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Effect of Biologic Mediators on Ciprofloxacin Accumulation by Gingival Fibroblasts

Swati Y. Rawal* and John D. Walters*

*Section of Periodontology, College of Dentistry, The Ohio State University Health Sciences Center, Columbus, OH

Abstract

Background—Human gingival fibroblasts actively accumulate fluoroquinolone antimicrobials. Because fibroblasts are prevalent in gingiva, they may help sustain therapeutic fluoroquinolone levels at that site. The purpose of this study was to determine whether mediators associated with infection or injury can enhance ciprofloxacin accumulation by gingival fibroblasts.

Methods—Quiescent fibroblast monolayers were treated for 1, 6, or 24 hours with several concentrations of tumor necrosis factor (TNF)- α , transforming growth factor (TGF)- β 1, platelet-derived growth factor (PDGF)-BB, fibroblast growth factor (FGF)-2, or insulin-like growth factor (IGF)-1. Transport was assayed by measuring cell-associated fluoroquinolone fluorescence.

Results—All mediators significantly enhanced ciprofloxacin transport in a dose dependent manner ($P < 0.05$; ANOVA). Except for TNF, this enhancement was associated with a decrease in the K_m of ciprofloxacin transport. Maximal enhancement was observed with 10 ng/ml PDGF or FGF and 30 ng/ml TNF, TGF, or IGF. Brief (1 hour) treatment with TNF or FGF upregulated ciprofloxacin accumulation by a maximum of 13% to 14%, whereas TGF, PDGF, and IGF enhanced this process by 19% to 24%. All of the mediators enhanced ciprofloxacin accumulation by a maximum of 19% to 24% after 6 hours and 30% to 38% after 24 hours. The accumulation of other fluoroquinolones (e.g., gatifloxacin) was also slightly enhanced.

Conclusions—Gingival fibroblasts treated with cytokines or growth factors accumulate significantly more ciprofloxacin than untreated controls. This provides a mechanism by which ciprofloxacin could be preferentially distributed to gingival wound or inflammatory sites, yielding local therapeutic levels that are more sustained than in serum.

Keywords

Antibiotic; fibroblast growth factor; insulin-like growth factor; platelet-derived growth factor; transforming growth factor; tumor necrosis factor

Periodontitis is caused by specific bacteria, which trigger inflammatory and immunologic changes in the periodontal tissues that result in destruction of connective tissue and bone.^{1,2} Scaling and root planing can eliminate most periodontitis-associated bacteria, but the pathogens *Actinobacillus actinomycetemcomitans* (Aa) and *Porphyromonas gingivalis* (Pg) are difficult to eliminate by mechanical therapy alone. Both pathogens possess virulence factors that frustrate the host response and thwart conventional therapeutic efforts. Aa can invade epithelial cells and enter the underlying connective tissue, whereas Pg can invade epithelial cells and linger inside them.^{3,4} Antibiotics can be used to help eliminate these pathogens as part of the treatment for aggressive and recurrent forms of periodontitis.^{5,6} Recent studies

demonstrate that fluoroquinolones are effective in the treatment of *Aa*-associated periodontitis.^{7,8} Ciprofloxacin exhibits bactericidal activity against a wide range of Gram-negative microorganisms and is also effective against Gram-positive bacteria.⁹ Human gingival fibroblasts possess transporters that actively accumulate ciprofloxacin and other fluoroquinolones, and it has been suggested that fibroblasts serve as a reservoir for these agents.¹⁰ Consistent with this possibility, recent studies have shown that ciprofloxacin's steady-state concentration in gingival fluid is more sustained than in serum.^{11,12} Moreover, there is evidence that serum ciprofloxacin levels fall below their steady-state levels in gingival connective tissue as they recede from their peak values.¹²

