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ORIGINAL ARTICLE

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Inhibition by doxycycline of angiogenesis in the chicken chorioallantoic membrane (CAM)

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Abstract Doxycycline, a tetracycline derivative, has many properties in addition to its antibiotic activity, including inhibition of matrix metalloproteinases (MMPs) and the ability to chelate divalent cations including Ca^{2+}. It has been shown to inhibit endothelial cell growth in vitro, and reduce the development of experimental tumours, especially bone metastasis in a model of breast cancer. We examined the effects of doxycycline on angiogenesis in the chicken chorioallantoic membrane (CAM) model, and showed that doxycycline will cause loss of the chorionic plexus in CAMs when applied at day 8 of incubation, and the duration of this inhibition was dose-dependent. Repeated doses prolonged the inhibition, but following removal of the doxycycline there was rapid recovery of the chorionic plexus. The effects of doxycycline are in part mimicked by the MMP inhibitor 1,10-phenanthroline, and more closely by the Ca^{2+}-chelating agent EGTA. Doxycycline was equally effective in causing loss of the chorionic plexus by day 11 in CAMs, a time at which the blood vessels are established. Doxycycline has important potential as an antiangiogenic treatment. It is capable of inhibiting angiogenesis in an in vivo model, including the removal of comparatively mature endothelial cells. The response is sensitive to the dosing regimen and the effect is rapidly reversible.

Keywords Doxycycline · Anti-angiogenesis · Calcium chelation MMP inhibition · CAM model

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Introduction

The pharmacological effects of modified tetracyclines, including doxycycline, range beyond antimicrobial activity, and include inhibition of matrix metalloproteinases (MMPs), in particular its anticollagenase property [3, 4, 26], calcium and other cation chelating activity, a direct effect on cell proliferation [21, 40], and blocking inflammation-induced nitric oxide synthases [1]. Potential beneficial effects of doxycycline in a number of disease states have been identified including, as an anticancer agent [3, 4, 6, 7, 40], reducing intimal hyperplasia in a rat model [21], and, also in rats, reducing granulation, fibrous tissue formation, and vascularity in a model of tissue repair [26]. Regarding its possible role in cancer therapy, doxycycline decreases the tumour burden in a model of bone metastatic human breast cancer [3, 4], and inhibits the growth of a number of tumour cell lines including breast cancer cells [6], prostate cancer cells [7] and mesothelioma cells [40].

Tetracycline derivatives including doxycycline are considered to be antiangiogenic and this property has been largely attributed to anti-MMP activity [6, 13, 16, 18, 34, 43, 47], and to a lesser extent to a direct effect on endothelial cell proliferation [16]. However an MMP-independent mechanism has been suggested in an in vitro assay using aortic sprouting in fibrin gels [10]. Inhibition of angiogenesis in vivo by doxycycline has been demonstrated by the reduction of vascularization in the rabbit cornea in response to VX2 tumour cells [47], and the reduction of VEGF-induced vascular endothelial growth in the cerebrum of mice [27]. Antiangiogenesis was considered to be important in the reduction in tumour growth in mice [32], and in a patient with pulmonary capillary haemangiomatosis [11]. Doxycycline inhibits angiogenesis in vitro in a tube-forming assay and a chemically modified tetracycline, COL-3, inhibits endothelial cell proliferation [8], but a precise mechanism for this inhibition was not indicated. A novel possibility for the effect of doxycycline on angiogenesis

was provided by the observation that, at low concentrations doxycycline reverses the downregulation of thrombospondin (TSH), the natural angiogenic inhibitor expression in a tumorigenic mouse fibroblasts by suppressing the expression of H-ras. However, induction of TSH and suppression of H-ras by doxycycline is not seen under hypoxic conditions [24].

To better define the mechanism of the antiangiogenic action of doxycycline in vivo, and to evaluate the importance of dose regimens, we examined the effects of doxycycline on angiogenesis in the chick chorioallantoic membrane (CAM) model.

Methods

The experimental protocol for the use of chick embryos was consistent with the guidelines of the Canadian Council for Animal Care and received approval from the Animal Research Ethics Board, McMaster University. Fertile eggs from Stone White hens were obtained from a local supplier (Flemmings, Beamsville, Ontario). Eggs were cracked on day 4 of incubation (day 4) and each one cultured in a covered glass dish at 37°C in an atmosphere of air containing 3% CO_2 and saturated humidity.

