polymicrobial UTI and catheter-unrelated monomicrobial UTI, respectively, (Fisher's exact test: $P = 0.0020$).

Discussion

Enterococci are an important cause of nosocomial infections [3-5]. Although *E. faecalis* in the urinary tract rarely causes serious infectious symptoms, the frequency of isolation of *E. faecalis* from the urinary tract of hospitalized patients has risen [6-8]. One of the reasons, we suspect, is that the number of patients with various urinary stents and catheters is increasing with the progress of endourology in the urology ward. Therefore, it is important to understand biofilm formation and the pathogenicity of *E. faecalis* infections in the urinary tract. Biofilms are surface-associated, sessile bacterial communities. A mature biofilm is formed when planktonic cells initially colonize a surface, aggregate and/or grow into multicellular colonies, and embed themselves in an exopolysaccharide matrix [29, 30]. Enterococci have been associated with biofilms on various kinds of indwelling medical devices [29]. An understanding of the bacterial factors that foster enterococci in the nosocomial environment or at infection sites is only recently emerging.

The incidence of virulence factors in *E. faecalis* clinical isolates has been studied [31-33]. In 1995, Coque *et al.* [31] reported that frequencies of Hln, Gel and *asa1* in *E. faecalis* urine isolates were 13, 53 and 67 %, respectively. In 2002, Vergis *et al.* [32] reported that frequencies of Hln, Gel and *esp* in *E. faecalis* blood isolates were 11, 64 and 32%, respectively. In our study, frequencies of Hln, Gel, *asa1* and *esp* in *E. faecalis* urine isolates were 63/352 (17.9%), 167/352 (47.4%), 291/352 (82.7%) and 254/352 (72.2%), respectively. Our data indicated that *E. faecalis* isolates possessing both *asa1* and *esp* were predominant. As shown in Fig. 2, *E. faecalis* isolates possessing *asa1* were more likely to contain *cylA* and/or *aac(6')/aph(2")*. This may be due to the expression of *asa1*-encoded Agg facilitating the subsequent exchange of genetic material between *E. faecalis* isolates. The additional presence of virulence factors may enhance the ability of pathogenic *E. faecalis* to persist in the clinical environment.

Horizontal gene transfer is important for the evolution and genetic diversity of natural microbial communities [34]. The prevalence of plasmids in bacteria from diverse habitats is well established, and gene transfer by conjugation is one of the best understood mechanisms for dissemination of genetic information. Since most bacteria in natural settings reside within biofilms, it follows that conjugation is a likely mechanism by which bacteria in biofilms transfer genes within or between populations. In this study, we chose *E. faecalis* isolates possessing the 3 genes *asa1*, *cylA* and *aac(6')/aph(2")* to examine gene transfer from one *Enterococcus* to another. These 3 genes have been reported to be encoded on pheromone-responsive *E. faecalis* plasmids [23, 35]. Our data indeed demonstrated the existence of highly conjugative virulence genes and antimicrobial resistance genes in *E. faecalis* isolates from patients with UTI.

With regard to biofilm formation, there were contrasting reports on the role of Esp and Gel. A strong correlation between the presence of Esp and the ability of an enterococcal strain to form biofilms *in vitro* has been reported [13]. In the same study, however, it was suggested that additional determinants in *E. faecalis* may also contribute to biofilm formation. More recently, Kristich *et al.* [14] demonstrated that an *esp*-negative strain can form biofilms on abiotic surfaces independently of Esp. Mohamed *et al.* [15] also demonstrated that *esp* was not required to form biofilm, but that its presence was associated with higher amounts of biofilm. In the same study, several genes of *E. faecalis* that influenced primary attachment and biofilm formation (*epa*, *atn*, *gelE*, and *fsr*) were identified. Most recently, Tendolkar *et al.* [17] defined Esp as a key contributor to the ability of *E. faecalis* to form biofilms in a glucose-dependent manner. In addition, Kristich *et al.* [14] reported that Gel enhanced biofilm formation by *E. faecalis*, whereas Tendolkar *et al.* [17] did not find a synergistic effect between Gel and Esp on biofilm formation. In our study, biofilm-forming capacities were significantly higher in *esp*-positive isolates than in *esp*-negative isolates (Table 3). On the other hand, there were no significant differences between *gelE/sprE*-positive, Gel producing isolates and *gelE/sprE*-negative, Gel non-producing isolates on biofilm-forming capacities (Table 3).

In our study, *cylA*-positive, Hln producing *E. faecalis* isolates formed biofilms at rates significantly higher than those of *cylA*-negative, Hln non-producing isolates ($P = 0.0116$ and $P = 0.0384$, respectively). To our knowledge, there has been no report on Cyl of *E. faecalis* implicating it in biofilm formation. Caiazza *et al.* [36] showed that Hla, a 34-kDa protein of *Staphylococcus aureus* that causes host cell lysis by heptamerizing upon insertion into eukaryotic cell membranes, plays a

primary role in cell-to-cell interactions during biofilm formation. They were initially surprised to find that a secreted toxin had such a dramatic impact on biofilm formation. More recently, we showed that the biofilm-forming capacities of MRSA isolates were higher in *hla*- and *hlb*-positive isolates than in *hla*- and *hlb*-negative isolates, respectively [37]. These toxins may be bifunctional enzymes and cause tissue damage of urinary epithelium.

The *fsr* quorum-sensing system has been shown to regulate 2 proteases, Gel and serine proteases [1, 10–12]. More recently, Hancock *et al.* [16] showed that the *E. faecalis fsr* quorum-sensing system controls biofilm development through the production of Gel. However, our data do not support this finding since Gel non-producing isolates can form biofilms (Table 3). In our previous study [24], a 23.9-kilobase chromosomal deletion containing the *fsr* gene cluster region was found to be present in the majority of Gel non-producing isolates. An understanding of the process of biofilm formation by *E. faecalis* is only now beginning to emerge, and the results appear to be contradictory [38].

In this study, there were no statistically significant differences between biofilm-forming capacities and clinical background (catheter-related and catheter-unrelated cases, polymicrobial and monomicrobial cases, febrile and non-febrile cases). Biofilm formation by enterococci occurs not only with indwelling devices but also in response to any bacterial factor that mediates adherence to components of the extracellular matrix of the host [1, 39, 40]. No single factor predominated as the major predictor of virulence, and their effects appeared to be cumulative [37]. The relative importance of host factors versus bacterial virulence determinants in disease pathogenesis is unknown. Host factors for *E. faecalis* disease are likely to include a genetic predisposition via one or more susceptibility genes and acquired factors such as the presence of intravenous devices, surgical wounds, and other events that perturb normal host defenses.

In summary, our study indicates that *E. faecalis* isolates that have accumulated virulence genes are apt to form persistent biofilms in the urinary tract.

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Original Article

Synergistic Effect of Fosfomycin and Fluoroquinolones against *Pseudomonas aeruginosa* Growing in a Biofilm

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Ulifloxacin is the active form of the prodrug prulifloxacin and shows a highly potent antipseudomonal activity. In this study, we examined the combined effect of fosfomycin and ulifloxacin against *Pseudomonas aeruginosa* (*P. aeruginosa*) growing in a biofilm using a modified Robbins device with artificial urine, and compared it to that of the combination of fosfomycin and ciprofloxacin or levofloxacin. An ATP bioluminescence assay was used to evaluate the antibacterial activity of the agents against sessile cells in a mature biofilm developed on a silicon disk. The total bioactivity of *P. aeruginosa* growing in a biofilm that had not been fully eradicated by fosfomycin or any of the fluoroquinolones alone at 10 times the MIC decreased after combination treatment with fosfomycin and fluoroquinolones. Morphological changes occurred in a time-dependent fashion; namely, swollen and/or rounding cells emerged within a couple of hours after combination treatment, marking the initial stage in the process leading to the destruction of the biofilms. We could not find any difference among the 3 fluoroquinolones with regard to their synergistic effects when administered with fosfomycin. The combination treatment of fosfomycin and fluoroquinolones with highly potent antipseudomonal activities was effective in eradicating sessile cells of *P. aeruginosa* in the biofilm and promises to be beneficial against biofilm-associated infectious diseases.

