

## SCIENTIFIC REPORT

## Doxycycline—a role in ocular surface repair

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**Background/aims:** Doxycycline is a broad spectrum antibiotic that chelates metal ions and is frequently used as part of the treatment of ocular surface diseases. Its therapeutic value has been ascribed to an ability to inhibit matrix metalloproteinase (MMP) activity and both MMP and IL-1 synthesis. The aim of this study was to evaluate the role of doxycycline as an inhibitor of corneal MMPs and assess its contribution to ocular surface repair mechanisms.

**Methods:** Corneal epithelial cell and keratocyte cultures were grown to confluence and incubated with IL-1 $\alpha$ , LPS, doxycycline, or doxycycline and LPS in serum free medium for 4 days. The cells were either harvested and assayed for caspase-3 activity or stained with either AE5 or antivimentin antibodies. Media samples were concentrated and assayed for MMP activity by zymography or using a fluorogenic substrate. ELISA was used to quantify IL-1 $\alpha$ , MMPs -1,-2,-3,-9, and TIMPs -1 and -2.

**Results:** IL-1 $\alpha$  and LPS had no effect on MMP/TIMP production by cultured corneal epithelial cells and keratocytes. Corneal MMP-2 inhibition by doxycycline was partially [Ca<sup>2+</sup>] dependent but irreversible. At the minimum inhibitory concentration, 100  $\mu$ m, doxycycline had no apparent effect on MMP and TIMP production, but ultimately caused the death of keratocytes and some of the epithelial cells that detached from their basement membrane. Caspase-3 activity was not detected in dead or dying keratocytes. The mechanism of cell death in cultured corneal epithelial cells was not caspase-3 related apoptosis as the activity of this enzyme, normally detectable, was lost. The epithelial cells that survived doxycycline treatment did not bind antivimentin antibody and compared with controls, reacted less with the AE5 antibody. They were probably transient amplifying cells.

**Conclusions:** Doxycycline irreversibly inhibits corneal MMP-2 activity by chelating the metal ions that are catalytically and structurally essential. Corneal MMP/TIMP production *in vitro* is not modulated by IL-1 $\alpha$ , LPS, or doxycycline. The therapeutic value of doxycycline may depend upon its effective concentration at the ocular surface and probably relates to its chelating properties.

Doxycycline ( $\alpha$ -6 deoxy-5-hydroxytetracycline) is a metal ion chelator and a broad spectrum antibiotic that prevents access of acyl t-RNA to the acceptor site on the mRNA-30s ribosomal subunit complex. In this role it is known to be more effective against bacteria than mammalian cells because the former actively transport the compound through their cytoplasmic membranes. No such carrier systems exist on the cytoplasmic membranes of mammalian cells.<sup>1</sup>

In addition to these established functions, other properties that have recently been ascribed to doxycycline include differential inhibition of the activity of members of the matrix metalloproteinase (MMP) family,<sup>2-5</sup> inhibition of

MMP synthesis,<sup>6-9</sup> inhibition of interleukin-1 synthesis,<sup>10 11</sup> inhibition of activated B cell function,<sup>12</sup> inhibition of nitrous oxide (NO) synthesis by lipopolysaccharide (LPS) activated macrophage,<sup>13 14</sup> and inhibition of collagen synthesis by cultured chondrocytes.<sup>15</sup>

Doxycycline is an orally administered drug. With varying degrees of success it has been used to treat patients with rheumatoid arthritis.<sup>4 16</sup> It is also a recommended treatment for patients with ocular surface diseases, particularly recurrent epithelial cell erosion,<sup>9 17</sup> rosacea,<sup>18</sup> and keratitis sicca.<sup>19</sup> In the latter cases, successes have been attributed to the inhibition of tear film MMP and interleukin-1 (IL-1) synthesis.<sup>3 20</sup>

The aim of this study is to evaluate the effect of doxycycline on the synthesis and activity of corneal MMPs and their inhibitors (TIMPs) and on the viability of cultured corneal epithelial cells and keratocytes.

## METHODS

## Tissue culture

Normal corneas, which proved to be unsuitable for transplant but were received with research permission, were obtained from the Bristol CTS Eye Bank. All cell cultures were prepared in a class 11 safety cabinet and incubated at 36°C under 5% CO<sub>2</sub>.

Keratocytes were cultured from central corneal stromal explants placed in 25 ml tissue culture flasks with Dulbecco's MEM medium (Invitrogen, Paisley, UK) containing an antibiotic, antimycotic cocktail (Sigma, Dorset, UK), L-glutamine and foetal calf serum (FCS, 10% v/v) as described previously.<sup>21</sup> The cells were trypsinised during the logarithmic phase of growth, aliquoted into 6 well plates and grown to confluence for experimentation.