All pharmacological agents were dissolved in avian Ringer's solution enriched with glucose [42] and applied to the surface of the CAM in 50-µl aliquots within each of two rings cut from Silastic tubing (Dow Corning; OD 10 mm, ID 8 mm) placed on the surface of each CAM. Control CAMs were exposed to avian glucose Ringer's solution with no added pharmacological intervention. For each time-point, at each concentration, and for each treatment a minimum of five CAMs were evaluated by en face analysis, and three by cross-section analysis (unless otherwise stated).

Recording images of the CAM

Digital images of the live CAM (instrumental magnification ×0.63, ×2 and ×4) were captured using a binocular dissection microscope (Leica MZ8, Heerbrugg, Switzerland) and a colour video camera (Sony Model DXC-950; Sony Corporation, Japan) interfaced with a frame grabber card (CG-7 PCI; Scion Corporation, Frederick, Md.), and saved as .tif files using a PC (Pentium II, 450 MHz). Images were recorded within each ring, and from an outside untreated area immediately prior to application of the test substance, and daily thereafter.

Tissue processing

At the end of the experiment, each embryo was killed by cervical dislocation and the CAM surface flooded with 2% glutaraldehyde buffered in 0.1 *M* sodium cacodylate, pH 7.5. Areas (2×2 mm) of the fixed CAM from each of the Silastic rings were removed, postfixed in 1% aqueous osmium tetroxide, dehydrated in a graded series of ethanol, and embedded in Spurr's resin. Sections of thickness 1 µm were mounted on glass slides and stained with toluidine blue, and digital images were captured using the camera and computer system described above, interfaced with a Zeiss light microscope (Carl Zeiss Canada, Guelph, Ontario). Images were recorded at instrumental magnifications of ×10 and ×40.

Application of doxycycline to the CAM

Doxycycline (Sigma-Aldrich Canada, Oakville, Ontario) was freshly prepared for each experiment and the solution protected from exposure to light.

Dose-related response

On day 8 of incubation 50 µl per ring of 25, 50, or 250 µ*M* doxycycline was applied to the CAM surface. The response was monitored daily from en face images and the CAMs were fixed and prepared for cross sections after 24 h (day 9), 48 h (day 10). The response was monitored en face until day 14, 6 days after the initial treatment.

Effect of repeated applications

Doxycycline (50 µ*M*) was applied to the surface of day-8 CAMs as above and repeated once on day 9, or three times (days 9, 10 and 11). The CAMs were killed on day 14, 15 or 16.

Effect of age of CAM

Doxycycline (50 µ*M*) was applied to the surface of day-11 CAMs as above, and the response monitored until day 14.

Application of 1,10-phenanthroline monohydrate

The general MMP inhibitor 1,10-phenanthroline (Sigma-Aldrich Canada) (50 µ*M* in avian glucose Ringer's solution) was applied to the surface of the CAM as above, and the response monitored for 48 h after which the embryo was killed and the CAM fixed and prepared for cross sections.

Application of calcium chelator, EGTA

EGTA [ethylene glycol-bis(oxyethylenenitilo)tetraacetic acid, 50 µ*M* in avian glucose Ringer's solution) was applied to the surface of the CAM on day 8 or day 11 in

two rings, using the same method as used with doxycycline. CAMs were observed for 48 h, fixed and prepared for cross sections.

Application of penicillin and streptomycin

Glucose avian Ringer's solution containing penicillin G (120 μg/ml) and streptomycin (100 μg/ml) (Gibco-Invitrogen, Burlington, Ontario) was applied to the surface of the day-8 CAM as above, and the response monitored for 48 h.

Morphometric evaluation

En face live images

A method previously described [19, 38, 42] was used to evaluate changes in the capillary plexus. Briefly, using digital images of the live CAM, magnification ×75, multiple areas (each approximately 0.2 mm^2 containing 100×200 pixels) were identified in which the illumination was even and which included only capillary plexus; approximately five such areas were identified in each micrograph. These images were converted to binary mode and the percentage of area containing red pixels (i.e. blood) was quantified using Adobe Photoshop (version 5.0; Adobe Systems, San Jose, Calif.) with the Image Processing Tool Kit (version 3.0; John. C. Russ, Reindeer Games, Raleigh, N.C.). The mean of these percentage values (mean capillary area, MCA) was calculated for every treatment at each time point.