Key words: urinary tract infection, *Pseudomonas aeruginosa*, biofilm, ulifloxacin, fosfomycin

Bacterial biofilms play an important role in the development and persistence of various chronic intractable infectious diseases, including catheter-associated urinary tract infections (UTI) [1-4]. The isolation frequencies of *Pseudomonas aeruginosa* (*P. aeruginosa*), a major pathogen in biofilm-associated infection, are also elevated in cases of complicated UTI [5]. The sessile type of *P. aeruginosa* in biofilms is protected by an extracellular polymeric substance (glycocalyx) from various host defense mechanisms and is susceptible to antibiotics at 100 to 1000 times lower levels than equivalent populations of planktonic bacteria [6-8]. The biofilm infection itself is an indolent infection, although the stability of biofilms is a major factor in the persistence of many chronic infections.

Fluoroquinolones not only exert antimicrobial activity against a broad spectrum of organisms isolated from the urinary tract, but they also exert bactericidal activity against non-growing bacteria [8, 9]. In addition, fluoroquinolones show a special killing effect on the sessile cells

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of *P. aeruginosa* growing in mature biofilms because of their penetrability through exopolysaccharides [8, 10]. However, the number of favorable cases is much smaller than might be expected. Fosfomycin (FOM: 1*R*-2*S*-epoxypropylphosphonic acid) is a widely prescribed antibiotic with a unique chemical structure effective against a broad spectrum of microbials [11]. FOM in combination with ofloxacin (OFLX) has been reported to exert clear synergistic effects against sessile cells of *P. aeruginosa* growing in biofilms, but combinations of FOM with other fluoroquinolones have not yet been studied [12, 13].

Ulifloxacin (UFX) is the active form of the prodrug prulifloxacin, a new fluoroquinolone antibacterial agent with a highly potent antipseudomonal activity [14]. UFX and ciprofloxacin (CPFX) have exhibited far stronger effects than levofloxacin (LVFX) in inhibiting DNA gyrase, the primary target of fluoroquinolones in *P. aeruginosa* [15]. Moreover, UFX is known to be accumulated in *P. aeruginosa* at higher concentrations than CPFX, with LVFX accumulating at the lowest concentration among the 3 agents [16]. At present, it remains unclear whether the antipseudomonal activity of fluoroquinolone against floating cells reflects the effectiveness of the eradication of sessile cells of *P. aeruginosa* in biofilms with or without FOM.

In this study, we focused on the combination treatment of FOM and UFX with respect to eradication of sessile cells of *P. aeruginosa* in biofilms using a modified Robbins device with artificial urine. At the same time, we observed time-dependent morphological changes by scanning electron microscopy (SEM) to assess the process leading to the destruction of the biofilms.

Materials and Methods

P. aeruginosa OP14-210 isolated from a patient with a catheter-associated UTI was used throughout this study [12, 13]. UFX is an active metabolite of PUFX and was provided by Nihon Shinyaku, Ltd. (Kyoto, Japan). FOM was supplied by Meiji Seika Kaisha (Tokyo, Japan). CPFX and LVFX were purchased from Sequoia Research Products Ltd. (Oxford, United Kingdom).

In the present study, the minimum inhibitory concentration (MIC) of each agent against *P. aeruginosa* OP14-210 was determined by the macrodilution tube broth method with a final inoculation of 5×10^5 colony-forming units (CFU)/ml using artificial urine supplemented with 0.4% (w/v) nutrient broth (AUB) (EIKEN CHEMICAL CO., LTD., Tokyo, Japan) or Mueller-Hinton broth (MHB) (DIFCO, BECTON DICKINSON, Sparks, MO, USA) [13]. The minimum bactericidal concentration (MBC) of each antibiotic using AUB was deemed to have been achieved when the number of CFUs per milliliter was $< 99.9\%$ compared with the initial inoculum size [17]. We studied the activities of FOM in combination with each fluoroquinolone against floating cells of *P. aeruginosa* OP14-210 by the checkerboard method to calculate a fractional inhibitory concentration (FIC) index using AUB. The results were interpreted as synergism, addition, indifference, or antagonism when the FIC indices were ≤ 0.5, 0.5 to 0.75, 1 to 4, and > 4, respectively [18].

The culture conditions for production of an adherent biofilm were essentially identical to those reported previously by Kumon *et al.* [13]. Briefly, AUB containing logarithmic-phase *P. aeruginosa* OP14-210 was pumped from a reservoir through a modified Robbins device by a peristaltic pump set to deliver 40 ml/h. After 16 h of contact with *P. aeruginosa* OP14-210 at time zero for the treatment period by antimicrobial agents, a thick biofilm developed on 10-mm silicon disks (Create Medic, Yokohama, Japan) in the device. At time zero, AUB containing *P. aeruginosa* was exchanged to AUB containing FOM, UFX, CPFX, or LVFX alone or a combination of FOM plus either UFX, CPFX, or LVFX at appropriate concentrations, and flowed through the modified Robbins device at 40 ml/h during the treatment period. Disks were removed from the device at 2, 4, 8, 24 and 48 h.

Instead of viable cell counts, an ATP bioluminescence assay was used as previously reported [19]. Briefly, silicon disks were removed, washed with distilled water, boiled at 100 °C for 8 min with 500 μl of distilled water, and subjected to ultrasonication, followed by centrifugation at 15,000 rpm for 10 min. Supernatants were kept at − 80 °C until use. For quantification of ATP contents, we used ATP Assay System LL-100-1 (TOYO B-Net, Co., LTD., Tokyo, Japan) with Fluoroskan Ascent FL L-5210520 (Labsystems, Helsinki, Finland). All assays were performed with 2 disks, and the values shown are the means of duplicate experiments.

The cells on each disk were fixed with 2.5% glutaraldehyde in phosphate-buffered saline, post-fixed with 2% tannic acid and 1% OsO_4, and dehydrated through an ethanol series. The specimens were then dried in a critical-point dryer (HCP-II: Hitachi, Tokyo, Japan),

coated with platinum-palladium, and observed with a JSM-6300F scanning microscope (JEOL DATUM LTD, Tokyo, Japan).

Results

Table 1 summarizes the MIC and MBC of FOM, UFX, CPFX and LVFX against floating cells of *P. aeruginosa* OP14-210 in AUB or MHB, as well as the results of checkerboard studies of the FOM-UFX, FOM-CPFX, and FOM-LVFX combinations. The effect of the FOM-fluoroquinolone combination against *P. aeruginosa* floating cells was additive (with FIC indexes between 0.5 and 0.75).

None of the fluoroquinolones alone at 10 times the MIC resulted in a detectable decrease in the total bioactivity of sessile *P. aeruginosa* OP14-210 in a mature biofilm, nor did FOM alone (Fig. 1A, B). In the case of UFX and LVFX, there was no effect even at 100

Table 1 Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and fractional inhibitory concentration (FIC) index of fosfomycin (FOM), ulifloxacin (UFX), ciprofloxacin (CPFX), and levofloxacin (LVFX) against *P. aeruginosa* OP14-210 in Mueller-Hinton broth (MHB) or artificial urine supplemented with 0.4% nutrient broth (AUB).

