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Cytofluorometric Analysis of Chondrotoxicity of Fluoroquinolone Antimicrobial Agents

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To better understand quinolone-related arthropathy, we conceived an experimental ex vivo model using cell cultures of articular chondrocytes issued from pretreated New Zealand White rabbits (NZW). Juvenile (4- to 5-week-old) NZW were orally dosed with ofloxacin or pefloxacin (300 mg/kg of body weight for 1 day) or with pefloxacin (300 mg/kg for 7 days). Adult (5-month-old) NZW were treated with pefloxacin (300 mg/kg for 1 day). Chondrocytes were enzymatically recovered from cartilage and were analyzed by cytofluorometry using 2′,7′-dichlorofluorescein diacetate (DCFH-DA) and dihydrorhodamine 123 (DHR), reflecting cellular respiratory-burst activity, and rhodamine 123 (Rh123) and 10-*N*-nonyl-acridine orange (NAO), specific for the mitochondrial activity and mass, respectively. A significant increase in the respiratory burst was detected by DCFH-DA and DHR in all treated groups of young animals, compared with untreated control groups. No significant increase of respiratory burst was noted in older treated rabbits. The 7-day treatment resulted in a decrease in mitochondrial uptake of Rh123 and an increase in NAO uptake. Fluoroquinolone arthrotoxicity seems to involve in its early phase the respiratory burst of immature articular chondrocytes.

Fluoroquinolone antimicrobial agents (FQ) are widely prescribed compounds, but their use so far has been restricted to adults because of the risk of arthropathy during teenage years (19, 36). However, in cases of serious bacterial infections, pediatricians are compelled to use these agents, most often with no articular complication (10, 26). Nevertheless, this drug-induced arthropathy, although usually reversible, can be in some instances of greater concern.

Several experimental studies have confirmed the peculiar susceptibility of growing animals to this side effect, whereas maturity seems to confer resistance (15, 36). Histologic lesions of articular cartilage appear very quickly after treatment initiation and begin in the intermediate cartilage zone. Ultrastructural studies have shown early changes within chondrocytes, consisting in swelling of mitochondria and the appearance of intracytoplasmic vesicles (5, 44).

Articular chondrocytes are responsible for matrix turnover or degradation by producing proteinases (47) and oxygen-derived reactive species (35, 45, 46) that accumulate into cytoplasmic vacuoles and that are known to alter matrix components hyaluronic acid, proteoglycans, and collagen (17).

Histologic manifestations of mitochondrial damage in chondrocytes issued from FQ-treated animals suggest that these drugs interfere with the functioning of these organelles. FQ act as inhibitors of bacterial DNA gyrase, and many experimental works have dealt with a possible action of these synthetic compounds upon mammalian topoisomerases, with no definite conclusion, particularly in the case of those originating from mitochondria (7, 14). Since articular chondrocytes thrive in low-oxygen tensions that other cell types would not tolerate, it is reasonable to postulate a particular mitochondrial activity in these as distinct from other cells. Additionally, mitochondria are known to be a major source of oxygen-derived free radicals (30).

All of these considerations prompted us to examine whether FQ induce modifications in chondrocytic mitochondrial and respiratory-burst activities.

For this purpose, we pretreated juvenile and older New Zealand White rabbits with either pefloxacin or ofloxacin and then studied their freshly enzymatically released articular chondrocytes. We used cytofluorometric techniques with four fluorochromes. Two are specific for the mitochondrial compartment. Rhodamine 123 (Rh123) accumulates in these organelles, depending on their transmembrane potential (9, 22). 10-*N*-Nonyl acridine orange (NAO) possesses a high affinity for the mitochondrial membrane, independently of its energetic state (25, 32). The two other vital tracers are 2′,7′-dichlorofluorescein diacetate (DCFH-DA) and dihydrorhodamine 123 (DHR). These allow an evaluation of intracellular production of oxidizing agents (39, 40). DCFH-DA crosses cell membranes and is firstly deacetylated by cellular esterases to give dichlorofluorescein (DCFH). Under the action of intracellular oxygen-derived reactive species (predominantly hydrogen peroxide), the nonfluorescent compounds DCFH and DHR are, respectively, converted into the fluorescent products dichlorofluorescein (DCF) and Rh123, the second being secondarily trapped within mitochondria. The resulting fluorescence gives an estimation of cellular respiratory burst.