Epithelial cell cultures were prepared by plating out three 2 mm diameter explants taken from the anterior limbus region of corneas in each of six well plates wetted with MEM containing 15% (v/v) FCS. Once attached, additional growth medium (2.5 ml) was added. The growth medium was replenished every 3–4 days and the cell cultures were used for experimentation when approximately 70% confluent.

## Experimental cell incubation conditions

Doxycycline is very sparingly soluble in aqueous solution and at concentrations exceeding 300  $\mu$ M it observably precipitates and/or pulls the MEM salts out of solution. For these reasons, stock solutions of solubilised doxycycline were prepared in dimethyl sulphoxide and the growth media solutions, made up to a maximal concentration of 200  $\mu$ M doxycycline, were not filter sterilised after its addition. Both epithelial cell and keratocyte cultures were incubated, minimally in triplicate, in serum free medium or serum free medium containing either

**Abbreviations:** FCS, fetal calf serum; HRP, horseradish peroxidase; LPS, lipopolysaccharide; MMP, matrix metalloproteinase; PBS, phosphate buffered saline; RFU, relative fluorescence unit

IL-1 $\alpha$  (10  $\mu\text{g/ml}$ ), lipopolysaccharide (LPS ex *E coli*, Sigma, 10  $\mu\text{g/ml}$ ), doxycycline (Sigma, 0–200  $\mu\text{M}$ ) or a combination of LPS (10  $\mu\text{g/ml}$ ) and doxycycline (100  $\mu\text{M}$ ). After 4 days the media was collected and centrifuged at 1500 rpm for 3 min to pellet detached cells. These were resuspended and counted using a haemocytometer. Small samples of the culture media were retained for zymographic analysis. Ammonium sulphate (80% w/v) was added to the remainders to precipitate the cell secreted proteins. After 16 h these were collected by centrifugation at 12 500 rpm, 4°C and after resuspension in 0.05 M Tris HCl, pH 7.4, stored at –80°C prior to analysis.

#### Cell growth and/or respiratory rate measurements

Alamar blue (BioSource Europe, SA, Nivelles, Belgium) was added to the cell culture media at a concentration of 5% (v/v). The kinetics of its reduction were followed fluorimetrically over 80 min (excitation 540 nm, emission 590 nm).

#### Protein concentration estimation

The concentration of the secreted proteins was measured at 280 nm in 96 well plates using solutions of BSA of known concentration as calibration references.

#### Apoptosis assays

Caspase-3 activity was measured in epithelial cells and keratocytes cultured in the absence and presence of doxycycline. After counting with a haemocytometer, the cells pelleted from media samples were combined with the cells that had remained attached to their matrix and required trypsinisation to release them. A fluorimetric (CASP-3-F) assay kit was purchased from Sigma Cell lysates were obtained and assayed for caspase-3 activity as directed.

#### MMP detection and activity assays

##### Substrate polyacrylamide gel electrophoresis

The protocol first described by Unemori and Werb<sup>22</sup> was followed. As described previously, all samples were applied to the gels without heating or reduction,<sup>23</sup> at fixed volume (culture media samples) or fixed protein concentration (concentrated protein samples). High molecular weight markers (210 000–25 000; Sigma) were routinely included on each gel.

##### Using Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH<sub>2</sub> as substrate

The Gly-Leu bond of peptides may be cleaved by almost all MMPs. This particular substrate (40  $\mu\text{M}$ ) was used to investigate the effects of IL-1 $\alpha$ , LPS and doxycycline on the activity of the MMPs secreted by cultured corneal epithelial cells and keratocytes because the molar extinction coefficient of the Mca-Pro-Leu product is extremely high at its emission wavelength. The reaction kinetics were followed fluorimetrically (excitation 325 nm, emission 393 nm) at 30°C in 0.05 M Tris HCl buffer pH 7.6 containing 0.2 M NaCl, 5 mM CaCl<sub>2</sub>, and 20  $\mu\text{M}$  ZnCl<sub>2</sub>.

#### Western blot immunoanalysis

For comparative purposes, all samples were of similar protein concentration and were loaded, alongside reference proteins, on 9% polyacrylamide gels after reduction (1% v/v mercaptoethanol) and heating in boiling water for 2 minutes.<sup>24</sup> After electrophoresis, the separated proteins were electroblotted onto immobilon PDVF membranes (Millipore Ltd Hertfordshire, UK) and probed with either polyclonal rabbit anti MMP-1, anti MMP-9, or anti IL-1 $\alpha$  antibodies, Chemicon, Hampshire, UK). The secondary antibodies were horseradish peroxidase (HRP) linked and diaminobenzidine

was used (0.075% v/v) as substrate to visualise the immunoreactive proteins.