Drug	MIC (μg/ml)		MBC (μg/ml)	FIC index (combined with FOM)
	MHB	AUB	AUB	AUB
FOM	32	64	128	—
UFX	0.25	2	4	0.75
CPFX	0.25	4	16	0.75
LVFX	1	8	16	0.56

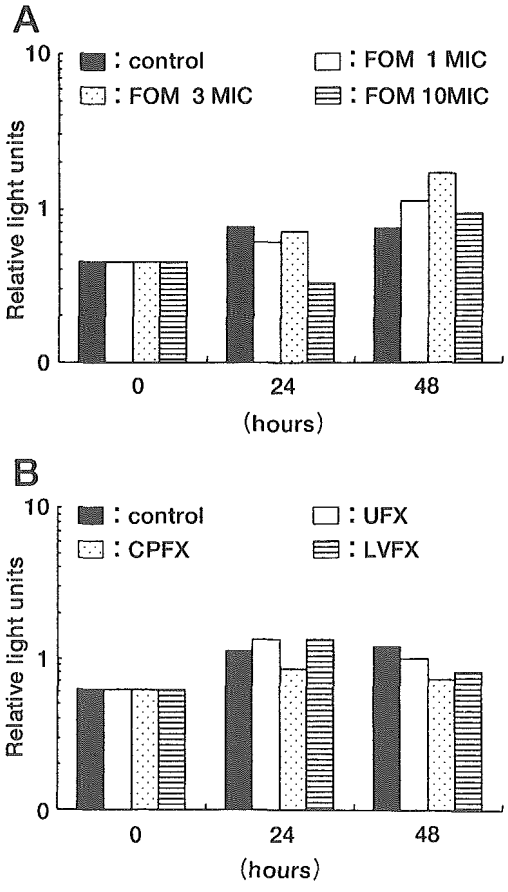

Fig. 1 Kinetics of *P. aeruginosa* eradication in a mature biofilm by (A) FOM at either 1 ×, 3 ×, or 10 × MIC, (B) either UFX, CPFX, or LVFX at 10 × MIC. The values are the means of duplicate experiments.

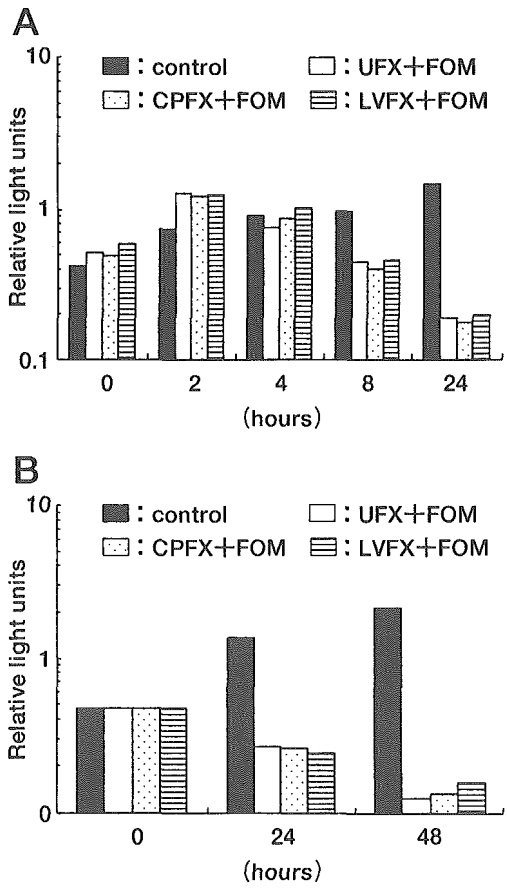

Fig. 2 Kinetics of *P. aeruginosa* eradication in a mature biofilm by either UFX, CPFX, or LVFX at 10 × MIC plus FOM at 3 × MIC. (A) within 24 h, (B) within 48 h. The values are the means of duplicate experiments.

times the MIC against biofilms (data not shown). However, the combination treatment of FOM and any of the 3 fluoroquinolones resulted in a decrease of total bioactivity of sessile cells in biofilms at the same concentrations at which the drugs had not been effective alone (Fig. 2A, B). There was no difference among the 3 fluoroquinolones in regard to their synergy with FOM. In the case of combination treatment with UFX and FOM, the ATP content of biofilm cells decreased in an FOM dose-dependent fashion (Fig. 3).

Fig. 4 shows ultrastructural changes of sessile cells of *P. aeruginosa* in a mature biofilm at 48 h after treatment with UFX and/or FOM. The presence of bloated cells was characteristic of treatment with FOM (Fig. 4B), and elongated and swollen cells were observed after treatment with UFX (Fig. 4 C). Swollen and/or bloated cells accompanied with destruction of the biofilms were observed after combination treatment (Fig. 4D). These morphological changes were observed within a couple of hours after the combination treatment (Fig. 5). Similar morphological changes were observed when sessile cells in biofilms were treated with FOM plus CPFX or LVFX, instead of UFX (Fig. 6).

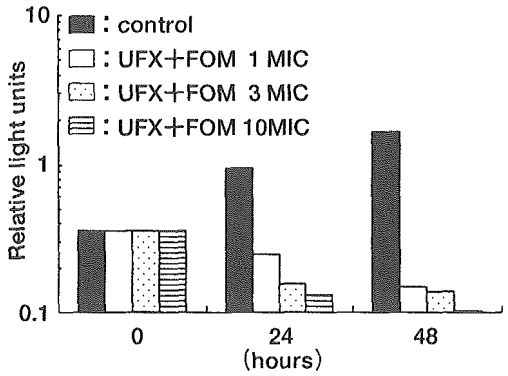

Fig. 3 Kinetics of *P. aeruginosa* eradication in a mature biofilm by UFX at 10 × MIC plus FOM at either 1 ×, 3 ×, or 10 × MIC. The values are the means of duplicate experiments.