The values for each experiment are presented as graphs (Figs. 1, 2, 3 and 4). The initial value (time 0) was calculated as 100%, and changes thereafter as percentage fractions of the initial value including the standard error (SE). In the graphs, inhibition (a value less than 100%) is shown as a negative value. Statistical comparisons were calculated from the raw data using Student's two-tailed *t* test; *P* values <0.05 were considered significant.

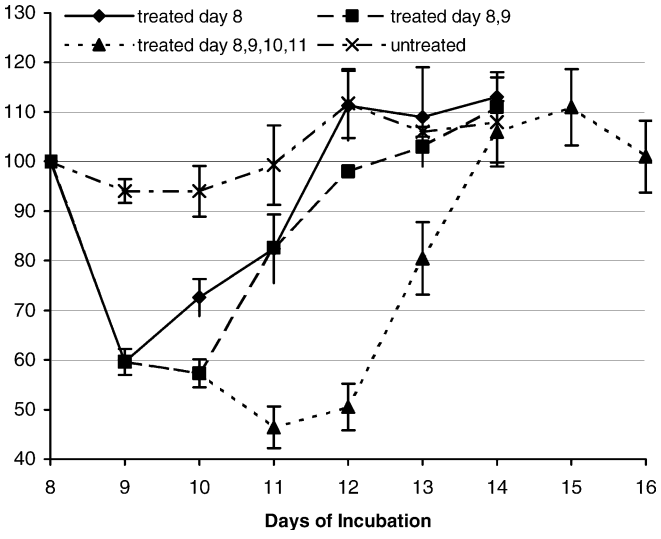

Fig. 2 Change in MCA (expressed as percent of value at beginning of experiment ±SE) following exposure to 50 μ*M* doxycycline on day 8, days 8 and 9, or days 9–11, and the response in untreated areas of the same CAMs ($n=5$ CAMs at all time points to day 13; $n=3$, days 14–16)

Cross sections

The length of the chorionic epithelium, and the total length containing capillaries were measured from cross sections, and the length containing capillaries expressed

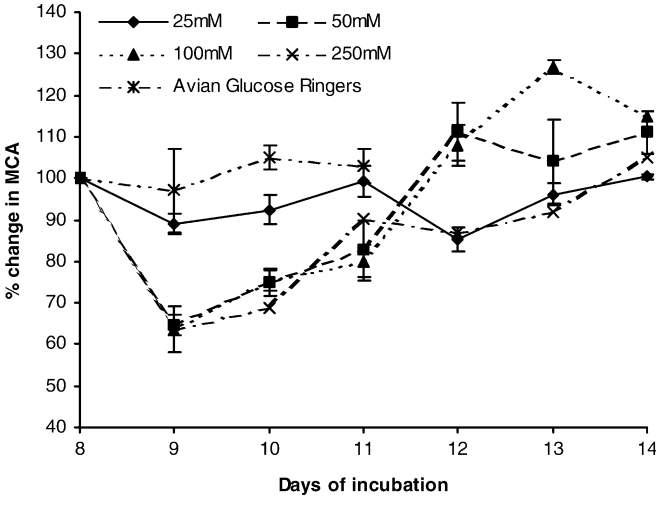

Fig. 1 Change in MCA (expressed as percent of value at beginning of experiment ±SE) following exposure of CAM surface to increasing concentrations of doxycycline ($n=5$ CAMs at all time points for each concentration)

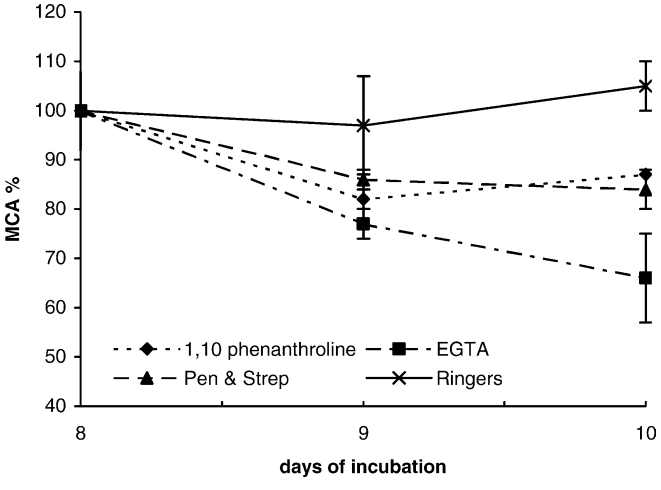

Fig. 3 Change in MCA (expressed as percent of value at beginning of experiment ±SE) following exposure to 50 μ*M* 1,10-phenanthroline, 50 μ*M* EGTA, penicillin and streptomycin, and avian glucose Ringer's solution applied to the CAM on day 8 ($n=5$ CAMs at all time points)