Fibroblasts can be activated by a wide variety of biologic mediators. Proinflammatory cytokines (e.g., tumor necrosis factor [TNF]- α) and growth factors (e.g., platelet-derived growth factor [PDGF]-BB, fibroblast growth factor [FGF]-2, transforming growth factor [TGF]- β 1, and insulin-like growth factor [IGF]-1) are released by epithelial cells, macrophages, platelets, endothelial cells, and fibroblasts in the inflamed or injured periodontium.¹³ The effects of these agents have been widely studied and include stimulation of proliferation, migration, and matrix protein synthesis.¹³⁻¹⁶ These biologic mediators induce wideranging cascades of intracellular signals in fibroblasts, which can alter gene expression, synthetic activity, intracellular pH, and the transport of biosynthetic precursors. If fibroblast ciprofloxacin transport were upregulated by these factors, it could enhance the distribution of antimicrobial agents to the gingiva and further sustain their therapeutic levels in the gingiva. In this study, we examined the effects of TNF- α and several growth factors on ciprofloxacin accumulation by cultured fibroblasts. The results suggest that these biologic mediators could enhance the accumulation of ciprofloxacin at areas of gingival wounding or inflammation.

MATERIALS AND METHODS

Culture and Treatment of Gingival Fibroblasts

Human gingival fibroblasts were isolated from healthy adult papillae and used between passage numbers 4 and 15.¹⁷ Cells were cultured at 37°C in 5% CO₂ in minimum essential medium[†] supplemented with 2 mM glutamine and 10% heat-inactivated fetal bovine serum[‡] (FBS). Cells were seeded into 24-well culture plates and grown into confluent monolayers. Fibroblasts were starved overnight in medium containing 0.5% FBS prior to treatment with biologic mediators. These experimental conditions were intended to enhance fibroblast responsiveness to the mediators while minimizing changes in cell number (as evaluated by cell DNA content¹⁸).

Fibroblast monolayers were treated for 1, 6, or 24 hours with biologically relevant concentrations of recombinant human growth factors and cytokines.[§] The range of treatment concentrations was adjusted for each mediator so that the highest concentration corresponded to the maximal response observed in vitro. Fibroblasts were treated with 1, 3, 10, and 30 ng/ml TNF- α , TGF- β 1, and IGF-1 and 0.3, 1, 3, and 10 ng/ml FGF-2 and PDGF-BB. Each multiwell plate was divided into one negative control (blank) well, three positive control wells, and three wells of each of the relevant concentrations of the particular growth factor being studied.

Assay of Fluoroquinolone Transport

Fluoroquinolone transport was assayed by measuring cell-associated fluoroquinolone fluorescence as previously described.¹⁰ Tissue culture plates containing confluent monolayer

[†]Invitrogen Life Technologies, Carlsbad, CA.

[‡]Atlanta Biologicals, Lawrenceville, GA.

[§]PeptoTech, Rocky Hill, NJ.

cultures were washed three times with warm Hanks balanced salt solution (HBSS), overlaid with 0.2 ml/well HBSS, and warmed to 37°C for 10 minutes prior to assay. At the indicated times, 0.2 ml HBSS containing twice the desired final fluoroquinolone concentration was added to each well, using multichannel pipettes that had previously been warmed to 37°C with multichannel pipettes. After incubation for 3 minutes at 37°C, the fluoroquinolone solution was removed, and each well was rapidly washed four times with HBSS. The adherent cells were removed into 1 ml 100 mM glycine (pH 3.0) and the resulting lysate was pelleted at $13,000 \times g$ for 4 minutes. The fluorescence of the supernatant was measured with a fluorescence spectrometer at excitation and emission wavelengths of 278 and 445 nm, respectively. Because the liver is the predominant site of ciprofloxacin metabolism,¹⁹ it is likely that little or no metabolism of ciprofloxacin occurred during the short duration of the assay. Transport data were expressed as a percentage of transport observed in untreated control fibroblasts for the dose response studies. For the determination of K_m , cells were incubated with several different concentrations of ciprofloxacin. Calibration plots were constructed to relate fluorescence values to antibiotic concentration. Line-weaver-Burk analysis was used to determine the affinity and maximal velocity of transport.