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MATERIALS AND METHODS

Animals. Juvenile (4- to 5-week-old) and adult (5-month-old) New Zealand White rabbits were obtained from L'Elevage des Pins (Epeigne sur Deme, France).

Chemicals and reagents. Ham's F-12 medium was from GIBCO (Cergy-Pontoise, France), and fetal calf serum was

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from Labsystems Flow (Les Ulis, France). Pancreatic porcine trypsin was from Biosys SA (Compiègne, France), and crude bacterial collagenase (*Clostridium histolyticum*) was from Worthington Biochemical Corp. (Freehold, N.J.). The fluorochromes propidium iodide (PI), Rh123, NAO, DCFH-DA, and DHR were purchased from Molecular Probes, Inc. (Eugene, Oreg.). Pefloxacin mesylate dihydrate solution was kindly supplied by Roger Bellon Laboratories (Neuilly, France). Ofloxacin powder was kindly provided by Roussel-Uclaf Laboratories (Romainville, France).

Treatments. A suspension of ofloxacin was prepared by dilution in a solution consisting of 0.2 g of carboxymethylcellulose per 100 ml. Pefloxacin mesylate dihydrate solution was used directly, with no further preparation. Four different groups of treated animals were defined as follows. Juvenile rabbits from groups 1 and 2 received, respectively, pefloxacin and ofloxacin, which were orally administered in two doses of 150 mg/kg of body weight repeated at 12-h intervals. Young rabbits from group 3 received a daily dose of 150 mg of pefloxacin per kg twice for 7 days. Group 4 was composed of adult rabbits, orally treated with two doses of pefloxacin, 150 mg/kg, repeated at 12-h intervals. Control animals were simultaneously given the same volume of a 5% glucose solution. Treatment and matched control groups were composed of seven animals each.

Chondrocyte isolation. Animals were killed 8 h after the last dose with an intracardiac injection of pentobarbital (Sanofi-Winthrop, Gentilly, France). Articular chondrocytes were enzymatically released from knee and shoulder joints by a modification of the procedure described by Green (16). Briefly, small slices of articular cartilage were aseptically cut off and immediately placed in Ham's F-12 medium. Then, enzymatic digestion began, first with a solution of 0.5 mg of trypsin per ml in Ham's F-12 medium for 30 min, and second with a 3-mg/ml collagenase solution for 30 min more. Afterward, articular cartilage pieces were incubated in a 0.6-mg/ml collagenase solution in Ham's F-12 medium supplemented with 10% fetal calf serum at 37°C in a 75% N_2–20% O_2–5% CO_2 atmosphere for 10 h. Enzymatically released chondrocytes were then isolated by medium centrifugation and filtration to eliminate collagenase as well as cellular and cartilage fragments. The average cell yields was 20×10^6 to 30×10^6 cells for young rabbits and 4×10^6 to 6×10^6 cells for adult rabbits, both pretreated or not.

Determination of FQ concentrations in articular cartilage. Concentrations of ofloxacin, pefloxacin, and its main metabolite, *N*-desmethyl pefloxacin (norfloxacin) were assayed on supernatants of cartilage matrix homogenates issued from rabbit tibial epiphysis by means of high-performance liquid chromatography (HPLC) with UV detection (adapted from Montay and Tassel [28]). Separations were performed on a reverse-phase column (100 by 4.6 mm) (Nucleosil C8 3μm; SFCC, Neuilly-Plaisance, France). The mobile phase (pH 4.8) was prepared by mixing 260 ml of acetonitrile, 2 g of sodium acetate trihydrate, 2 g of citric acid monohydrate, 4 ml of triethylamine, 2 ml of formic acid, and 740 ml of water. All HPLC measurements were carried out at room temperature with a 1-ml/min flow rate.

Preparation of fluorochromes. Fluorochromes were provided in powder presentation and were dissolved and maintained in solution in *N*-*N*-dimethylformamide (DMF), except for PI, which was kept in aqueous solution. Concentrations of stock solutions were 2 mg/ml for PI, 0.1 mg/ml for Rh123 and NAO, 1 mg/ml for DHR, and 5 mg/ml for DCFH-DA. From these stock solutions, we prepared cell coloration solutions by a 100-fold dilution in Ham's F-12 medium.