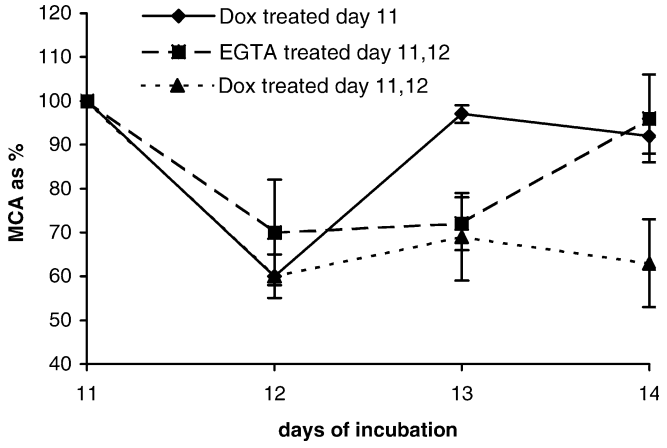

Fig. 4 Change in MCA (expressed as percent of value at beginning of experiment ±SE) of 11-day CAM following exposure to 50 μ*M* doxycycline or 50 μ*M* EGTA. There is significant inhibition (or removal) of the chorionic plexus after 24 h exposure to both treatments ($n = 5$ CAMs at all time points)

as a percentage of the total length. Morphological evidence for apoptosis was identified by light microscopy of plastic-embedded sections, and confirmed by transmission electron microscopy (TEM).

Results

Control CAMs

Consistent with previous observations [42], control CAMs, untreated or exposed to avian glucose Ringer's solution showed no significant change in the capillary plexus either en face (Figs. 1, 5d and 6e) or in cross section (Figs. 5h and 6f). By cross section the percentage of the chorionic surface which was occupied by the capillary plexus was 91 ± 1% ($n = 3$) on day 10, 98 ± 5% ($n = 5$) on day 11, and 89 ± 2% ($n = 4$) on day 14.

Dose-dependent response

The response to increasing doses of doxycycline is summarized in Fig. 1, and illustrated in Fig. 5a–c, e, f. Concentrations of 50 and 250 μ*M* doxycycline applied on day 8 of incubation resulted in a significant ($P > 0.001$) loss of capillaries after 24 h, but 25 μ*M* did not induce a significant reduction in the capillary plexus either en face or in cross sections on day 10 (capillaries 92 ± 2% ($n = 4$) of the chorionic surface). The inhibition after 24 h (day 9) was similar following application of 50 and 250 μ*M* doxycycline (Fig. 1). The trend towards recovery was seen on day 10, and by day 11 there was no significant difference between treated and control CAMs following either dose, but the rate of recovery following application of doxycycline at 250 μ*M* was slower than following application at 50 μ*M* (Fig. 1). The significant ($P > 0.01$) loss of capillaries induced by 50 μ*M* doxycycline seen on day 9 and day 10 in en face measurements was confirmed by cross section data: 42 ± 7% ($n = 3$) on day 9, 68 ± 6% ($n = 11$) on day 10, with total recovery to 94 ± 8% ($n = 2$) by day 14. Endothelial cells in apoptosis were observed in cross sections on days 9 and 10, and this was confirmed by TEM (Fig. 5d). Exposure to 250 μ*M* doxycycline caused a loss of capillaries in cross sections on day 10 of 61 ± 6% ($n = 8$).

Repeated applications of doxycycline

Following application of 50 μ*M* doxycycline on days 8 and 9, or days 8 to 11, a significant loss of capillaries ($P > 0.001$) was maintained throughout the period of application of doxycycline (Fig. 2, Fig. 6a, b). Repeated applications of 50 μ*M* doxycycline to the CAM continued to increase the amount of capillary loss compared to that seen following a single dose on day 8. Following removal of the doxycycline, recovery of blood flow to the chorionic epithelial surface was observed after 24 to 48 h (Fig. 6c, d). This was associated with growth of the large vessels, but this was not in the normal vascular pattern (Fig. 6c). Cross section data confirmed the regrowth of capillary vessels (< 96% for each treatment, Fig. 6d).