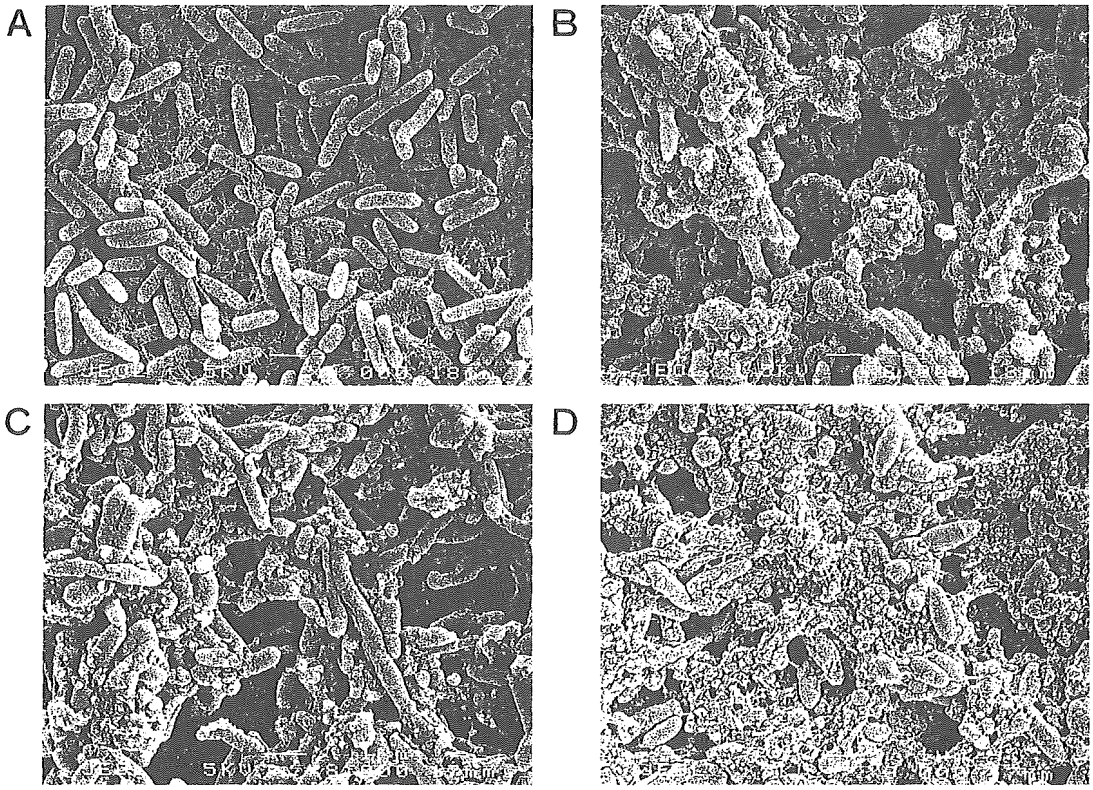

Fig. 4 Morphological changes of *P. aeruginosa* in a mature biofilm at 48 h after treatment with UFX and/or FOM. SEM, original magnification, × 8,000; Bar = 1 μm. A, control; B, FOM 3 × MIC; C, UFX 10 × MIC; D, UFX 10 × MIC plus FOM 3 × MIC.

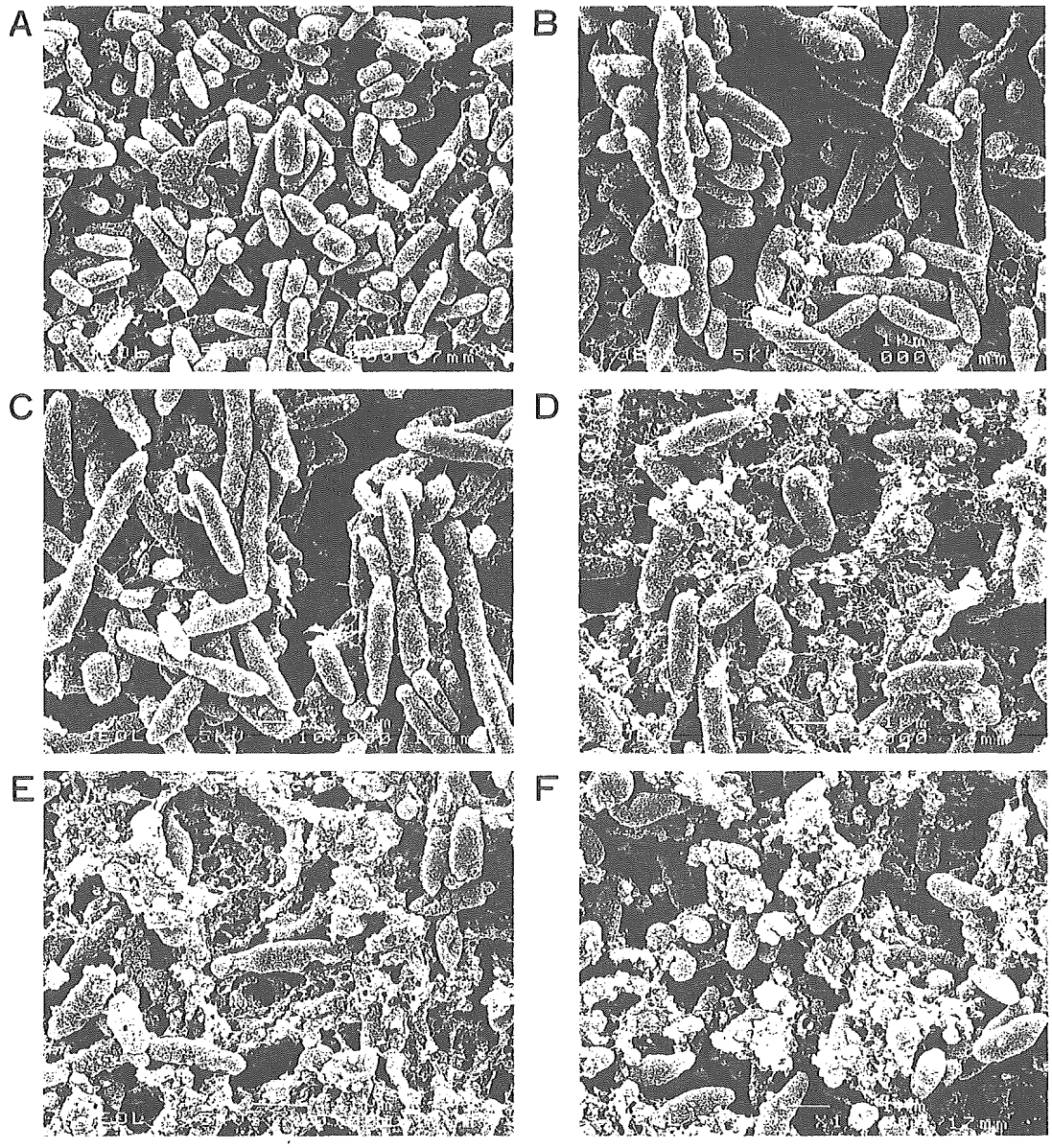

Fig. 5 Morphological changes of *P. aeruginosa* in a mature biofilm observed within 24 h after treatment with UFX 10 × MIC plus FOM 3 × MIC. SEM, original magnification, × 10,000; Bar = 1 μm. A, 0 h; B, 2 h; C, 4 h; D, 6 h; E, 8 h; F, 24 h.

Discussion

As the use of implant devices increases, the risk of biofilm infection tends to increase [3]. The isolation frequencies of non-uropathogenic bacteria which would not normally cause infection, like *P. aeruginosa*, have increased. *P. aeruginosa* biofilms are detected on the surface of indwelling catheters, calculi, scar tissue produced by endoscopic surgery or necrotic tissue associated with urothelial tumors in the case of complicated urinary tract infections [5].

In a short period, *P. aeruginosa* is capable of invading and adhering to the urinary tract to form a biofilm, accompanied with changes of gene expression. In the initial cell attachment phase, for example, alginate (exopolysaccharide) synthesis is up-regulated within a couple of minutes after adhesion to a solid surface [20]. Recently, it was observed that the expression of specific