Application of fluorochromes to chondrocytes. All incubations with fluorochromes were performed at 37°C. The final concentrations achieved in contact with cells and the durations of incubation were determined to be as follows: Rh123 at 0.1 μg/ml (0.26 μM) for 30 min, NAO at 0.1 μg/ml (0.21 μM) for 15 min, DCFH-DA at 5 μg/ml (10.2 μM) for 15 min, and DHR at 1 μg/ml (2.89 μM) for 15 min. With Rh123 only, we performed a subsequent centrifugation of cell suspensions to eliminate fluorochrome excess from the supernatant. The cell density per sample was always of the same order of magnitude for a single comparison analysis. The vitality of chondrocytes was controlled at the beginning and the end of every cytofluorometry session, with the PI exclusion test, with a final concentration of PI in cell samples of 2 μg/ml (3 μM). The average duration of a cytofluorometric session was 1 h and 30 min.

Confocal microscopy. We used a confocal microscope (Sarastro 2000; Molecular Dynamics, Sunnyvale, Calif.) to verify intracellular localization of each fluorescent compound and the morphological aspect of the resulting fluorescence.

Cytofluorometric analysis. Immediately at the end of each incubation phase with fluorochromes, chondrocyte populations were analyzed with a flow cytometer FACScan (Becton Dickinson, Grenoble, France) equipped with an argon laser emitting at 488 nm. We first studied forward low-angle light scattering and right-angle side scattering of cell populations, respectively, reflecting cell size and granulosity. Then, fluorescence intensity data were acquired in a logarithmic mode. Dead cells were excluded from analysis by using PI staining. DCF, NAO, and Rh123 green fluorescence was collected between 515 and 545 nm, and PI red fluorescence was collected between 563 and 607 nm. Each cell sample was analyzed in triplicate (5,000 events per analysis). For further analysis and comparison of data, the results were converted into linear fluorescence units by the following formula: linear fluorescence units = $10^{(\text{fluorescence log}) \times d/c}$ (d, the number of decades of the cytometer scale; c, the number of channels).

We could then calculate the fluorescence intensity ratio (FIR) between pretreated chondrocytes (PTC) issued from treated animals and control chondrocytes (CC) issued from matched untreated animals.

Kinetic fluorocytometric studies were previously systematically performed with Rh123, DCFH-DA, and DHR, with distinction between PTC and CC. With Rh123, a stable plateau of fluorescence intensity was obtained after 30 min of incubation. With NAO, DCFH-DA, and DHR, the plateau phase was reached after 15 min of incubation. At the Rh123 concentration that we used (0.26 μM), neither release of fluorochrome nor signs of cytotoxicity could be observed, even after prolonged incubation, in contrast to what has been described with higher Rh123 concentrations (27).

Statistical analysis. A paired Student's t test was used to compare FIR values to 1. P values of less than 0.05 were considered significant.

RESULTS

FQ concentrations in cartilage. In group 1, the ofloxacin concentration was 7.31 ± 2.78 μg/g of tissue (mean ± standard deviation). In groups 2 and 3, pefloxacin concentrations were respectively 17.53 ± 4.25 and 81.54 ± 8.21 μg/g of tissue (means ± standard deviations). The pefloxacin metabolite norfloxacin remained constantly under the detection level.

Confocal microscopy. Confocal microscopy gave precious information about fluorescence obtained with the four vital

TABLE 1. FIRs calculated for chondrocytes issued from treated and from control juvenile rabbits

Treatment drug (duration)[a]	FIR (mean ± SD)[b]	
	DCFH-DA	DHR
Ofloxacin (1 day)	1.52 ± 0.33	1.20 ± 0.19
Pefloxacin (1 day)	1.68 ± 0.20	1.30 ± 0.17
Pefloxacin (7 days)	1.88 ± 0.31	1.31 ± 0.20

[a] $n = 7$ per group.
[b] Results significantly different from 1 ($P < 0.05$) by paired Student's t test.

TABLE 2. FIRs calculated for chondrocytes issued from treated and from control juvenile rabbits

Treatment drug (duration)[a]	FIR (mean ± SD)	
	Rh123	NAO
Ofloxacin (1 day)	1.10 ± 0.14	0.83 ± 0.17
Pefloxacin (1 day)	1.01 ± 0.17	0.81 ± 0.21
Pefloxacin (7 days)	0.82 ± 0.12[b]	1.39 ± 0.26[b]

[a] $n = 7$ per group.
[b] Results significantly different from 1 ($P < 0.05$) by paired Student's t test.

tracers that we used. Rh123 was accumulated in small cytoplasmic vesicles (size, ≤2 μm) in a patchy distribution pattern. Addition of the uncoupling agent carbonylcyanine *m*-chlorophenyl hydrazone resulted in a dramatic lowering of fluorescence, thereby demonstrating the potential-dependent affinity of Rh123 for the mitochondrial compartment of chondrocytes. NAO-induced fluorescence was more homogeneous and corresponded well with a mitochondrial trapping. Both Rh123 and NAO were excluded from the cellular nucleus. With these two fluorochromes, there was no obvious difference of fluorescence intensity or distribution between PTC and CC, whether they originated from young or older rabbits.