MMP inhibitor 1,10-phenanthroline

Application of the MMP inhibitor 1,10-phenanthroline at the same concentration as doxycycline (50 μ*M*) had no significant effect on the chorionic capillaries at 24 h, but by 48 h there was a significant ($P > 0.01$) reduction (Fig. 3); the capillaries in cross section occupied 76 ± 5% ($n = 5$) of the chorionic surface on day 10.

EGTA

Application of the Ca^{2+} chelating agent EGTA in the same concentration as doxycycline (50 μ*M*) caused a significant reduction ($p > 0.01$) in the chorionic capillaries at 24 h, and at 48 h after treatment (Fig. 3), and this was confirmed in cross sections on day 10 (31 ± 14%, $n = 5$, $P > 0.01$).

Penicillin and streptomycin

Avian Ringer's solution containing penicillin and streptomycin (concentration to inhibit bacterial growth in tissue culture) applied to the surface of the CAM resulted in a small but not significant ($p = 0.09$) reduction in the capillary plexus (Fig. 3).

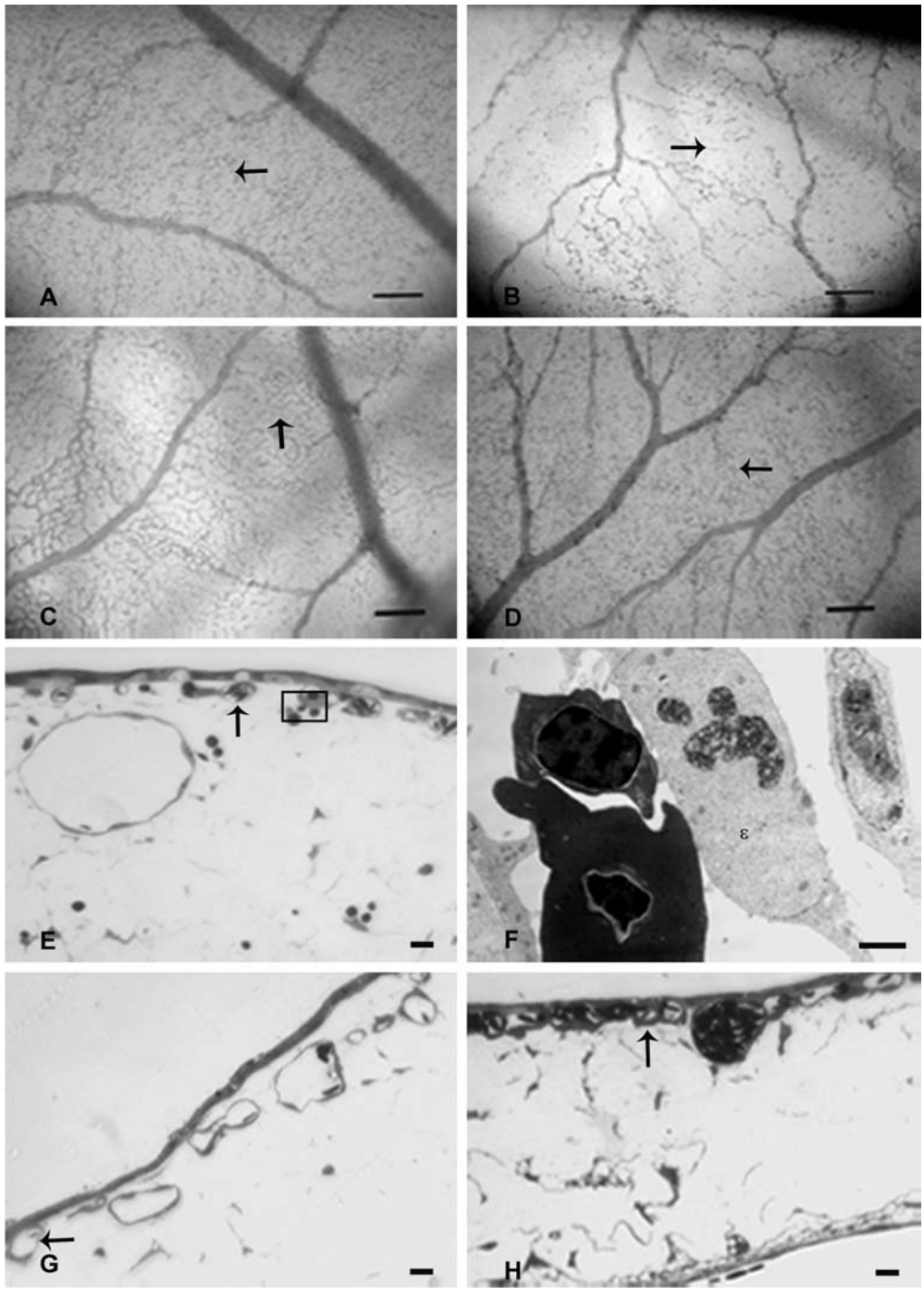

Fig. 5 En face images (**a–c**) and micrographs of cross sections (**e–g**) of CAMs treated with 50 μ*M* doxycycline (single application on day 8), and avian glucose Ringer's solution. **a** En face image on day 8 prior to application of doxycycline; **b** day 9 showing loss of capillaries; **d** day 10 showing recovery of