Effects of flunixin meglumine and dexamethasone on metalloproteinases in canine keratoconjunctivitis

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SUMMARY

To determine the effects of subconjunctivally injected flunixin meglumine (a nonsteroidal anti-inflammatory drug) and dexamethasone on matrix metalloproteinases (MMPs) in the tears of dogs with traumatic keratoconjunctivitis, six dogs with experimental keratoconjunctivitis were used. A 1cm incision was made in the corneal epithelium. Tear samples from each dog were collected prior to and one week after surgery under sterile condition. The dogs (group1) were treated with 1 mg/kg of subconjunctival flunixin meglumine (50 mg/mL). The dogs (group 2) were treated with 0.5 mg/kg of subconjunctival dexamethasone (8 mg/mL). Gelatine zymography was performed to determine enzyme activities in control and traumatic keratoconjunctivitis tear samples. Matrix metalloproteinases are present in tears of both normal and diseased eyes. All enzyme bands were totally inhibited by dexamethasone and only active MMP-9 partially inhibited by flunixin meglumine. Effect of flunixin meglumine and dexamethasone give insight into the treatment procedure and pathogenesis of these diseases and may provide useful information for other species, particularly man.

KEY WORDS : canine, metalloproteinases, keratitis, flunixin meglumine, dexamethasone.

INTRODUCTION

Ocular inflammation is a serious and frequently encountered problem in veterinary ophthalmology. Common causes include infection, trauma, immune-mediated problems and systemic disease [10]. A significant number of inflammatory corneal conditions are of unknown aetiology. For the successful management of ocular inflammation, the clinician must understand normal ocular anatomy and physiology, be able to recognize signs of ocular inflammation, identify the cause when possible, and select the appropriate therapy. In the early stages of chronic superficial keratitis, corneal epithelial cells proliferate and the superficial stroma is infiltrated by plasma cells and lymphocytes. As the disease progresses, melanocytes, histocytes and fibrocytes enter, with oedema and neovascularization occurring [21]. Macrophage production and secretion of large quantities of many MMPs after contact with matrix proteins is enhanced by surface determinants on activated T cells. T cells secrete much smaller amounts of MMPs, predominantly MMP-2 and 9 after stimulation by a range of chemotactic mediators [12]. Therefore, it is probable that damage caused to the eye is due to infiltrating polymorphonuclear neutrophils and associated inflammatory changes. Thus, it is reasonable to consider targeting the polymorphonuclear (PMNs) for therapy, or even the proteolytic enzymes they secrete such as metalloproteinases. These same enzymes, in disease, can lead to excessive destruction of the extracellular matrix. Elevated levels of MMP were found in the aqueous humour (AH) in humans with uveal inflammation and they may be important in tissue destruction and repair processes [11]. It was also demonstrated that MMP-2 and MMP-9 are present in tears of diseased eyes and increased levels of MMP were found in canine tears with traumatic keratoconjunctivitis [1].

The treatment of ocular inflammatory disease is varied and based on the segment of the eye involved, aetiology and severity of inflammation. Primary therapy should be directed toward removing or controlling the initial inciting cause, although concurrent nonspecific anti-inflammatory therapy is often indicated to prevent further ocular injury. Effect of steroids/non-steroids on MMPs in tear samples has not previously been investigated.

Corticosteroids are perhaps the most widely used ophthalmic medication. If used correctly they are an indispensable therapeutic modality in veterinary ophthalmology. However,
corticosteroids are often over used and used incorrectly [22]. Dexamethasone is one of the more potent topical ocular corticosteroids. Flunixin meglumine is used extensively in dog medicine to treat many inflammatory conditions [17].

The aim of this study was to determine the effects of subconjunctivally injected flunixin meglumine (a nonsteroidal anti-inflammatory drug) and dexamethasone on MMPs in the tears of dogs with traumatic keratoconjunctivitis.

Materials and methods

The dogs used were of unselected breed and of both sexes and weighed between 15 and 30 kg. Clinical examination confirmed the absence of ocular diseases. After one month quarantine and conventional health measures, all procedures have been performed in accordance with national local animal welfare legislation based on the European Council Directive [13,15]. They were anaesthetised with intravenous sodium thiopenthal (20mg/kg). The corneal epithelium (corneal limbus) was cut length (1 cm) and depth (0.05-0.08 mm) by sharp incision in the left side eyes. Right side eyes were used as control. Six dogs with experimental traumatic keratoconjunctivitis were used. They were divided into two groups. The dogs in group I were treated by flunixin meglumine and the dogs in group II were treated by dexamethasone.