#### ELISA quantification

Unless otherwise stated, antihuman polyclonal antibodies raised in rabbit and purchased from Chemicon were used for this investigation. A selection of the MMPs and TIMPs secreted by the cultured corneal epithelial cells and keratocytes were quantified by ELISA. All samples quantified together were of a similar protein concentration (40–100  $\mu\text{g}$ ). They were also assayed in triplicate alongside a dilution series of reference protein of known concentration. Each well of a 96 well plate was blocked with phosphate buffered saline (PBS) containing 5% FCS before sequentially adding the appropriate primary antibody and HRP linked secondary antibody. The plates were extensively washed with TBS/TBS-Tween/TBS-FCS before and after adding the antibodies. Tetramethylbenzidine (Sigma) dissolved in phosphate citrate buffer containing 7.5  $\mu\text{l}$  H<sub>2</sub>O<sub>2</sub> was used as the HRP substrate. The kinetics of reduction were followed at 370 nm, 30°C.

#### Immunohistology

AE5 is an antibody that binds cytokeratin 3 and is used as a marker of corneal epithelial cell differentiation.<sup>25</sup> Vimentin is a structural protein that is ubiquitously present in fibroblasts but may also be detected in limbal stem cells. Thus AE5 (ICN, Hampshire, UK) and antivimentin (Chemicon) antibodies were used to investigate the phenotypic composition of the cultured epithelial cells. These were fixed in situ using formal saline (10% v/v), permeabilised using Triton X-100 (0.25% in PBS) then sequentially incubated with either the AE5 or antivimentin antibodies, diluted 1:500 and the appropriate fluorescent secondary antibody diluted 1:100 for 1 hour at 37°C. After incubating with antibody, the cultures were washed three times with PBS containing Tween 20 (0.1%). The cultures were photographed using a digital camera mounted on a fluorescent microscope.

## RESULTS

### Effect of doxycycline on MMP-2 activity

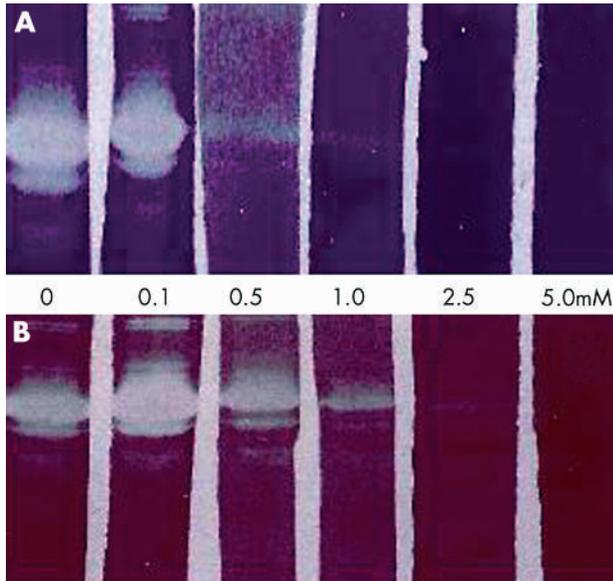
In initial experiments corneal MMP-2 activity was measured in the presence of doxycycline (0–200  $\mu\text{M}$ ) using the quenched fluorescent peptide as substrate. The results obtained (table 1) indicated that the activity of this enzyme was not inhibited at concentrations below 20  $\mu\text{M}$  and because doxycycline is yellow, the observed activity inhibition at the higher concentrations could have resulted from colour quenching. The zymographic MMP-2 activity data did not support this possibility. Furthermore, as MMP activity is Ca<sup>2+</sup> dependent and it was subsequently shown that the inhibitory effects of doxycycline were attenuated by the inclusion of excess Ca<sup>2+</sup> in the assay buffer (fig 1), metal ion chelation is the most likely mechanism of doxycycline inhibition.

Additional experiments in which doxycycline (0–200  $\mu\text{M}$ ) was included in the culture medium of corneal epithelial cells and keratocytes again indicated that the measurable activity

**Table 1** Effect of doxycycline on MMP-2 activity

Doxycycline ( $\mu\text{M}$ )	Corneal MMP-2 activity (RFU/sec)
0.0	32.0 (1.8)
1.0	35.9 (2.2)
20.0	34.6 (1.8)
100.0	21.2 (1.0)
1000.0	6.0 (0.3)

RFU, relative fluorescence units.