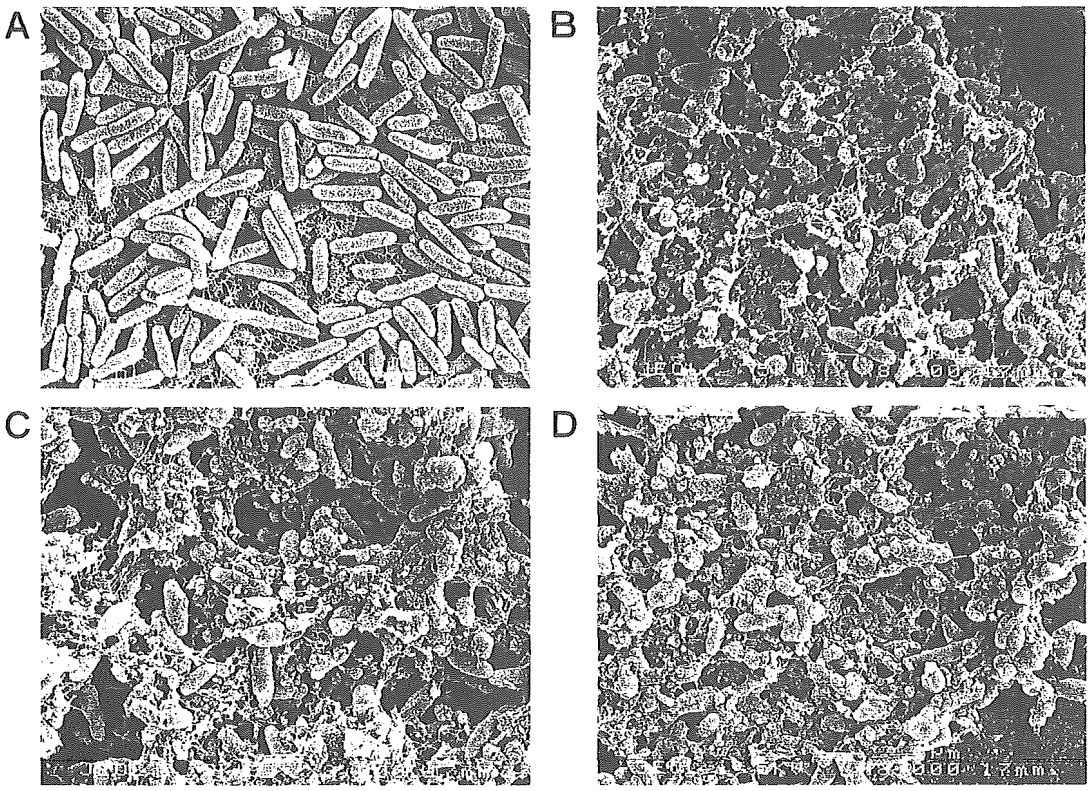

Fig. 6 Morphological changes of *P. aeruginosa* in a mature biofilm at 48 h after treatment with either UFX, CPFX, or LVFX plus FOM. SEM, original magnification, × 8,000; Bar = 1 μm. A, control; B, UFX 10 × MIC plus FOM 3 × MIC; C, CPFX 10 × MIC plus FOM 3 × MIC; D, LVFX 10 × MIC plus FOM 3 × MIC.

genes associated with biofilm formation was controlled by a quorum-sensing system, thereby emphasizing the significance of cell-to-cell interactions [21, 22].

Fluoroquinolones show a considerable effect on *P. aeruginosa* biofilms; however, it is still limited and insufficient, leading to failure of the clinical therapy as incomplete eradication means an easy return to the previous condition. We also failed to destroy the biofilms completely even after 48 h of treatment with UFX or LVFX at 100 times the MIC. Furthermore, the antimicrobial activities of some agents are sometimes reduced due to the biological characteristics of biofilms. Namely, cationic agents like aminoglycosides, which show a critical antimicrobial activity against floating bacteria, would be trapped by the anionic polysaccharide matrix, reducing the concentration of the free drug [23, 24]. In addition, aminoglycosides are less effective under the anaerobic condition within biofilms, compared to their efficacy under aerobic conditions [25].

Antimicrobial agents are not yet sufficiently effective against biofilm infection, especially in the chronic indolent phase. As things now stand, the only effective method of treatment is to correct the obstruction and directly remove the biofilm. In this regard, Kumon *et al.* demonstrated the significant effects of a combination treatment by OFLX and FOM against biofilms using a modified Robbins device *in vitro* [12, 13]. In pursuit of a more efficient method to eradicate sessile cells of *P. aeruginosa* in biofilms, we therefore evaluated the combination effects of FOM and UFX, which possesses a highly potent antipseudomonal activity.

In this study, we demonstrated the equivalent synergistic effects of UFX, CPFX, or LVFX plus FOM against sessile cells in a biofilm. Importantly, synergistic effects were confirmed at a concentration easily achievable in the urine of patients treated with clinical oral doses of these drugs. The urinary concentration of these agents just before the next administration was more than 10 times the MIC against *P. aeruginosa* OP14-210 [26-29]. In addition to the 3 fluoroquinolones studied here,

reports have demonstrated that other fluoroquinolones predominantly excreted via the kidney (*e.g.*, fleroxacin) acted synergistically with FOM against floating cells of *P. aeruginosa* [30]. At present, however, it is not clear whether these other combinations would exercise the same effect against sessile cells of *P. aeruginosa* in a biofilm.

The mechanism behind the synergistic effect of FOM and fluoroquinolones remains unknown. In preliminary experiments, we observed that treatment of UFX with the enantiomer of FOM with no bactericidal activity did not elicit any significant decrease of the ATP contents of bacteria growing in a biofilm (data not shown). Under the anaerobic conditions of cells embedded in a biofilm, the levels of *sn*-glycerol 3-phoshate transport, the transport system that delivers FOM into bacterial cells, will increase [31]. Therefore, FOM is still transported into cells in the stationary phase and can be expected to provide a potential effect against sessile cells with low growth rates. We also confirmed that FOM did not react with the negatively charged bacterial glycocalyx, implying that FOM is able to penetrate deeply into the multilayers of the biofilms [8].

As a general role in Gram-negative organisms, hydrophilic quinolones cross the outer membrane through porins while hydrophobic quinolones appear to enter via lipopolysaccharides (LPS) or cross the lipid bilayer [32]. Increased susceptibility to hydrophobic quinolones has been described in LPS-defective mutants. On this basis, we can postulate that the disruption and/or break of the outer membrane by FOM accelerates the quinolone uptake by passive diffusion [32]. In contrast, the hydrophilic fluoroquinolones UFX and CPFX do not alter the antimicrobial activity against LPS-defective mutants [33]. As *P. aeruginosa* initially accumulates these hydrophilic fluoroquinolones at higher concentrations than it accumulates LVFX, it may be possible to accelerate the accumulation in the presence of FOM. These observations suggested that the bactericidal activity of the combination of FOM and fluroquinolones might be sufficient to completely eradicate the sessile cells in a biofilm.

In the case of indolent infection with biofilm diseases, in general a sudden elevation of the pressure in the urinary tract caused by a mechanical obstruction introduces planktonic cells into the renal parenchyma and blood vessels, despite the presence of mucosal host defense systems [5, 34]. Under these severe life-threatening conditions, which are uroseptic, selective use of carbapenems as a potential empiric antibiotic is justified.

However, treatment with a carbapenem alone would fail to completely destroy the biofilm, even if it could be rescued. Furthermore, insufficient eradication of biofilms would cause repeated life-threatening infections. Combination therapy using fluoroquinolones and FOM after carbapenem treatment appears to be effective in completely eradicating biofilms and promises to be of much help in obtaining satisfactory results against biofilm-associated infectious diseases.

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第39回
緑膿菌感染症研究会
講演記録

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緑膿菌バイオフィルムに対するフルオロキノロン系薬とホスホマイシンの併用効果

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I. はじめに

カテーテル留置複雑性尿路感染症をはじめとする慢性感染症において、細菌バイオフィルムの存在はその難治性の要因となっている。我々は、複雑性尿路感染症に対する *in vitro* の実験モデル系として、従来、岡山大学式ロビンスデバイスを用いて、治療実験を重ねてきた[1-3]。また、新しいバイオフィルム実験モデル系であるキャピラリーフローセルシステムを用いて、抗菌薬の有効性評価に関する実験も開始した[4]。今回は、それぞれの実験系で得られた新しい知見を報告する。

II. 対象と方法

1. 使用菌株および抗菌剤

カテーテル留置複雑性尿路感染症患者由来の緑膿菌 *Pseudomonas aeruginosa* OP14-210 株を用いた。今回はフルオロキノロン系薬のなかで、新規のプルリフロキサシンに着目して、シプロフロキサシン(CPFX)とレボフロキサシン(LVFX)との比較検討をロビンスデバイスの実験系で行い、さらにホスホマイシン (FOM) との併用効果についても検討を加えた。本実験ではプルリフロキサシンの活性本体であるウリフロキサシンを用いたので、以下 UFX と記載した。キャピラリーフローセルシステムでは、LVFX と FOM の併用効果について検討を加えた。人工尿における浮遊菌に対する各種抗菌薬の MIC は、UFX 2 μg/ml、CPFX 4 μg/ml、LVFX 8 μg/ml、FOM 64 μg/ml であった。