Tear samples from each dog were collected with a 50 µL pipette that was placed in the lower conjunctival fornix. Care was taken to cause as little conjunctival trauma as possible during collection, prior to and one week after surgery under sterile conditions. Tears samples were also collected after treatment procedures. All tear fluids were centrifuged to remove cells and fragments; supernatants were stored as aliquots at -20°C until required. The dogs in group I were treated subconjunctively with 1 mg/kg with flunixin meglumine (50 mg/mL) once a day for five days. The dogs in group II were treated with a 0.5 mg/kg of subconjunctival melogine (50 mg/mL) once a day for five days. The dogs were divided into two groups. The dogs in group I were treated by flunixin meglumine and the dogs in group II were treated by dexamethasone.

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Gelatine zymography was performed to determine enzyme activities in control and traumatic keratoconjunctivitis tear samples. The assay was carried out at the Liverpool Veterinary School, Department of Veterinary Pathology.

GELATINE ZYMOGRAPHY

Gelatine zymography was performed as described by Coughlan and Clegg [5, 6, 8, 9]. Briefly, tears were diluted 1:20 in a Tris/HCl sample buffer containing 1.5% sodium dodecyl sulphate (SDS), 5% glycerol and 0.005% bromophenol blue, (pH 6.8) and incubated at 37°C for one hour prior to electrophoresis. Each sample (7.5 µL) was added to the gel (polyacrylamide gels containing 0.25% gelatine). The 64 kDa gelatinolytic band in an aliquoted batch of 10% foetal calf serum (FCS) was used as an internal standard on each gel (7.5 µL of a 1:10 dilution loaded per well). The effects of including 10mM, 50mM and 100mM ethylenediaminetetraacetic acid (EDTA) (MMP inhibitor) or 50mM phenylmethanesulfonyl fluoride (PMSF) (serine proteinase inhibitors) in the standard reactivation buffer (0.05 Tris/HCl, 1M NaCl, 0.005 CaCl2, 0.05 % v/v Bril 35, 0.02 % w/v NaN3) were also investigated.

QUANTIFICATION OF MMP ACTIVITY

Image analysis was used to quantify enzyme activities on the zymography gels. Gel images were captured and evaluated with image analysis software (NIH image 1.44). For each enzyme band, the area of substrate clearing and the mean intensity of the enzyme band were calculated. The activity value of the band was determined by multiplying the band area by its intensity. To allow comparison between gels, this value was divided by the activity of the standard foetal calf serum (FCS) which was applied to each gel, to give a relative activity value.

GELATINE DEGRADATION ELISA

An assay was developed to assess the breakdown of gelatine by MMP. Briefly, gelatine was coated onto a 96 well ELISA plate (Dynatech) by adding 100 µL of gelatine (0.5 mg/mL) /phosphate buffered saline (PBS) to each well and incubating for 1 hour at 37°C and 24 hours at 4°C. The solution was decanted and the plate washed with PBS. Tears, diluted in Tris/HCl (0.05 M), CaCl2 (0.005 M), Brij 35 (0.05 %) pH 7.6, were added (dilution of 1:40) and the plate incubated at 37°C for 2 hours. The samples were removed, the plate washed with PBS/Tween and a rabbit anti-gelatine antiserum was added at a dilution of 1:4000 in PBS/Tween and incubated at 37°C for 1 hour. After incubation the plate was washed with PBS/Tween. Anti-rabbit IgG-alkaline phosphatase conjugate was then added at a dilution of 1:4000 in PBS/Tween and incubated for one hour. The conjugate was removed, the plate washed with PBS/Tween, and Sigma 104 phosphatase substrate (1 mg/mL) in glycine buffer (Glycine 14.0 g, MgCl2 203 mg, ZnCl2 136 mg, NaOH 1M) (pH 10.3) added and the colour change read at 405nm between 30-60 minutes later.

STATISTICAL ANALYSIS

The data were analysed using the Mann-Whitney U test.

Results

GELATINE ZYMOGRAPHY

Normal tears

At 1:20 dilution, normal tears contained a single gelatino-lytic band at 62 kDa, of relatively low intensity. This was due to MMP-2 activity (Fig. 1), as defined by the band molecular weight.

Keratoconjunctivitis tears

There was an increase in the activity of the 62 kDa enzyme in keratoconjunctivitis tears, compared to the control group. Enzyme activity was seen at 94 kDa (MMP-9 monomer) and
and the 88 kDa band is due to active form of MMP-9. The 239 kDa band is due to dimer of MMP-9 in all of the keratitis samples (Fig. 1). These increases were statistically significant (p<0.001) (Fig 1).