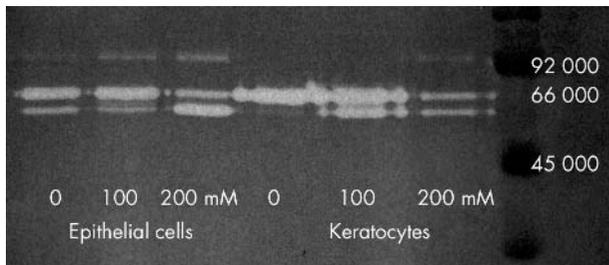


**Figure 1** Effect of doxycycline on the zymographic activity profile of corneal MMP-2 when included at varying concentration in incubation buffer containing 2 mM Ca<sup>2+</sup> (A) or 10 mM Ca<sup>2+</sup> (B).

of the secreted MMPs, assayed with the quenched fluorescent peptide substrate, was reduced by doxycycline only at concentrations exceeding 50 μM (data not shown). However, except in the case of the keratocytes incubated with 200 μM doxycycline, it was apparent from the zymographic activity profiles (fig 2) that the compound effected the appearance of the 62 000 M<sub>r</sub> species of MMP-2 and also that the observed reduction in activity was not a result of suppressed enzyme synthesis.

**Effect of doxycycline on MMP and TIMP synthesis in cultured corneal epithelial cells and keratocytes**

The zymographic MMP-2 activity data suggested that doxycycline, at concentrations not exceeding 100 μM, had little effect on MMP-2 production. To confirm this and determine the overall effect of the compound on corneal MMP and TIMP synthesis, the epithelial cell and keratocyte secreted proteins were quantitatively assayed for MMP-1, MMP-2, MMP-3, MMP-9, TIMP-1, and TIMP-2. The experimental data obtained (table 2) provided no evidence that doxycycline selectively or otherwise inhibited MMP and TIMP synthesis. MMP-3 was either absent or present at very low concentrations in both epithelial cell or keratocyte protein samples. Additionally, because the ELISA estimates of the concentrations of MMP-1 in the samples of epithelial cell secreted protein and MMP-1 and MMP-9 in the samples of keratocyte secreted protein were low, and because western



**Figure 2** Effect of doxycycline on the zymographic activity profile of MMP-2 secreted by cultured corneal epithelial cells and keratocytes.

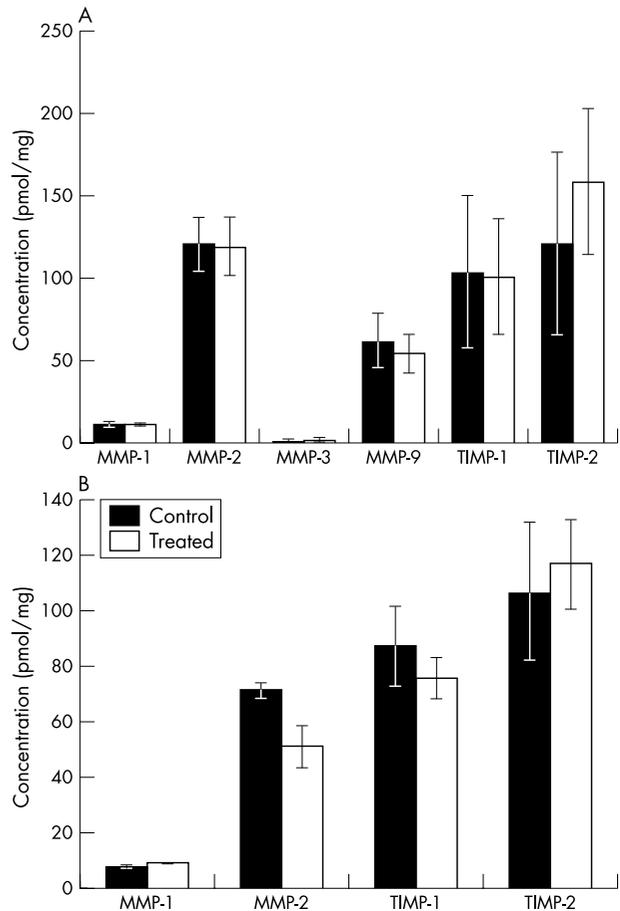
**Table 2** Effect of doxycycline on MMP and TIMP synthesis in corneal cell cultures

Doxycycline (μM)	MMP-1	MMP-2	MMP-9 (pmol/mg)	TIMP-1	TIMP-2
Epithelial cells*					
0	8.2 (0.8)	92.0 (25.2)	53.7 (14.3)	98.2 (11.9)	227.8 (22.1)
100	7.7 (0.6)	87.7 (27.8)	51.3 (19.9)	108.0 (10.8)	226.5 (19.1)
200	5.8 (1.6)	79.0 (28.9)	41.6 (12.8)	98.0 (38.0)	192.0 (25.4)
Keratocytes*					
0	8.3 (1.9)	97.2 (20.2)	<2.0	131.2 (18.1)	202.0 (20.7)
100	7.6 (1.4)	80.8 (9.4)	<2.0	122.3 (15.0)	205.0 (25.2)

\*Culture numbers = 5.

blots of the keratocyte secreted proteins probed with anti MMP-1 and anti MMP-9 antibody picked up many high molecular weight proteins but none corresponding to MMP-1 and MMP-9 reference proteins (data not shown), the existence of these proteins in the samples is also questionable.