RESULTS

Gingival fibroblasts cultured for 1, 6, and 24 hours in the presence of TNF- α (1 to 30 ng/ml) exhibited a significant dose-dependent enhancement of ciprofloxacin accumulation compared to untreated controls ($P \leq 0.001$; repeated measures ANOVA; Fig. 1A). TNF- α (30 ng/ml) enhanced ciprofloxacin uptake by 14% at 1 hour, 19% at 6 hours, and 33% at 24 hours. TGF- β 1 (1 to 30 ng/ml) also significantly enhanced fibroblast ciprofloxacin accumulation ($P < 0.001$; ANOVA; Fig. 1B). Treatment with 30 ng/ml TGF- β 1 enhanced ciprofloxacin accumulation by 23% after 1 hour, 19% after 6 hours, and 35% after 24 hours.

PDGF-BB (0.3 to 10 ng/ml) significantly upregulated fibroblast ciprofloxacin accumulation after treatment for 1, 6, and 24 hours ($P < 0.001$; ANOVA; Fig. 2A). Treatment for 1 hour with PDGF (10 ng/ml) enhanced ciprofloxacin accumulation by 23%, whereas treatment for 6 and 24 hours resulted in enhancements of 24% and 35%, respectively. Fibroblasts cultured in the presence of FGF-2 (0.3 to 10 ng/ml) resulted in enhanced ciprofloxacin accumulation after 1, 6, and 24 hours ($P < 0.001$; ANOVA; Fig. 2B). FGF-2 (10 ng/ml) increased ciprofloxacin accumulation by 12% at 1 hour, 25% at 6 hours, and 38% at 24 hours.

IGF-1 (1 to 30 ng/ml) induced an increase in ciprofloxacin accumulation at the 1-, 6-, and 24-hour time points ($P \leq 0.001$; ANOVA; Fig. 3A). IGF-1 (30 ng/ml) enhanced ciprofloxacin accumulation by 19% at 1 hour, 22% at 6 hours, and 30% at 24 hours. In addition to its effects on accumulation of ciprofloxacin, IGF-1 (30 ng/ml; 6 hours) enhanced the accumulation of other fluoroquinolones, including gatifloxacin (16% increase), moxifloxacin (11% increase), and levofloxacin (9% increase) (Fig. 3B).

The mechanism of transport enhancement by biologic mediators was investigated by analyzing the kinetics of transport. None of the mediators enhanced ciprofloxacin accumulation by inducing a significant increase in maximal transport velocity (not shown). However, all of the mediators except TNF- α induced a statistically significant decrease in the K_m of transport after 6 hours of treatment ($P \leq 0.01$; paired t test; Table 1).

Many effects of the mediators used in this study are triggered by signaling through mitogen-activated protein (MAP) kinases. We utilized the p42/p44 MAP kinase inhibitor PD 98059²⁰ and the p38 MAP kinase inhibitor SB 203580²¹ to evaluate the role of these MAP kinases in the enhancement of fibroblast ciprofloxacin transport by TNF, TGF- β , PDGF-BB, FGF-2, and IGF-1. When fibroblasts were pretreated for 20 minutes with an effective dose of

PD 98059 (5 μ M) or SB 203580 (3 μ M) prior to incubation with these mediators for 6 hours, the effects of all five mediators were significantly inhibited (Table 2). PD 98059 abrogated more than 75% of the enhancement produced by TNF, TGF- β 1, and PDGF-BB, whereas SB 203580 blocked more than 75% of the enhancement produced by TNF, PDGF-BB, FGF-2, and IGF-1.