capillaries. **e** Cross section from a day-9 CAM after 24 h exposure; **f** TEM of the area identified in **e** which includes cells with pyknotic nuclei, showing morphology consistent with apoptosis. **g** Cross section of a CAM on day 10 after 48 h exposure to doxycycline; **h** a CAM on day 10 after 48 h exposure to avian glucose Ringer's solution (en face images, *bar* 100 μm; cross sections, *bars* 10 μm; TEM *bars* 1 μm; *arrows* capillary, ϵ endothelial cell)

Effect of age of CAM

Application of doxycycline (50 μ*M*) to the chorionic epithelium on day 11 induced a significant ($P < 0.01$) loss in chorionic capillaries (Fig. 4), similar in magnitude to that seen following treatment on day 8 with either a single application on day 11 or two applications on days 11 and 12 (Fig. 2).

Application of EGTA (50 μ*M*) on days 11 and 12 also resulted in a significant ($P < 0.05$) loss of chorionic capillaries.

Apoptosis

In all cross sections from CAMs in which a significant loss of capillaries had been observed en face, endothelial cells which displayed pyknotic nuclei were observed (Fig. 5e). On examination by TEM, typical examples of these cells showed morphological features of apoptosis (Fig. 5f). Sections of samples from CAMs in which no loss of capillaries was observed did not appear to contain cells with apoptotic features.