Although doxycycline does not appear to inhibit MMP synthesis directly, a number of reports have suggested that doxycycline may inhibit MMP synthesis as an indirect consequence of inhibiting IL-1α synthesis.<sup>10 11</sup> Thus experiments were carried out to determine whether IL-1α, added to



**Figure 3** Effect of IL-1α on cultured corneal epithelial cell (A) and keratocyte (B) MMP and TIMP production.

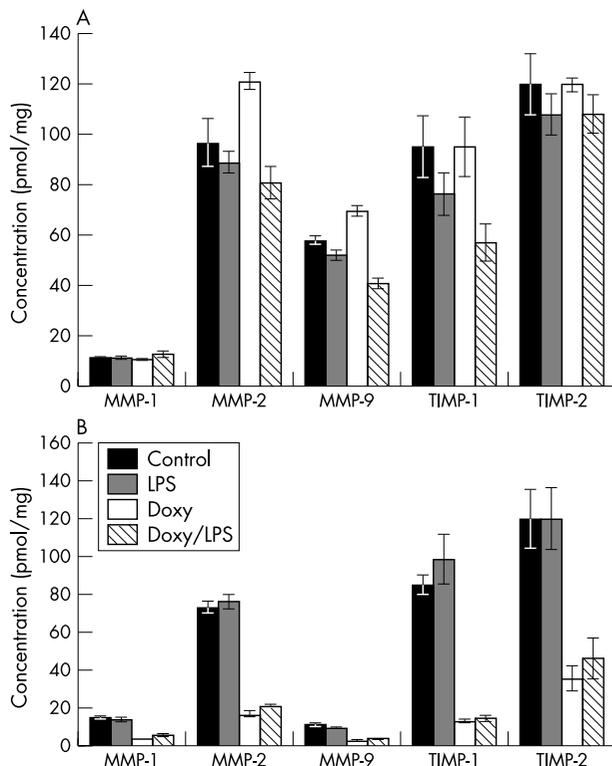
the culture medium of corneal epithelial cells and keratocytes, upregulated MMP or TIMP synthesis. The data presented in fig 3, pertaining to a series of experiments in which the concentration of exogenous IL-1 $\alpha$  was 10  $\mu$ M, indicated that this was not the case; similar results were also obtained at a 10-fold higher concentration of the cytokine.

Whereas LPS may stimulate the production of proinflammatory cytokines including IL-1, doxycycline apparently inhibits their synthesis.<sup>26</sup> Thus, further experiments were carried out to determine whether IL-1 $\alpha$  could be detected in the culture medium of corneal epithelial cells and keratocytes incubated in the presence or absence of lipopolysaccharide (LPS, 10  $\mu$ g/ml) and/or doxycycline (120  $\mu$ M) for 4 days and secondly whether these treatments modulated MMP and TIMP synthesis. Neither IL-1 $\alpha$  quantification by ELISA (detection limit 45 pmol/mg) or western blot immunoanalysis of the concentrated culture media samples (data not shown) provided positive evidence that this cytokine was secreted by the cultured corneal epithelial cells or keratocytes in response to treatment with LPS. Although the LPS and doxycycline also had no effect on the quantity of MMP-1, -2, -3, and -9 and TIMP-1 and -2 recovered in the samples of epithelial cell secreted protein, this was not the case for the samples of keratocyte secreted protein. However, although doxycycline did cause a significant reduction in the MMP and TIMP content of these samples (fig 4), this was considered to be a consequence of cytotoxicity.

### Toxic effects of doxycycline on epithelial cells and keratocytes

#### Effect of doxycycline on cell viability

Although the concentration of doxycycline required to significantly inhibit MMP-2 activity (100  $\mu$ M) was apparently insufficient to inhibit MMP synthesis *in vitro*, after three or four days of incubation with the compound, it became apparent that around this concentration doxycycline



**Figure 4** Effect of doxycycline and LPS on MMP and TIMP secretion by corneal epithelial cells (A) and keratocytes (B).

affected the cells, particularly the keratocytes (fig 5). In subsequent experiments the rate of alamar blue reduction, usually used as a method of determining viable cell number, was measured before and after a 4 day incubation with/without doxycycline (120  $\mu$ M) and/or LPS (10  $\mu$ M). The data from these experiments, shown in table 3, confirmed that doxycycline added at a concentration of 120  $\mu$ M killed keratocytes. At this concentration, it also effected the detachment of (some) of the cultured epithelial cells but given that the rate of alamar blue reduction actually increased in these cultures, the cells were not apparently dead or dying.

