to the results of previous reports.[9-19]

Response and Survival

The objective tumor responses are summarized in Table 2. The ORR and disease control rates were 66% (90% CI, 51% to 80%) and 90% (90% CI, 80% to 99%), respectively. Posthoc subset analyses performed to examine the difference in response rate between certain clinical factors (sex, smoking status, PS) and the type of *EGFR* mutation revealed no relationship between these factors and gefitinib response.

The median follow-up time was 17.8 months (range, 12 to 26 months), and 12 patients were alive at the data cutoff point. The median PFS was 6.5 months (Fig 2A). The median survival time (MST) was 17.8 months, and 1-year survival rate was 63% (Fig 2B). There were no statistical differences in OS and PFS between patients with PS 1 to 2 (frail older adult patients) and patients with PS 3 to 4.

Of the 29 patients treated with gefitinib, five patients received postgefitinib chemotherapy. Although an ORR of their second-line chemotherapy was not reviewed, the MST of the five patients from the time of gefitinib failure was 11.7 months. Conversely, MST of the other 24 patients with BSC alone after gefitinib failure was 8.2 months. There was no significant difference of MST after gefitinib failure between patients treated with second-line chemotherapy and those treated with BSC alone ($P = .122$).

Table 1. Patient Characteristics

Characteristic	No. of Patients
Enrolled patients	30
Treated patients	29*
Sex	
Male	6
Female	23
Age, years	
Median	72
Range	50-84
Performance status	
1	3†
2	4†
3	17
4	5
Smoking status, pack-years	
0	22
1-19	2
≥ 20	5
Histology	
Adenocarcinoma	27
Adenosquamous	1
Undifferentiated	1
Stage	
IV	27
Others	2‡
Metastatic site	
Brain	11
Meninges	1
Lung	18
Bone	12
Liver	2
Others	2
Malignant effusion	
Malignant pleural effusion	10
Malignant pericardial effusion	4
Sample used to detect mutations	
Sputum	4
Pleural effusion	11
Bronchial washing	7
Paraffin embedded tissue	7
Type of mutations	
Deletion	18
L858R	10
L861Q	1

*One patient did not receive gefitinib due to pneumonia and rapid disease progression.
†All ≥ 80 years of age.
‡Includes one patient with stage IIIA disease and one patient with postoperative recurrence.

Table 2. Response to Treatment

Response	No. of Patients	Response Rate (%)	90% CI
Complete response	1	3	—
Partial response	18	62	—
Stable disease	7	24	—
Progressive disease	2	7	—
Not assessable	1	3	—
Overall response	19	66	51 to 80
Disease control rate	26	90	80 to 99

Improvement of PS

F3 Figure 3 shows the change in PS for each patient during this study. Twenty-three (79%) of 29 patients (90% CI, 67% to 92%) had improved PS after gefitinib treatment. This improvement was highly significant by Wilcoxon signed rank test ($P < .00005$). In particular, an improvement from PS 3 to 4 at baseline to PS 0 to 1, which we considered clinically valuable, was observed in 68% of patients. Some patients with PS 4 experienced a dramatic improvement in systemic advanced disease shortly after the initiation of gefitinib treatment.

Toxicity

The most frequent adverse event with severity of grade 2 or worse was AST/ALT elevation (Table 3), and all affected patients improved T3 within a few months of modification of the treatment. One patient