DISCUSSION

Fluoroquinolones inhibit bacterial DNA topoisomerase II and produce bactericidal effects against a broad spectrum of bacteria. Ciprofloxacin and levofloxacin are second-generation compounds with anti-Gram-negative potency, plus some limited anti-Gram-positive activity. Gatifloxacin and moxifloxacin are third-generation compounds with enhanced Gram-positive activity. Most *Aa* strains are highly susceptible to ciprofloxacin.^{7,22} All of these fluoroquinolones exhibit excellent absorption and tissue penetration, and their distribution profile differs markedly from that of β -lactams.²³ They achieve tissue-to-serum ratios in excess of 2:1 at many tissue sites,¹⁹ whereas β -lactams seldom attain tissue-to-serum ratios of greater than 0.4:1. To reach a tissue-to-serum ratio in excess of 1:1, ciprofloxacin must cross the plasma membrane and accumulate within the cell. This property makes ciprofloxacin and other fluoroquinolones particularly useful in treating pathogens that have invaded host cells.

Fibroblasts are the most abundant cellular compartment of healthy human gingival connective tissue, and the volume of the fibroblast compartment increases nearly three-fold in the presence of inflammation.²⁴ Previous work has shown that gingival fibroblasts accumulate fluoroquinolones through an active transport process that exhibits Michaelis-Menten kinetics. When incubated in medium containing 1 μ g/ml ciprofloxacin, fibroblasts attain a steady-state intracellular ciprofloxacin concentration of 8 μ g/ml.¹⁰ Transporters are capable of moving ciprofloxacin in a forward or reverse direction to maintain this relationship between intracellular and extracellular concentrations.¹⁰ As extracellular levels decrease, ciprofloxacin moves across the plasma membrane into the extracellular environment. Efflux from gingival fibroblasts could potentially maintain relatively high antimicrobial levels in the interstitial fluid after blood levels decrease. This ability of the fibroblast compartment to function as a reservoir could help explain why ciprofloxacin appears to reach higher levels in gingival fluid than in blood serum when sampled after blood levels recede from their peak values.^{11,12}

During periodontal inflammation and wound healing, a variety of cytokines and growth factors are released. Among them are TNF- α (which is an inflammatory mediator and growth factor for human fibroblasts), TGF- β (which stimulates collagen synthesis and inhibits matrix degradation), PDGF (a potent fibroblast mitogen), FGF-2 (which stimulates angiogenesis and fibroblast proliferation), and IGF-1 (which stimulates collagen and matrix synthesis and is also a fibroblast mitogen).^{15,25} The purpose of this study was to determine whether biologic mediators associated with wound healing or inflammation can enhance the accumulation of ciprofloxacin by gingival fibroblasts. At treatment doses similar to those utilized in other in vitro studies of gingival fibroblasts,²⁶⁻²⁹ these factors produce a time- and dose-dependent enhancement of fibroblast ciprofloxacin accumulation. The enhancement is manifest within 1 hour, with TGF- β 1, PDGF-BB, and IGF-1 producing the largest effects and TNF- α and FGF-2 producing effects that are statistically significant but smaller in magnitude. Their effects were sustained for at least 24 hours and were associated with a decrease in the K_m (increase in affinity) of ciprofloxacin transport. The early (1 hour) effects appear to be mediated by signaling for a decreased K_m of preexisting transporters, since several hours are required to induce the expression of newly synthesized transporters. Experiments with selective MAP kinase inhibitors suggest that p42/p44 and p38 MAP kinases are involved in the upregulation of ciprofloxacin transport by most of the mediators, implying that these kinases could be useful targets for therapeutic modulation of fluoroquinolone accumulation in gingiva. After 24 hours,

the degree of enhancement produced by TNF- α , TGF- β 1, PDGF-BB, FGF-2, and IGF-1 was similar.

CONCLUSIONS

Biologic mediators found in wounded or inflamed gingiva could enhance the ability of the fibroblast compartment to serve as a ciprofloxacin reservoir, which may enhance distribution of systemic ciprofloxacin to the gingiva and help maintain therapeutic levels at that site. This could benefit the host by enhancing the local control of bacterial infections, which disrupt wound healing. Moreover, cytokine-induced increases in intracellular ciprofloxacin accumulation could potentially enhance the elimination of invasive pathogens associated with aggressive periodontitis. Together, these effects could have a favorable impact on periodontal therapy.