### Caspase-3 activity assays

The results of assaying the cultured keratocytes and epithelial cells and for caspase-3 activity after incubation with doxycycline are given in table 4. In all keratocyte samples the activity of this enzyme was on the limits of detection. By contrast, the observation that caspase-3 activity was highest in the population of epithelial cells that had not been exposed to doxycycline indicated that the caspase-3 apoptotic pathway is the mechanism by which corneal epithelial cells are normally turned over in culture, but it is not induced or enhanced by doxycycline. Furthermore, the doxycycline concentration dependent reduction in caspase-3 activity probably indicates that for both epithelial cells and keratocytes, the compound initiates cell death by inhibiting protein synthesis *in toto*.

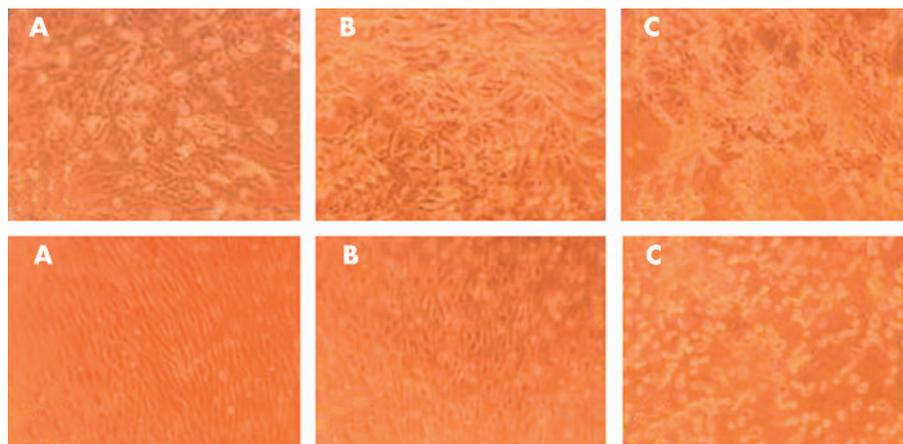
### Immunohistological investigation of cell cultures treated with doxycycline and LPS

Despite being expanded from limbal explants, the corneal epithelial cultures contained two visually distinguishable types of cell. The smaller of these were considered to be the basal cells or transient amplifying cells (TACS) and the larger cells the superficial wing cells. Although LPS had no apparent effect upon the morphological appearance of the epithelial cell cultures, the majority of the cells that remained matrix bound after doxycycline treatment were of the smaller variety. Negative results were obtained after probing control epithelial cell cultures or those treated with doxycycline and/or LPS with antivimentin antibody and the lack of fluorescent cells indicated that the cultures neither contained putative stem cells nor were contaminated with keratocytes. By contrast, after probing with AE5 fluorescent images of the epithelial cells were observed but compared with the control or LPS treated cultures, there was a marked reduction in the fluorescence intensity of those that had been treated with doxycycline and remained matrix bound (fig 6). This observation provides further evidence that doxycycline treatment has no effect on basal cells but induces the detachment of the more highly differentiated epithelial cells.

### DISCUSSION

Doxycycline is an effective metal ion chelator and not surprisingly therefore, inhibits MMP activity. Claims have been made that doxycycline also inhibits the synthesis of these proteases and that this is an indirect consequence of inhibiting the synthesis/secretion of the proinflammatory cytokine IL-1.<sup>10 11 27 28</sup> Thus, in an investigation of the anti-inflammatory properties of doxycycline, the effect of this compound on MMP activity and the interrelation between IL-1 $\alpha$  and corneal MMP/TIMP systems were examined.

MMP-2 is the major MMP secreted by corneal keratocytes. Partially activated preparations of this enzyme, visualised by zymography and assayed using a quenched fluorescent peptide as substrate, were used to determine the inhibitory concentration range of doxycycline. The results obtained indicated that a minimum concentration of 50  $\mu$ M was



**Figure 5** Effect of doxycycline on the morphological appearance of cultured epithelial cells (top row) and keratocytes (bottom row) A=0, B=100µM, C=200µM.