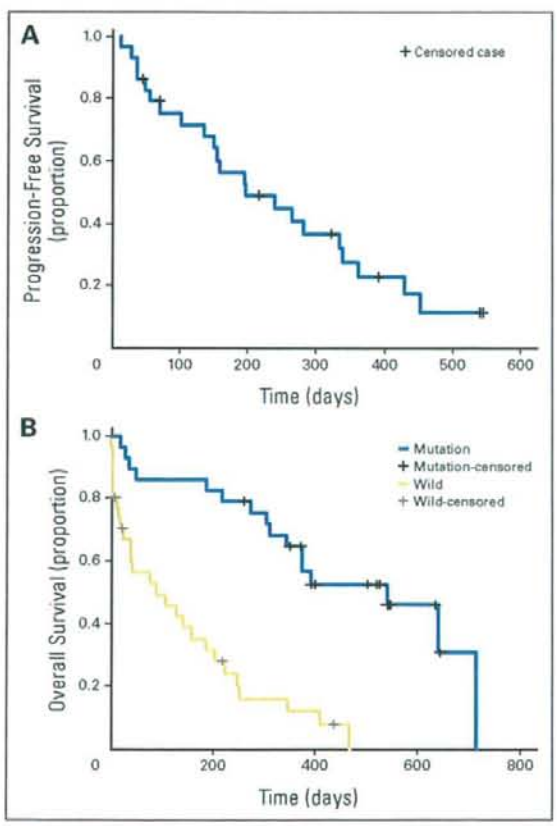

Fig 2. Progression-free survival (PFS) and overall survival (OS) of patients. (A) PFS of all patients in the study. (B) OS for all the patients with sensitive epidermal growth factor receptor (*EGFR*) mutations (green line). Vertical bars indicate censored cases at the data cutoff point. The median PFS, median survival time, and 1-year survival rate of the patients with sensitive *EGFR* mutations were 6.5 months, 17.8 months, and 63%, respectively. B also shows a survival curve of the 31 patients without *EGFR* mutations (red line; Fig 1). Their median survival time was 3.5 months.

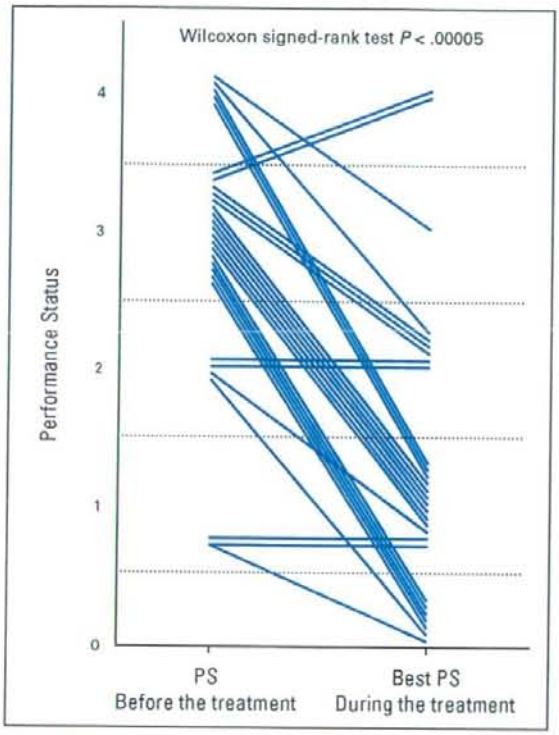

Fig 3. Change of performance status of each patient during treatment. Each line shows the change of performance status (PS) of a patient from baseline to best status during the treatment. A clinically valuable improvement in 68% of patients was observed (ie, improvement from PS 3 to 4 at baseline to PS 0 to 1).

Table 3. Grade 2 or Worse Adverse Events

Toxicity	No. of Patients				
	Grade 2	Grade 3	Grade 4	Total With ≥ Grade 3 Toxicity	
				No.	%
Hematologic					
Anemia	3	2	0	2	7
Neutropenia	2	0	0	0	
Nonhematologic					
Pneumonitis	0	0	1	1	3
AST/ALT	4	3	0	3	10
Anorexia	0	1	0	1	3
Rash	4	0	0	0	
Diarrhea	2	0	0	0	
Hypoalbuminemia	2	0	0	0	
Vomiting	1	0	0	0	
Pain	1	0	0	0	
Hyperkalemia	1	0	0	0	

experienced grade 4 interstitial lung disease that resolved after corticosteroid pulse therapy (methylprednisolone 1 g/d for 3 days). Most of the other toxicities observed were mild or moderate. There was no treatment-related death. Two patients died within 30 days after initiation of gefitinib as a result of rapid disease progression. Toxicity was comparable to that observed in patients with PS 0 to 2 in the previous studies and was considered acceptable.

DISCUSSION

There have been few previous reports of successfully treating patients with advanced NSCLC and extremely poor PS (especially PS 3 to 4). A recent randomized trial comparing gefitinib with BSC for unselected AQ: I patients with NSCLC having poor PS also did not show a clinical benefit.[25] By selecting patients with sensitive *EGFR* mutations, the present phase II study of first-line gefitinib for patients with NSCLC having poor PS could be the first to demonstrate promising efficacy.