2. ロビンスデバイスの実験系

Pseudomonas aeruginosa OP14-210 株を人工尿中で 37℃、4 時間静置培養後、ロビンスデバイスに 37℃、16 時間、40 ml/hr で灌流させ、シリコンディスク上にバイオフィルムを形成させた。薬剤無添加と薬剤作用後のシリコンディスク上のバイオフィルムについて、ATP 量の変化を指標にして薬効を評価した。ATP 測定はシリコンディスク上のバイオフィルムを超音波で破砕後、菌の総 bioactivity を反映する ATP 量を bioluminescence assay で測定した。また走査型電子顕微鏡(SEM)を用いて形態変化を観察した。

3. キャピラリーフローセルシステム

Pseudomonas aeruginosa OP14-210 株の菌液をガラスキャピラリー中に注入して、37℃、2 時間放置したのち、人工尿を 20 ml/hr で灌流させバイオフィルムを形成させた。薬剤無添加と薬剤作用後のバイオフィルムについて、蛍光染色キットを用いて生菌(緑色)と死菌(赤色)を染め分け、共焦点レーザー走査型顕微鏡で観察した。また、GFP(green fluorescent protein) 産生株 *Pseudomonas aeruginosa* OP14-210(pMF230)が形成したバイオフィルムについても検討を行った。

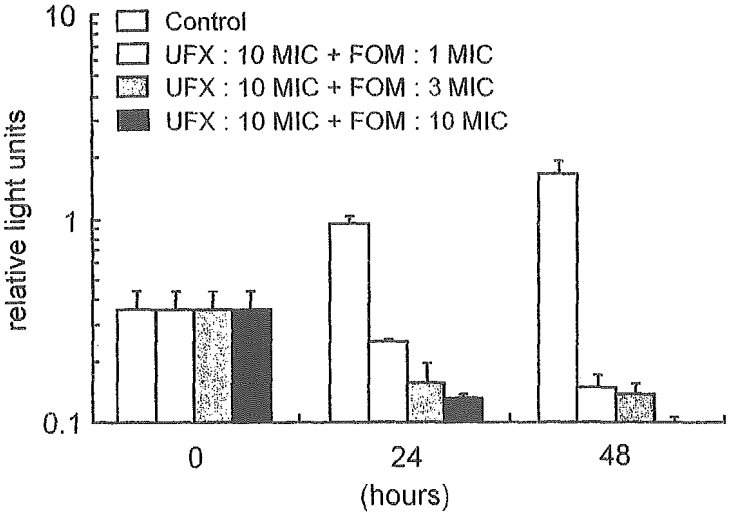

Fig. 1. Synergistic effects of ulifloxacin 10 x MIC plus fosfomycin either 1 x, 3 x, or 10 x MIC against 16-h *P. aeruginosa* biofilms. The values are the means of duplicate experiments

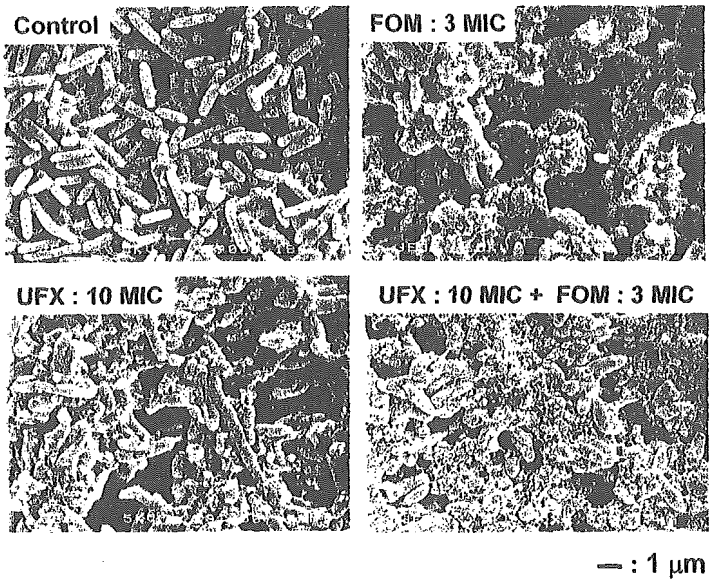

Fig. 2. Morphological changes of 16-h *P. aeruginosa* biofilms after 48-h treatment with ulifloxacin and/or fosfomycin. SEM: original magnification, x 8,000

III. 結 果

1. ロビンスデバイスの実験系

10 x MIC の UFX・CPFX・LVFX を単独で、また 1 x ・3 x ・10 x MIC の FOM を単独で作用させた場合、形態的な変化を認めたものの、総 ATP 量は薬剤無添加と同程度であった。それぞれのフルオロキノロン系薬(10 x MIC)に FOM(3 x MIC)を併用すると、いずれの場合も経時的に ATP 量が減少した。UFX(10 x MIC)と FOM(1 x MIC・ 3 x MIC・ 10 x MIC)を併用したところ、FOM の濃度依存的に ATP 量が減少し、併用効果が増強した(Fig. 1)。形態的には、UFX(10 x MIC)を単独で 48 時間作用させると伸長化・膨潤化した菌、FOM(3 x MIC)を単独で 48 時間作用させると球形化した菌が観察された(Fig. 2) 。UFX (10 x MIC)と FOM(3 x MIC)を 48 時間併用させると、膨潤化・球形化した菌を認めたが、伸長化した菌は観察されなかった (Fig. 2)。また、CPFX(10 x MIC)

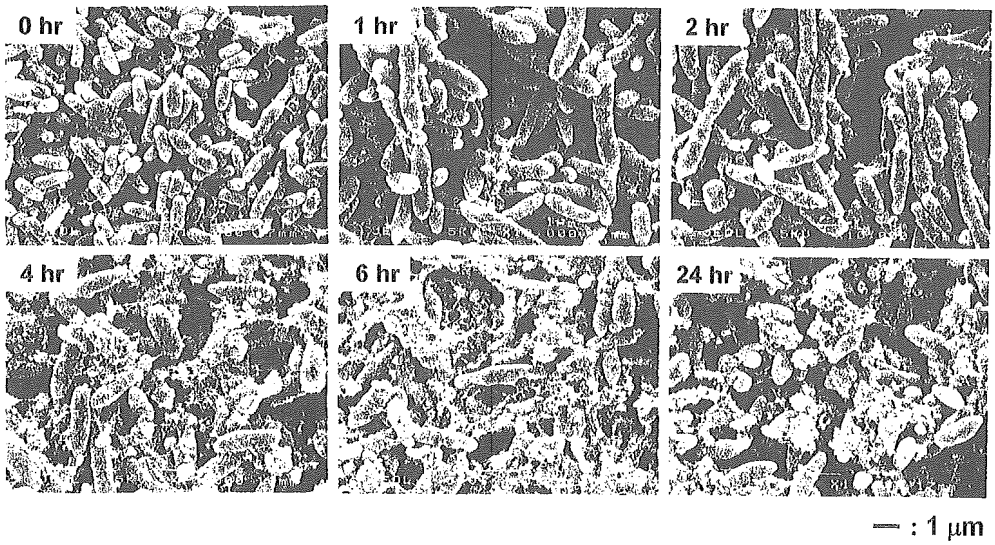

Fig. 3. Morphological changes of 16-h *P. aeruginosa* biofilms observed within 24 h after treatment with ulifloxacin 10 x MIC plus fosfomycin 3 x MIC. SEM: original magnification, x 10,000