required to detect activity inhibition in the presence of 5 mM Ca<sup>2+</sup>. Given that the role of this metal ion is to hold the enzyme in its catalytically active conformation and that the Ca<sup>2+</sup> dependence of corneal MMP-2 is reversible, the further observation that this could be attenuated by increasing the concentration of exogenous Ca<sup>2+</sup> was compatible with the fact that doxycycline is a chelating agent. Similar inhibition kinetics were however observed when the activity of the MMP-2, recovered from the media of keratocytes incubated with doxycycline (0–200 µM), was assayed. The results of these experiments showed that the activity inhibition was neither reversible on removal of doxycycline nor related to a reduction in the quantity of MMP-2 secreted. It was therefore considered likely that doxycycline partially extracted the tightly bound catalytic zinc ion from the active site of the protein. This is known to be an irreversible process for, after treating corneal MMP-2 with the zinc ion chelator phenanthroline, activity cannot be restored with exogenous Zn<sup>2+</sup>.<sup>21 29</sup>

Zymographically MMP-2 presents in two forms, one of *M<sub>r</sub>* 66 000, believed to be the inactive or latent form of the enzyme,<sup>30</sup> the other of *M<sub>r</sub>* 62 000. The proportion of the latter is significantly increased by adding doxycycline to the culture medium of either keratocytes or epithelial cells and may visually account for the reduction in MMP-2 activity assayed directly with the quenched fluorescent peptide substrate: Though the *M<sub>r</sub>* 62 000 species of MMP-2 has been considered to be the activated form of MMP-2,<sup>31 32</sup> we have previously shown that it differs from the *M<sub>r</sub>* 66 000 species only in conformation. Moreover Ca<sup>2+</sup> depletion, which would result from doxycycline treatment, favours the *M<sub>r</sub>* 62 000 species, which may be the conformer cleaved to generate activated enzyme but is actually of lower specific activity than that of the *M<sub>r</sub>* 66 000 species.<sup>33</sup>

Some MMPs are inducible enzymes<sup>34 35</sup> and can be pathologically activated. As examples MMP-1 is abnormally

found in corneas of patients with rheumatoid peripheral ulcerative keratitis<sup>36 37</sup> and in corneas with chemical or thermal burns.<sup>38 39</sup> The regulatory agents are growth factors and cytokines. Doxycycline, in addition to inhibiting MMP activity apparently inhibits MMP synthesis and the synthesis of proinflammatory cytokines, particularly IL-1, which may induce the synthesis of MMP-9 in cultured corneal epithelial cells and MMP-1, -3, and -9 in cultured keratocytes.<sup>10 40 41</sup> Thus, to investigate the direct effect of doxycycline on MMP and TIMP synthesis and on the IL-1α dependent MMP and TIMP synthesis in cultured corneal epithelial cells and keratocytes, the quantities of MMP-1, MMP-2, MMP-3, MMP-9, TIMP-1, and TIMP-2, secreted by these cells over a fixed time period, were determined. In these experiments it was shown that the epithelial cell protein preparations contained MMP-2 and MMP-9 but little or no MMP-3 and MMP-1, and the keratocyte protein preparations contained MMP-2 which is constitutively produced in vivo and in vitro and little or no MMP-3, MMP-1, and MMP-9. It was also shown that doxycycline, included in the culture medium of corneal epithelial cells and keratocytes in the concentration range 0–100 µM, did not significantly inhibit the synthesis of MMP-9 and/or MMP-2 respectively. These findings were contrary to expectation in view of a previous report<sup>10</sup> that doxycycline inhibits MMP-9 synthesis at a concentration of only 20 µM, but does not prevent MMP-3 from cleaving and activating corneal epithelial cell MMP-9 at a concentration of 200 µM.

Furthermore, in the subsequent experiments in which IL-1α was added to culture medium at a concentration of either 10 µM or 100 µM with or without doxycycline (100 µM) the cytokine neither upregulated or induced MMP-1, MMP-2, MMP-3, or MMP-9 synthesis in keratocytes or corneal

**Table 3** Effect of doxycycline on the rates of alamar blue reduction by cultured epithelial cells and keratocytes (n = 6)

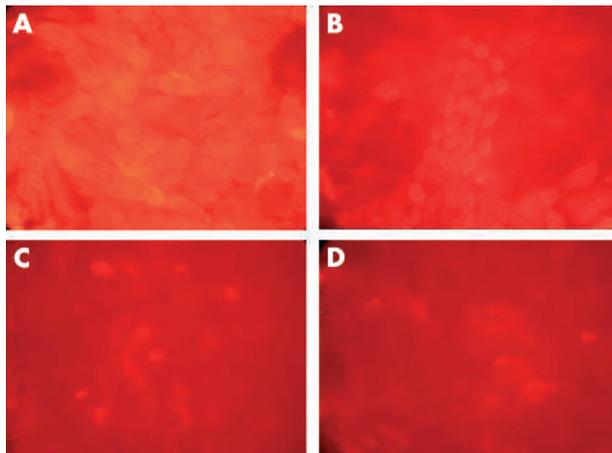
Culture conditions	Rate of alamar blue reduction (RFU/min)		
	Epithelial cells		Keratocytes
	Day 1	Day 4	Day 4
Control	0.9 (0.3)	1.1 (0.1)	4.8 (0.4)
+ LPS (10 µg ml <sup>-1</sup> )	0.9 (0.2)	0.9 (0.2)	5.1 (0.4)
+ Doxycycline (120 µM)	0.9 (0.3)	1.5 (0.4)	1.6 (0.1)
+ Doxycycline + LPS	0.8 (0.2)	1.6 (0.4)	1.3 (0.1)