DCFH-DA-induced fluorescence accumulated in cytoplasmic vesicle-shaped organelles, which appeared to be more numerous and fluorescent in PTC than in CC. DHR gave a fluorescence aspect similar to that of Rh123, with no apparent morphologic difference between PTC and CC. With both of these respiratory-burst markers, there was no nuclear fluorescence.

Flow cytometric analysis. The morphometric studies based on low-angle light scattering and right-angle side scattering measurements gave no significant differences between PTC and CC. In all groups of rabbits, analysis of chondrocyte vitality performed by the PI exclusion test gave a mean percentage of dead cells of less than 5% at the beginning of a cytometric session and less than 6.5% at the end.

In the 24-h treatment groups of juvenile rabbits (groups 1 and 2), we registered a significant enhancement of cellular processing of the vital tracers DCFH-DA and DHR within isolated PTC, compared with CC (Table 1). This resulted from an accumulation of the oxidized and fluorescent compounds DCF and Rh123 in PTC that was higher than that in CC. The mean fluorescence of chondrocytes isolated from pefloxacin-treated animals appeared higher than in the ofloxacin-treated group, but without a significant difference.

In groups 1 and 2, we failed to measure any significant treatment-induced modification of cellular incorporation of Rh123 and NAO.

In the 7-day treatment group (group 3), we similarly found a significant fluorescence increase in PTC with the respiratory-burst tracers DCFH-DA and DHR but also significant treatment-induced modifications of cellular incorporation of Rh123 and NAO. At the mitochondrial level, smaller amounts of Rh123 were incorporated into PTC than into CC, whereas NAO-induced cellular fluorescence, on the contrary, was significantly higher in PTC (Table 2).

In chondrocytes issued from older rabbits treated with pefloxacin for 24 h, we did not find any significant increase of respiratory burst. Cellular uptake of mitochondrial probes was not significantly modified.

DISCUSSION

Under physiological or pathological conditions, chondrocytes are involved in an active way in cartilage matrix turnover or degradation, by the mean of production of proteolytic enzymes, proinflammatory prostaglandins, and oxygen-derived reactive species (17, 31, 45, 47).

Recently, Tiku et al. (46) used the vital tracer DCFH-DA to demonstrate the production of hydrogen peroxide by articular chondrocytes in vitro. The authors noted an enhancement of resulting cellular DCF fluorescence after stimulation with interleukin-1, gamma interferon, or tumor necrosis factor alpha. Using spectrophotometry, Stadler et al. showed the ability of articular chondrocytes to synthesize nitric oxide in response to interleukin-1 or lipopolysaccharide (43).

DCFH-DA and DHR are nonfluorescent compounds that give, after cellular processing and oxidation, two fluorescent products, respectively, DCF and Rh123, the latter accumulating afterward in mitochondria (1, 39). The level of cellular fluorescence is correlated with the intracytoplasmic production of oxidizing agents, e.g., hydrogen peroxide and free-radical metabolites. Besides hydrogen peroxide, nitric oxide seems capable of transforming DCFH into DCF (34).

The present study confirms the ability of articular chondrocytes to produce oxidizing agents, apparently contained in cytoplasmic vesicles. Secondly, our results suggest that oral administration of either pefloxacin or ofloxacin to juvenile rabbits induces an early stimulation of respiratory burst in articular chondrocytes. This phenomenon could easily explain the early fissures of articular cartilage. The oxidizing agents produced thereby could act as activators of latent metalloproteinases or could be directly toxic against cartilage matrix components, in situ or during their synthesis (17). This might explain the inhibition of synthesis of collagen type II or proteoglycan by articular chondrocytes exposed to various quinolones (4, 18, 41).

Pefloxacin seemed to diffuse to articular cartilage better than ofloxacin, and the level of stimulation of respiratory burst with the first molecule appeared slightly higher than that with the second one, although without significant difference. However, besides the FQ type itself, penetration of FQ in cartilage could be an independent risk factor for arthrotoxicity.