The ORR (66%) was comparable to that observed when gefitinib was given to *EGFR* mutation-positive patients with PS 0 to 2.[14-19] Median PFS (6.5 months) was markedly better than that expected in patients managed with only BSC.[25] The PFS in this study was even better than that reported previously for standard chemotherapy in patients with PS 2, and that observed in unselected patients with PS 2 AQ: J treated with erlotinib (median PFS, 1.9 to 2.9 months).[26,27,30]

The PS results were also remarkable because they met the important study end point for improvement of PS, which suggests the clinical usefulness of gefitinib for this population. A clinically valuable improvement in 68% of patients was observed (ie, they improved from PS 3 to 4 at baseline to PS 0 to 1). Namely, these patients enjoyed the rest of their lives with a good quality of life instead of spending the end of their lives in bed. As for other secondary end points, MST of patients who had sensitive *EGFR* mutations and were treated with gefitinib (17.8 months) was markedly longer than that of 31 patients without *EGFR* mutations (3.5 months; Fig 2B). The results also indicated that the MST of 17.8 months was much longer than that of patients managed with BSC alone,[25] as well as that of patients with PS 2 treated with standard chemotherapy (MST, 8.0 months; 1-year survival rate, 31%).[26,27] Although there is little evidence on prognosis of untreated patients with NSCLC having *EGFR* mutations, we consider that even if *EGFR* mutated tumors have somewhat indolent behavior, MST of more than 1 year is a rare and represents a promising result for patients with advanced NSCLC having poor PS.

Although some studies have indicated that clinical factors, including adenocarcinoma and/or nonsmoker status, are enough to identify a responder to gefitinib,[28] there is much evidence that a number of patients with nonadenocarcinomas or who are heavy smokers also have *EGFR* mutations and show a response to gefitinib.[11-13] In the present study, if these clinical factors had been used in the eligibility criteria, 11 (38%) of 29 patients would not have been treated with gefitinib. Assessment of *EGFR* mutation status before treatment is thus a reasonable strategy to increase the accuracy of predicting a response. Stressful examination procedures such as open-lung biopsy are often contraindicated in patients with poor PS or who are frail and older in age. The present study found that the PNA-LNA PCR clamp method, which has been commercially available in Japan for more than 2 years (US$200), could detect both sensitive and insensitive *EGFR* mutations from patients with poor PS (Table 1). With this test, the strategy of first-line gefitinib is readily applicable to the clinical setting.

Currently, patients ≥ 80 years of age with advanced NSCLC are often treated only with BSC, because no standard chemotherapy regimen has been established for such older patients. Although the number of ≥ 80-year-old patients treated with first-line gefitinib was small in this study, the efficacy observed was at least comparable to that achieved with standard chemotherapy regimens given to younger patients, which indicates that a large-scale study of first-line gefitinib for *EGFR* mutation-positive older patients is warranted. In contrast, we are conducting a phase III trial to compare first-line gefitinib with standard chemotherapy, carboplatin and paclitaxel, for patients with NSCLC and PS 0 to 2 with *EGFR* mutation. However, in patients with sensitive *EGFR* mutations with extremely poor PS, the difference in benefit with or without gefitinib treatment is so marked that a randomized phase III study to compare gefitinib with BSC alone may not be justified. Additional studies to confirm our results are considered to be appropriate.

In conclusion, *EGFR* mutation-positive patients with extremely poor PS benefit from first-line gefitinib. Previously, these patients have usually been treated with BSC alone because no standard treatment has been established. Examination of *EGFR* mutations as a biomarker is strongly recommended for these patients, because it identifies patients for whom salvage therapy with gefitinib may be beneficial, which is especially relevant to Asian and South European patients with advanced NSCLC who have high possibilities of harboring *EGFR* mutations.[29]

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest. AQ: K

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A—AUTHOR: No affiliations were included with the manuscript. I have added the affiliation for the corresponding author, but if other authors are located at other institutions, please add those institutions to the footnote, including department, institution, city, and country.

B—AUTHOR: Per Journal style, information about the group was removed from the byline and added as a footnote.

C—AUTHOR: Correct to italicize EGFR throughout as a gene? (Per Journal style, all genes, oncogenes, and proto-oncogenes should be italicized. Proteins, antigens, and receptors should not be italicized.)

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