RFU, relative fluorescence units; LPS, lipopolysaccharide.

**Table 4** Caspase-3 activity of corneal epithelial cells and keratocytes treated with doxycycline

Sample	Caspase-3 activity (RFU/sec)		
	Doxycycline (µM)		
	0	100	200
Epithelial cells (E)*	0.46 (0.02)	0.36 (0.04)	0.12 (0.02)
E + caspase	2.00 (0.02)	1.81 (0.00)	1.23 (0.02)
E + caspase inhibitor	0.01	0.01	0.01
Caspase	1.30	—	—
Keratocytes (K)†	0.02	0.01	0.01
K + caspase	0.01	0.01	0.01

\*Approx 2 × 10<sup>5</sup> cells/assay; †approx 5 × 10<sup>5</sup> cells/assay. RFU, relative fluorescence units.



**Figure 6** Immunostained epithelial cell cultures. (A) Control; (B) cultured with doxycycline (120  $\mu$ M), immunostained with AE5 antibody; (C) cultured with doxycycline (120  $\mu$ M), immunostained with antivimentin antibody; (D) control, primary antibody omitted.

epithelial cells and no inhibitory effects of doxycycline on the activity of these enzymes were noted.

LPS may stimulate IL-1 $\alpha$  synthesis in inflammatory cells and also in corneal epithelial cells and keratocytes. In these cells the intracellular IL-1 is apparently present in the mature form whereas the precursor form predominates in their growth medium.<sup>10</sup> To test the possibility that it is LPS induced intracellular IL-1 that in turn induces MMP synthesis, the concentrations of the MMPs, TIMPs, and IL-1 $\alpha$  secreted by corneal epithelial cells and keratocytes incubated with LPS and/or doxycycline were also compared. These data also indicated that neither LPS nor doxycycline affected the quantities of MMP and TIMP recovered and, if present, the quantities of secreted IL-1 $\alpha$  were also very low. In the absence of any supportive evidence therefore, it was concluded that doxycycline, directly or indirectly, does not inhibit MMP synthesis at the minimum concentration required to inhibit corneal MMP-2 activity (100  $\mu$ M).

Although doxycycline (100  $\mu$ M) did not inhibit MMP production when included in the growth medium of corneal epithelial cells or keratocytes, after 3–4 days, morphological changes in the cultured cells became apparent and significant numbers of the epithelial cells in particular, lifted from their matrix. Though not apparently dead, these cells appeared to be differentiated epithelial cells (wing cells) that reacted strongly with the AE5 antibody. A plausible explanation for their detachment was that doxycycline, acting as a chelating agent, abstracted the Ca<sup>2+</sup> from hemidesmosomes and other Ca<sup>2+</sup> dependent junctional proteins. At concentrations exceeding 100  $\mu$ M, doxycycline caused cell death, but not, apparently, by the caspase-3 linked apoptotic mechanism as the activity of this enzyme, generally detectable in extracts of cultured epithelial cells was lost rather than increased. The keratocyte cultures were significantly more sensitive than the epithelial cell cultures and the apparent inhibition of MMP-2 production observed at 200  $\mu$ M doxycycline was simply a consequence of its cytotoxicity.

In conclusion, it has been shown that the minimum concentration of doxycycline required to inhibit the activities of corneal MMPs (100  $\mu$ M) is similar to that required to inhibit these enzymes in other tissues. If this concentration is achievable in the tears of patients treated systemically for ocular surface disorders then, in addition to inhibiting MMPs that have been pathologically activated, it may kill migratory keratocytes or fibroblasts responsible for the formation of scar tissue, promote complete coverage of the ocular surface

with epithelial basal cells and consequentially the development of a stable, stratified epithelium. Clinical reports suggest that doxycycline is most effective in the treatment of inflammatory ocular conditions. The results presented in this paper, though not supporting a hypothesis that the mechanism is inhibition of IL-1 synthesis in corneal epithelial cells, do not preclude the possibility that doxycycline either kills cells of the immune system or inhibits the synthesis/release of their cytokines and MMPs.

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