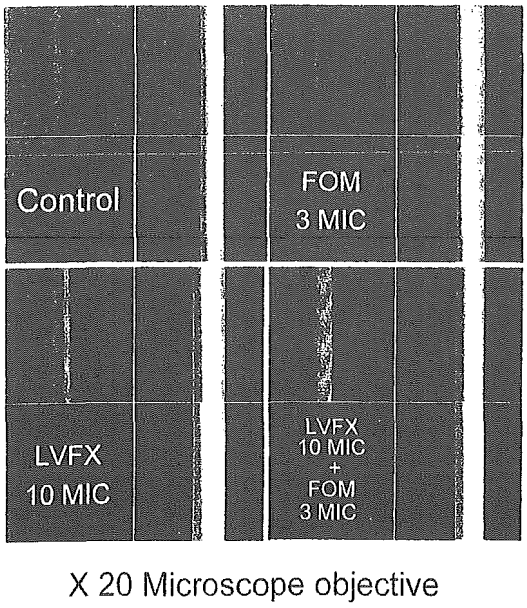

Fig. 4. Reconstructed three-dimensional images of 2-day *P. aeruginosa* biofilms after 18-h treatment in the absence and presence of antimicrobial agents (levofloxacin 10 x MIC and fosfomycin 3 x MIC), either alone or in combination. Confocal laser scanning microscopy (Zeiss LSM 510) was used to examine biofilms after staining with two-color fluorescence. Green and red signals are images for live and dead cells, respectively

およびLVFX(10 x MIC)をFOM(3 x MIC)と併用すると、UFXとFOMの併用の場合と同様に膨潤化・球形化した菌が観察された。UFX(10 x MIC)とFOM(3 x MIC)の両薬剤を作用させた後、短時間での形態変化を観察すると、1時間目から伸長化し、4時間目以降には膨潤化・球形化した菌が観察された(Fig. 3)。

2. キャピラリーフローセルシステム[4](Figs. 4, 5)

ガラスキャピラリー中に形成されたバイオフィルムの厚さそのものは薬剤作用により大きく変化

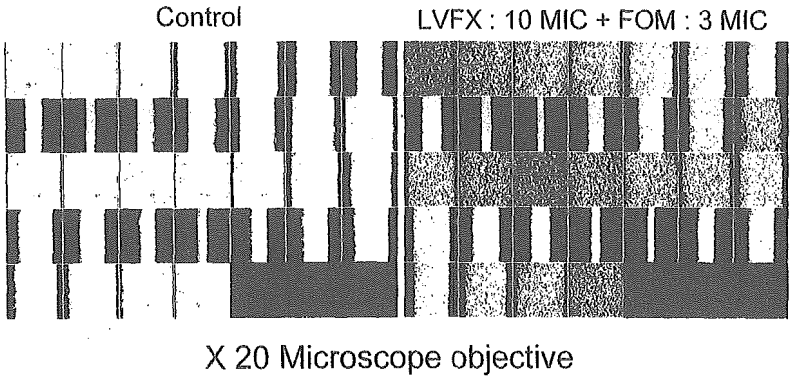

Fig. 5. Reconstructed three-dimensional images of GFP producing 1-day biofilms after 24-h treatment in the absence and presence of antimicrobial agents (levofloxacin 10 x MIC plus fosfomycin 3 x MIC). Confocal laser scanning microscopy (Zeiss LSM 510) was used to examine biofilms

しなかった(50～100 μm)。薬剤無添加では緑色が大部分を占め、生菌の分布が確認された。LVFX (10 x MIC)単独では、死菌の存在を示す赤色がバイオフィルムの浅層部で観察された。FOM(3 x MIC)単独では、薬剤無添加と同程度の緑色であった。LVFX(10 x MIC)と FOM(3 x MIC)併用では、バイオフィルムの深層部まで赤色が観察され、併用効果が認められた。GFP 産生株でも LVFX と FOM の併用効果を認めた。

IV. 考 察

近年、細菌バイオフィルムは臨床各科領域の難治性感染症に関与する病態概念として注目されており、病態の解明や治療方法の開発など基礎的・臨床的研究が展開されている[5,6]。尿路バイオフィルム感染症は、通常臨床症状に乏しく比較的穏やかな感染症であるが、一旦、尿流障害を合併すると敗血症に移行し、宿主を重篤化させる[1-3]。また、除菌が困難であるため感染が持続し、院内感染の感染源となっている。特に、緑膿菌はバイオフィルム形成能が高く、尿路バイオフィルム感染症の主たる原因菌である。細菌バイオフィルムの関与が強い留置カテーテルのある複雑性尿路感染症においては、緑膿菌がもっとも高頻度に分離され、現時点でもっとも監視が必要とされているメタロ-β-ラクタマーゼ産生緑膿菌の分離頻度も増加している。尿路バイオフィルム感染症の持続が宿主とそれを取り巻く環境におよぼす影響を考えると、除菌を目的とした治療方法の考案と積極的な予防策を講じる必要性はきわめて高いと考える。

フルオロキノロン系薬は緑膿菌バイオフィルムに対してある程度の効果を示すものの、単剤での効果は十分であるとは言えない。これまでの岡山大学式ロビンスデバイスを用いた検討では、緑膿菌バイオフィルムに対するオフロキサシンと FOM の併用効果について報告した[7]。また、その作用機序は、細胞壁合成阻害剤である FOM によるフルオロキノロン薬の菌体内取り込み量の増加であることも示した[8]。今回の検討では FOM は LVFX のみならず、UFX、CPFX とも併用効果を示し、その程度は、3 薬剤間で差異のないことが明らかになった。以上の検討において、通常の臨床投与量で十分尿中に到達しうる薬剤濃度で効果が認められたことが重要な点である。

In vitro 実験系は、臨床での併用効果のメカニズム解明と抗菌剤投与計画のエビデンス創出に有用であると考えられる。尿路の細菌バイオフィルムはカテーテル留置、尿路結石、尿路上皮の壊死部などを伴う症例で形成される。カテーテル留置複雑性尿路感染症ではカテーテル留置時と発熱時が化学療法の適応時期であり、経口フルオロキノロン薬は、軽症～中等症例、重症例では注射用抗菌薬による治療後、使用される。フルオロキノロン薬と FOM の併用により短期間に解熱、菌陰性化が得られる可能性があり、今後の有効症例の蓄積が期待される。

以上より、1) 使用した薬剤濃度は、それぞれの

薬剤が通常の臨床投与量で十分尿中に到達しうる濃度であり、緑膿菌バイオフィルムに対して、フルオロキノロン系薬と FOM の併用は有効な治療法となる可能性が示唆された。2) キャピラリーフローセルシステムは、抗菌薬を含む抗バイオフィルム剤開発のための新しい実験・評価系になるものと考えられた。

V. 文 献

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Synergy between Fluoroquinolones and Fosfomycin against *Pseudomonas aeruginosa* Biofilms

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To examine the synergistic effects of antimicrobial agents against sessile cells in a mature biofilm, *in vitro* experimental models of complicated urinary tract infections (UTI) are utilized. *P. aeruginosa* OP14-210 isolated from a patient with catheter-associated UTI was used. Fluoroquinolones (ulifloxacin which is the active form of prodrug prulifloxacin, ciprofloxacin and levofloxacin) and fosfomycin, either alone or in combination, were tested. An ATP bioluminescence assay was employed to evaluate the antibacterial activity of the applied agents against sessile cells in a mature biofilm developed on a silicon disk in a modified Robbins device with artificial urine. Combination treatments with fluoroquinolones at 10 times the MIC plus fosfomycin at 3 times the MIC decreased the total bioactivity of the sessile *P. aeruginosa* in a mature biofilm that had not been fully eradicated by fosfomycin or any of the fluoroquinolones alone at 10 times the MIC. As observed by scanning electron microscopy, swollen and/or rounding cells emerged within a couple of hours after the combination treatment, marking the initial stage in the process leading to the destruction of the biofilms. We could not find any difference among three fluoroquinolones in regard to the synergy with fosfomycin or the kinetics of killing or morphological changes of the sessile cells in the biofilm.