Whether the respiratory-burst stimulation is due to a direct effect of FQ toward chondrocytes or to a complex phenomenon involving other cell types, such as synoviocytes or circulating immune cells, remains unclear. Synoviocytes, lymphocytes, and macrophages are able to produce proinflammatory cytokines, such as interleukin-1 or tumor necrosis factor, that may act upon chondrocytes and indirectly stimulate their production of proteolytic enzymes and oxygen-derived reactive species (17, 31). Several hypotheses concerning a stimulation of proinflammatory cytokines by FQ have been studied so far, with frequently contradictory results (42). In the case of FQ-related arthropathy, the early onset of histologic articular

lesions argues against indirect physiopathological mechanisms involving circulating immune cells and cytokines.

Of interest is that we failed to detect any significant increase of oxidative burst in mature chondrocytes issued from adult rabbits pretreated with pefloxacin at a dosage that appeared significantly effective for immature chondrocytes. This is in good agreement with in vivo experimental studies and human clinical data that have already shown a particular susceptibility of growing organisms to FQ arthrotoxicity and the apparent resistance acquired with maturity (36). The explanation of this age-dependent toxic effect remains to be determined. In any case, these negative results reinforce the relevance of our experimental model.

The antibacterial activity of quinolones is based on an inhibition of bacterial DNA gyrase, resulting in inhibition of DNA synthesis and bacterial death (20, 21). Conflicting results have been published concerning targeting of these drugs toward mammalian topoisomerases, which are closely related to DNA gyrases (13). Mammalian nuclear topoisomerases do not seem to be inhibited in vitro by quinolones at relevant concentrations. Mammalian mitochondria resemble bacteria in numerous ways and are thought to possess their own DNA topoisomerases, independent from nucleus-originated ones (6, 11, 12). The function of these putative mammalian enzymes has been tested as a potential target of DNA gyrase inhibitors, with no convincing result (7, 37). Hildebrand et al. noted a reduction of mitochondrial dehydrogenase activity in isolated articular chondrocytes under the action of different quinolones (18). Recent experimental data have shown damages caused in vitro by FQ to mitochondria of the flagellate *Euglena gracilis* (23, 33) and the yeast *Saccharomyces cerevisiae* (29).

Chondrocyte metabolism in vivo is unique, because of the low oxygen tension in articular cartilage. In comparison with those of other cell types, the mitochondrial activity of chondrocytes seems to be very particular (8). Effectively, chondrocytes maintain in vivo a predominantly fermentative metabolism, with a low level of respiration (oxygen consumption 20 to 50 times reduced, compared with that of other tissues).

The fluorochrome Rh123 is trapped within mitochondria, depending on *trans*-membrane potential (22). If the phospholipid-to-protein ratio stays constant, mitochondrial incorporation of NAO is predominantly mass dependent and is weakly influenced by membrane potential (24, 25, 32). The combination of simultaneous cytofluorometric analysis with these two fluorochromes allows an estimation of mitochondrial membrane area and energy state (3, 38). Cytofluorometry with these two vital tracers has already been employed to study chondrocytes (2) and the evolution of their mitochondrial activity in rabbit articular chondrocytes under culture conditions, after their isolation from cartilage (8).

The results obtained in the present study with Rh123 and NAO seem to indicate a mitochondrial alteration that is detected secondarily in PTC, with respect to respiratory-burst stimulation. The diminution of mitochondrial incorporation of Rh123 is highly suggestive of an injury in this organelle's membrane, while NAO uptake enhancement suggests an increase in membrane area. This confirms anterior ultrastructural data that have shown swelling of mitochondria in chondrocytes issued from dogs pretreated with the fluoroquinolone compound difloxacin (5, 44).

No significant difference in the uptake of Rh123 and NAO could be seen in the 24-h treatment groups between CC and PTC. This negative result can be interpreted in different ways. A delayed and direct toxicity of FQ against the mitochondria of chondrocytes is possible. On the other hand, we must consider the possibility that mitochondrial alterations correspond to a global cytotoxic effect, induced by an oxidative stress.

In conclusion, our results seem to indicate that immature, but not mature, articular chondrocytes are precociously and actively involved in quinolone-related arthropathy. The first drug-induced phenomenon could be the stimulation of cellular respiratory burst, resulting in the production of oxygen-derived reactive species, highly toxic for cartilage matrix components.

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