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Figure 1.

Effect of TNF- α and TGF- β 1 on fibroblast ciprofloxacin accumulation. Confluent fibroblast cultures were treated with the indicated mediator concentrations for 1, 6, and 24 hours. The medium was then removed and replaced with HBSS. After rewarming to 37°C, 50 μ g/ml ciprofloxacin was added, and uptake was assayed as described in Materials and Methods. The conditions indicated by * failed to induce a significant increase in ciprofloxacin accumulation compared to untreated controls ($P > 0.05$; Dunnett's test). The data represent the mean $\pm$ SE of five experiments. **A)** TNF- α produced a significant treatment effect at all time points ($P \leq 0.001$; repeated measures ANOVA). **B)** TGF- β 1 produced significant enhancement of ciprofloxacin accumulation at all time points ($P \leq 0.001$; ANOVA; $N = 4$).

Figure 2.

Stimulation of fibroblast ciprofloxacin accumulation by PDGF-BB and FGF-2. The experiments were performed as described in Figure 1. Conditions that failed to produce a significant increase in ciprofloxacin accumulation compared to controls ($P > 0.05$; Dunnett's test) are indicated by *. **A)** PDGF-BB enhanced ciprofloxacin accumulation in a dose-dependent manner after 1, 6, and 24 hours as compared to untreated controls ($P < 0.001$; ANOVA; $N = 5$). **B)** Fibroblasts treated with FGF-2 exhibited a dose-dependent enhancement of ciprofloxacin uptake at all time points as compared to untreated controls ($P < 0.001$; repeated measures ANOVA; $N = 5$).

Figure 3.

A) Stimulation of fibroblast ciprofloxacin accumulation by IGF-1. Experiments were conducted as described previously. IGF-1 induced a significant dose-dependent enhancement of gingival fibroblast ciprofloxacin uptake compared to untreated controls at all time points ($P < 0.001$; repeated measures ANOVA; $N = 6$). Conditions that failed to produce a significant increase in the accumulation of ciprofloxacin compared to controls ($P > 0.05$, Dunnett's test) are indicated by *. **B)** Effect of IGF-1 (30 ng/ml; 6 hours) on accumulation of several fluoroquinolones by gingival fibroblasts. There were significant differences in accumulation of these fluoroquinolones ($P = 0.012$; ANOVA). The condition that is not significantly different from ciprofloxacin ($P > 0.05$; Dunnett's test) is indicated by +.

Table 1
Effects of Mediators on the Km of Fibroblast Ciprofloxacin Transport

Treatment	Effect on Km [*]	Significance [†]
TNF- α	9.3% decrease	$P = 0.48$
TGF- β 1	32% decrease	$P = 0.003$
PDGF-BB	26% decrease	$P = 0.01$
FGF-2	32% decrease	$P < 0.001$
IGF-1	42% decrease	$P < 0.001$

* Results were determined from Lineweaver-Burk analysis and compared to results obtained with untreated control cells.

[†] P values were derived from a paired t test.

Table 2

Effects of PD98059 and SB203580 on Enhancement of Fibroblast Ciprofloxacin Transport by Biologic Mediators

Mediator	Inhibition by 5 μ M of PD98059	Inhibition by 3 μ M of SB203580
TNF- α	84.0 $\pm$ 14.7%	100 $\pm$ 26.5%
TGF- β 1	77.5 $\pm$ 13.5%	51.5 $\pm$ 9.5%
PDGF-BB	86.2 $\pm$ 16.8%	75.2 $\pm$ 14.9%
FGF-2	57.0 $\pm$ 12.4%	86.5 $\pm$ 28.8%
IGF-1	63.5 $\pm$ 24.3%	90.0 $\pm$ 18.6%

Results are expressed as mean $\pm$ SE. The inhibitory effects were statistically significant ($P < 0.03$; paired t test; $N \geq 5$).