**INHIBITION STUDIES**

All four enzyme bands (62, 88, 94 & 239 kDa) were partially inhibited by 10 mM EDTA and totally inhibited by 50 and 100 mM EDTA but not by 50 mM PMSF (specific serine proteinase inhibitors). This result indicates that the enzymes are MMP [1,8,9].

**Effect of Treatment**

From a subjective clinical assessment, both drugs appeared to be equally effective in reducing both the corneal and the keratoconjunctivitis. The dogs showed no sign of blepharospasm, an ocular discharge or hyperemia. Subconjunctival dexamethasone treatment inhibits all four enzyme bands (62, 88, 94 & 239 kDa). MMP-2 (62 kDa). The active form of MMP-9 (88 kDa) was partially inhibited by 50 mg of flunixin meglumine. On the other hand, proenzyme form of MMP-9 (94 kDa) was not inhibited by flunixin meglumine (Fig. 2).

**GELATINE DEGRADATION BY CANINE TEAR FLUIDS**

Tears from normal dogs demonstrated little degradation of gelatine as measured by the gelatine degradation ELISA. MMP-2 and active form of MMP-9 were partially inhibited by flunixin meglumine (p<0.05). Subconjunctival dexamethasone treatment inhibit all four enzyme bands (p<0.01).

**Discussion**

MMPs are considered to play a major role in the proteolytic degradation of extracellular matrix [12,19]. Changes of the matrix metalloproteinase family has been documented in different species with eye problems such as rabbits, dogs and man[1,2,4,11]. MMP-2 (65 kDa), which is the proenzyme structure of gelatinase is synthesised by the cells of the normal corneal stroma. Inactive forms of these enzymes are secreted by fibroblasts, macrophages, neutrophils and chondrocytes together with tissue inhibitors (TIMP) [19, 20]. Normal tears showed a single gelatinolytic band at 62 kDa, of relatively low intensity compared to disease group. There was a large increase in activity of the 62 kDa enzyme in keratoconjunctivitis tears, compared to the control group. Enzyme activity was seen at 94 kDa (MMP-9 monomer) and 239 kDa (MMP-9 dimer) in all keratitis samples. These increases were statistically significant (p<0.001). Whilst MMP-9 is produced by monocytes and macrophages, the main source appears to be neutrophils [3].

It has been hypothesised that MMP-2 performs a surveillance function in normal cornea, catalysing degradation of collagen molecules that occasionally become damaged [18]. After tissue injury this enzyme appears to participate in the prolonged process of collagen remodelling in the corneal stroma that eventually results in functional regeneration of the tissue [20].

Flunixin meglumine is used extensively in many inflammatory conditions [7, 14, 17]. Therefore, it was selected for this study. It is known to act by inhibiting cyclo-oxygenase the enzyme responsible for formation of prostaglandins E<sub>2</sub> [16]. The latter is considered to be particularly important in acute inflammatory responses [17]. It is generally believed that high doses of flunixin may inhibit many enzymes, even though there was not any report on inhibition of MMPs in tear samples until now. In this study, MMP-9 are partially inhibited by 50 mg of flunixin meglumine. This result shows that only active MMP-9 (94 kDa) was totally inhibited by flunixin. It is most likely that, in keratitis, the increased MMP-9 originates from infiltrating neutrophils [11]. In the eye, treatment with topical flunixin meglumine suppresses the breakdown of the blood-aqueous barrier and reduces vascular permeability, inhibits leukocyte migration, stabilizes cell membranes and inhibits platelet aggregation [13].

Corticosteroids inhibit inflammation through the inhibition of arachidonic acid (AA) production by phospholipase A2 [22]. This results in the suppression of the production of prostaglandins (PGs), leukotrienes (LTS) and other mediators of inflammation. In this study, dexamethasone inhibits all enzymes (MMP-2 and MMP-9). Corticosteroids decrease...
exudation of inflammatory cells and fibrin, supresses fibroblast activity, decrease vascularization and inhibits collagen formation. Effective use of topical corticosteroids will result in a decrease of photophobia, blepharospasm, discharge, keratitis, and flare and a return to normal of intraocular pressure to normal in patients with ocular hypotony.

An ELISA using antibodies against MMP has been developed by COUGHLAN [8,9]. It can only give information on the level of biologically active enzyme activity within a sample. The gelatine degradation ELISA is a form of bioassay and furthermore measures the level of biologically active enzyme activity within a sample.

In conclusion, effect of flunixin meglumine and dexamethasone give insight into the treatment procedure of pathogenesis of these diseases. It may provide useful information for other species, including man.

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REFERENCES