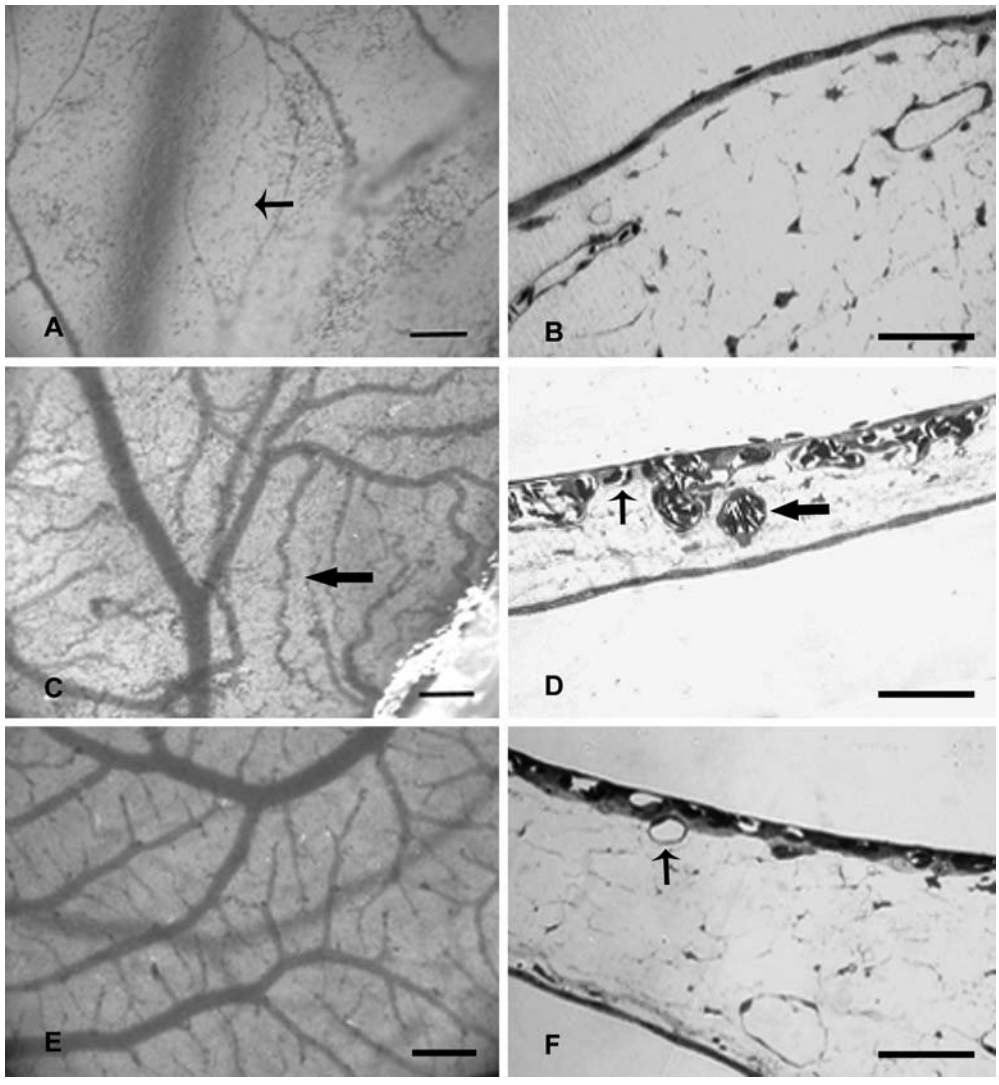

Fig. 6 En face images (**a, c**) and cross sections (**b, d**) of a CAM treated on four consecutive days (days 8–11) with 50 μ*M* doxycycline. **a** CAM en face on day 11 (after 4 days of exposure) showing marked inhibition of capillary growth, and (**b**) in cross section. **c** En face image on day 14 of a CAM treated on four consecutive days (days 8–11) with 50 μ*M* doxycycline, and (**d**) in cross section. There is a marked recovery including an abnormal pattern of blood vessel growth compared to the day-14 CAM shown in **e** and **f** which was treated with avian glucose Ringer's solution for 4 days

Discussion

Doxycycline inhibits angiogenesis in the CAM model as shown by a reduction in the chorionic capillary plexus viewed either en face or in cross section. The use of the CAM to examine doxycycline-induced antiangiogenesis provides a number of unique advantages over other systems [39, 43], and includes the ability to observe and quantify changes on an ongoing basis, to easily manipulate dose regimens, and to evaluate effects in rapidly growing or in comparatively mature blood vessels. Moreover, the CAM is known to contain MMPs [2, 37], making it especially relevant in this study. In contrast the CAM ex ovo exhibits a high rate of attrition in the early days of incubation, and there is some variation in response. It is necessary to correlate the en face observations with cross section data, but in all cases in the present study this correlation was good.

In this study, the capillary plexus of the CAM was reduced by approximately 50% by 24 h after application of doxycycline to the chorionic epithelial surface and there was morphological evidence of endothelial cell apoptosis. Increasing the concentrations of doxycycline prolonged the rate of recovery. Inhibition was maintained during repeated applications of doxycycline for up to 4 days, but there was recovery upon cessation of treatment. The inhibitory effect was not reduced in CAMs over 10 days of age, and this is in contrast to the effect of other antiangiogenic agents, including human angiostatin [42].

There was a minimal antiangiogenic effect of penicillin and streptomycin at the same concentrations used to sterilize tissue culture, excluding an antibiotic effect in the antiangiogenic response to doxycycline. In addition to its antibiotic activity, doxycycline will chelate cations, and is an inhibitor of MMPs 2 and 9 [41]. It is considered antimitotic to smooth muscle cells, especially those in G_0 [21]. When we compared the antiangiogenic effects of an MMP inhibitor (1,10-phenanthroline) and the chelating agent EGTA with the effects induced by doxycycline, all at the same molar concentration (50 μ*M*), both agents induced a significant loss of

capillaries, but that following EGTA was greater. These observations are consistent with doxycycline exerting its antiangiogenic activity through mechanism(s) related to Ca^{2+} sequestration as well as by inhibiting the action of MMPs.