More recently, we began using a capillary biofilm system as an *in vitro* model. *P. aeruginosa* OP14-210 biofilms were grown in glass capillary tubes under continuous flow conditions with artificial urine, and were observed by confocal laser scanning microscopy. BacLight staining was applied to assess the effects of treatment on the number of viable cells, and their distribution in biofilms. A GFP (green fluorescent protein)-producing strain was also used. The thickness of 2-day biofilms did not vary markedly after 18-h treatment with levofloxacin (10 times the MIC; 80 μg/ml) or fosfomycin (3 times the MIC; 192 μg/ml), either alone or in combination. After combined treatment with levofloxacin and fosfomycin, live and dead cells were distributed throughout the vertical profile of the biofilms, while a higher proportion of dead cells was observed in the upper third of the biofilms after treatment with levofloxacin alone. Evenly distributed live cells dominated after no treatment or treatment with fosfomycin alone. GFP-producing 1-day biofilms were irregular after 24-h treatment with both levofloxacin and fosfomycin. Our previous findings regarding the synergy between fluoroquinolones and fosfomycin were confirmed using the capillary biofilm system.

The combination treatment of fosfomycin and fluoroquinolones against *P. aeruginosa* biofilms was effective and would appear beneficial against biofilm-related infections. This synergy was confirmed at concentrations easily achievable in the urine of patients treated with clinical oral doses of these agents.

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腸球菌性尿路バイオフィルム形成に関する検討

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要　旨：尿路感染症における *Enterococcus faecalis* のバイオフィルム形成能と病原性との関連性について検討した．岡山大学泌尿器科で1991年から2002年までの12年間に，複雑性尿路感染症患者より分離された *E. faecalis* 352株（1症例1株）を対象とした．病原性に関与する遺伝子（*asa1*, *esp*, *cylA*, *gelE-sprE*）の保有状況，バイオフィルム形成能，ヘモリジンおよびゼラチナーゼの産生性を検討し，臨床的背景との関連性について検討を加えた．*E. faecalis* 352株のうち315株（89.5%）が，付着に関与するとされる *asa1* もしくは *esp* を保有していた．ヘモリジン産生63株およびゼラチナーゼ産生167株のうち，*asa1* および *esp* 両遺伝子保有株はそれぞれ59株（93.7%），94株（56.3%）であった．*asa1* および *esp* 両遺伝子保有株のバイオフィルム形成能は，いずれも保有しない株に比べて有意に高かった（P = 0.038）．*asa1*, *esp* のいずれかを保有する株は，カテーテル留置症例および非留置症例のいずれからも分離されていた．*asa1* は高頻度伝達プラスミド上にコードされており，遺伝情報交換の中心的役割を担っている．病原性遺伝子を集積した *E. faecalis* はバイオフィルム形成能が高く尿路に定着するものと考えられた．

Key words : *Enterococcus faecalis*, complicated urinary tract infection, biofilm formation, pathogenicity, gene transfer

序　文

Enterococcus faecalis はヒトの正常腸内細菌叢の一菌種であるが，院内感染の主な原因菌種の一つでもある[1～4]．尿路における *E. faecalis* の病原性は必ずしも高くないと考えられているが，その分離頻度は増加傾向ある[5]．*E. faecalis* の主な病原性因子としては，aggregation substance（Agg），extracellular surface protein（Esp），cytolysin（Cyl），gelatinase（Gel）などが報告されており，これらの病原性因子はクォーラムセンシング（菌密度依存的遺伝子発現制御）により相乗的に細胞を傷害すると考えられている，[1,6～9]．フェロモン反応性プラスミド上の *asa1* がコードするAggは，菌の凝集，プラスミドの伝達および生体への付着を促進することが報告されている[10]．染色体上の *esp* がコードするEspは尿路への定着およびバイオフィルム形成に関与するとされている．フェロモン反応性プラスミドもしくは染色体上に存在する *cylA* を含むオペロンがコードするCylは，腸球菌性感染症を重症化させると報告されている．染色体上の *gelE* がコードするGelは種々の細胞外蛋白を加水分解する働きをもつ．

本研究では，尿路における *E. faecalis* のバイオフィルム形成に関する臨床的意義について検討した．岡山大学泌尿器科における複雑性尿路感染症患者由来 *E. faecalis* において，病原性に関与する遺伝子（*asa1*, *esp*, *cylA*, *gelE/sprE*）の保有状況を検討した．またバイオフィルム形成能，hemolysin（Hln：cytolysinの一機能）およびGelの産生性について検討し，臨床的背景との関連性についても検討を加えた．さらに，対象株を付着因子（Agg, Esp）をコードする *asa1*, *esp* の保有の有無により4群に分類して解析を行った．

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対象と方法

1. 対象

岡山大学泌尿器科で1991年から2002年までの12年間に，複雑性尿路感染症患者（尿培養で菌数が10^4CFU/ml以上）352症例より分離された*E. faecalis* 352株を対象とした．

2. PCR法

*E. faecalis*をTodd Hewitt Broth（THB）に一晩培養したものを遠心後，調製した菌液を熱処理し，再遠心後の上清をDNA調製液として使用した．文献11）に記載されたプライマーを用い，熱変性，アニーリング，伸長反応は各プライマーペアにおける最適条件で行った．*TaKaRa Taq* ™を用い，PCR buffer, dNTP, プライマー，*Taq* DNA polymeraseの混液を調製し，DNA調製液を加え反応を開始した．各反応液を電気泳動後，エチジウムブロマイドで染色し，UV transiluminatorにて撮影した．

3. Hln産生株の検出法

5%ウサギ血液加Todd Hewitt Agar（THA）に画線して37℃で48時間培養後，溶血が認められた場合をHln産生株とした．

4. Gel産生株の検出法

3%ゼラチンを含む寒天培地[12]上で*E. faecalis*を37℃で48時間培養した後，飽和硫酸アンモニウム溶液を滴下して産生性を調べた．

5. バイオフィルムアッセイ

*E. faecalis*を0.25%グルコース添加Tryptic Soy Brothで一晩培養し，100倍に希釈し，滅菌した

Table 1 Relationship between biofilm-forming capacities and virulence factors/clinical background

	Number of isolates	OD_{570} (mean ± SD)	*P* value (Mann-Whitney's *U* test)
Total isolates tested	352	0.36 ± 0.37	
Virulence determinants			
asa1-positive	291	0.38 ± 0.38	0.0176
asa1-negative	61	0.27 ± 0.27	
esp-positive	254	0.40 ± 0.41	0.0276
esp-negative	98	0.26 ± 0.18	
cylA-positive	164	0.41 ± 0.41	0.0116
cylA-negative	188	0.32 ± 0.32	
gelE/sprE-positive	306	0.36 ± 0.35	0.0915
gelE/sprE-negative	46	0.35 ± 0.46	
Extracellular enzymes			
hemolysin producing	63	0.47 ± 0.48	0.0384
hemolysin non-producing	289	0.34 ± 0.33	
gelatinase producing	167	0.35 ± 0.31	0.1376
gelatinase non-producing	185	0.37 ± 0.42	
Clinical background			
catheter-related	107	0.33 ± 0.34	0.0582
catheter-unrelated	245	0.38 ± 0.38	
polymicrobial	202	0.35 ± 0.34	0.5505
monomicrobial	150	0.37 ± 0.40	
febrile	60	0.31 ± 0.32	0.1267
non-febrile	292	0.37 ± 0.38	