There are a number of possible explanations for an effect through Ca^{2+} sequestration. It is unlikely that the chelation of Ca^{2+} induces apoptosis of endothelial cells, since apoptosis is associated with a sustained rise in cytosolic Ca^{2+} [46]. However, a reduction in intracellular Ca^{2+} signalling may initiate or mediate some of the cellular effects of endostatin and angiostatin because exposure to these antiangiogenic compounds results in a reduction in the elevation in Ca^{2+} in endothelial cells seen in response to VEGF or bFGF [22]. Ca^{2+} influx into endothelial cells induced by the ligation of the integrin $\alpha v\beta_3$ by extracellular matrix proteins, influences the release of vasoactive substances [25], and may affect the response to the interaction of this integrin with growth factors such as bFGF [5]. Ca^{2+} is involved in other cell surface receptors including platelet endothelial cell adhesion molecule (PECAM), and chelation of Ca^{2+} by EGTA results in the inhibition of PECAM activity and PGI_2 release [17]. Ca^{2+} chelation inhibits VEGF-induced activation of p38 MAPK [30]. High concentrations of doxycycline and another tetracycline derivative, minocycline, inhibit leukocyte functions as a result of the chelation of Mg^{2+} or Ca^{2+} [12]. However, since tetracyclines exert their bacteriostatic activity by preventing the binding of the aminoacyl t-RNA to the ribosome, and through a parallel mechanism interfere with the synthesis of mitochondrial protein in mammalian cells [41], the consequent increase in permeability and disruption of the mitochondrial outer membrane can lead to apoptosis [15]. Although it is not known to what extent mitochondrial damage may be involved in the process of antiangiogenesis, it is likely that it is an important factor.

The importance of the inhibition of MMP activity in angiogenesis is well recognized [2, 9, 14, 23, 28, 35, 36, 44], and the MMP inhibitor, 1,10-phenanthroline, used in this study caused a significant loss of capillaries. Generally it has been considered that the antiangiogenic effect of tetracycline derivatives is related to the ability to inhibit MMP-2 and -9, and this is consistent with our observations. However, there are paradoxical reports including low plasma levels of MMP-9 associated with an increase in tumour angiogenesis through the decreased synthesis of angiostatin [33], and the induction of endothelial apoptosis consequent on the detachment of endothelial cells from the basement membrane as a result of activation of MMPs [48].

The antiangiogenic affects of doxycycline in the CAM reported here appear to involve both Ca^{2+} sequestration, and the inhibition of MMPs. The importance of connective tissue collagen [20, 31] and fibronectin [37] and MMP-2 activity [2, 37] have been demonstrated in vasculogenesis in the CAM. Both minocycline [16] and doxycycline [45] inhibit collagenase, consistent with MMP inhibition being involved in the antiangiogenesis observed here. However, other mechanisms, including Ca^{2+} sequestration, appear to be involved in the inhibition of angiogenesis by doxycycline, consistent with observed effects on vascular sprouting in aortic rings [10]. In the day-8 CAM it is likely that the MMP levels were low, permitting doxycycline to sequester Ca^{2+} in addition to the Zn of the MMPs. Further, Ca^{2+} is involved in the activation of MMP-2 by pulmonary smooth muscle cells [29], and thus it is possible that doxycycline is able to exert an inhibitory effect on basement membrane degradation via both its anti-MMP and a Ca^{2+}-chelating ability.

The response to repeated applications of doxycycline for 2 or 4 days to the CAM showed that the inhibitory effect was maintained during the time of application, but recovery began on cessation of the treatment. This recovery included random growth of large vessels, resulting in bending and distortion and a loss of the normal branching pattern. The precise explanation for this reaction is unknown. It may be linked to an interaction between VEGF, integrins, connective tissue and Ca^{2+} [5, 25, 49].

The apparent ability of doxycycline and EGTA to induce a loss of chorionic capillaries in CAMs by day 11 of incubation is unusual and important. At this age the rate of growth of CAM blood vessels is minimal [39, 42]. Thus the loss of capillaries is consistent with removal of comparatively mature endothelial cells, and this is in sharp contrast to the effects of human angiostatin [42].

These observations indicate the potential for doxycycline as an antiangiogenic agent, even in the context of mature endothelial cells. The combined anti-MMP activity as well as the Ca^{2+}-chelating activity of doxycycline present a potent inhibitory mechanism, and removal of the doxycycline would permit complete recovery. The importance of dose regimen in its administration is illustrated. These features are consistent with doxycycline as an effective anti-cancer agent.

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