Destructive periodontal diseases affect 10–15% of the world population, and are a major cause of tooth loss. There is increasing evidence that the disease occurs in a predisposed group of the population that has an aberrant inflammatory/immune response to the microbial plaque that accumulates around the gingival margin. This exaggerated response is known to result in inadvertent or collateral host tissue damage. The predominant inflammatory cell (96%) within the healthy connective tissues and epithelium of the gingiva is the polymorphonuclear leucocyte (PMNL). In aggressive and chronic forms of periodontitis, PMNLs appear to be functionally activated and exhibit increased production of reactive oxygen species (ROS). These molecules are reported to be capable of inducing periodontal tissue destruction and are associated with osteoclastic bone resorption. The degree to which ROS influence the progression of periodontal diseases is as yet unclear, but their role cannot be considered in isolation, given the range of antioxidant species that protects cellular reduced glutathione (GSH). Alterations in antioxidant defence have also been detected in the cervix, with reduced tissue levels being detected in cases of intraepithelial neoplasia.

In aggressive and chronic forms of periodontitis, polymorphonuclear leucocytes appear to be functionally activated and exhibit increased production of reactive oxygen species.

The epithelial surfaces most involved in the periodontal diseases are the junctional and crevicular epithelia, which are bathed in a plasma derived fluid, called gingival crevicular fluid (GCF). Although the crevicular epithelium is stratified squamous epithelium, the junctional epithelium has a uniquely differentiated structure, which, although separating the microbial plaque biofilm on the tooth surface from the underlying gingival connective tissues, is relatively permeable.
GCF continuously flows through this epithelium and enters the gingival crevice at a very slow rate (0.24–1.56 µl/minute at non-inflamed sites). Similarly, neutrophils migrate into the crevice by the same route. Therefore, GCF, which can be conveniently collected by non-invasive methods on paper strips, provides a sample whose contents reflect the status of the underlying periodontal tissues.

To date, there has been little published data on the role of free radicals or antioxidants in the pathogenesis of the periodontal diseases. The purpose of our study was to determine possible changes in GCF antioxidant defense in adult periodontal disease using an enhanced chemiluminescent (ECL) assay for total antioxidant capacity, and to investigate the nature of the local radical scavenging mechanisms, with particular reference to the presence and role of reduced glutathione.

MATERIALS AND METHODS

Chemicals
Chemicals were obtained from Sigma Aldrich Chemical Co. (Poole, Dorset, UK) unless otherwise stated.

Patients and periodontally healthy controls
Ten patients with clinically advanced stages of periodontal disease (mean age, 46.1 years) were age and sex matched (five men and five women) with periodontally healthy control subjects (mean age, 46.9 years). All volunteers were non-smokers and otherwise in good health, as confirmed by a detailed medical history. Inclusion criteria were the complete absence of vitamin supplements and no use of anti-inflammatory or antibiotic medication in the previous three months. Patients were categorised on the basis of accepted clinical criteria, and had mean pocket depths of 2.9 mm (SD, 0.59; range, 1–9) with a mean 21.6% (SD, 8.2%) of sites bleeding on probing. All volunteers gave written informed consent and participants were starved overnight before clinical sampling. Ethical approval for our study was obtained from the south Birmingham local research ethical committee (number LREC 0405).

ECL assay for total antioxidants
Determination of the total antioxidant capacity of biological samples was performed as described previously, using a BioOrbit 1250 luminometer (Labtech International, Uckfield, Sussex, UK), interfaced with a data acquisition software that allows simultaneous recording/display of up to 5000 data points over a period of up to one hour. Antioxidant capacities of test samples were derived from standard curves produced using the water soluble vitamin E analogue, 6-hydroxy-2,5,7,8-tetramethylethlen-2-carboxylic acid (Trolox®; assay range, 0.0625–1.6 nmol). Standard curves were run both before and after test samples. Results were expressed as a concentration (µM Trolox equivalents/litre; Teq/litre) and, for GCF samples, the amount of antioxidant activity for each 30 second sample (nmol/30 second sample).

Biological sample collection and preparation for ECL antioxidant assay
GCF samples were collected on paper strips over 30 seconds (Periopaper™ strips; Oralflow, Plainview, New York, USA), and volumes were measured using a precalibrated Periotron 8000 (Oralfow), as reported previously. Samples were then eluted for 30 minutes before strip removal and stored under liquid nitrogen. Strips were eluted into assay buffer (phosphate buffered saline (PBS), pH 7.6, containing 50 mg/litre bovine serum albumin; one strip for each 100 µl) for subsequent antioxidant assay or into 3.5% (vol/vol) perchloric acid (Fischer Scientific, Loughborough, UK; six strips for each 300 µl) transport medium for high performance liquid chromatography (HPLC) analysis.

For the cross sectional study, a total of 12 GCF samples for each subject were collected, with pooled eluates from six strips being used for antioxidant and six strips for HPLC analyses. Filtration experiments to investigate the molecular weight of specific GCF antioxidants were performed on 12 GCF samples collected from a periodontally healthy volunteer and eluted into a total volume of 1200 µl assay buffer. The eluate was assayed with and without filtration through Microcon 30 kDa (10 000 g; 12 minutes), 10 kDa (10 000 g; 30 minutes), and 3 kDa (10 000 g; 60 minutes) microconcentrators (Amicon Inc, Beverley, Massachusetts, USA) with the unfiltered, control aliquots being centrifuged in Eppendorf tubes at the same time as the filtered samples.

To examine the contribution of thiol groups to the antioxidant activity of GCF, pooled samples from healthy controls (n = 10) were assayed with and without preincubation with N-ethylmaleimide (200 µM; 5 minutes). All assays of GCF eluates were performed on 100 µl sample volumes.

Venous blood was collected from the antecubital fossa into lithium/heparin or plain Vacutainers®. Blood was allowed to stand at room temperature for 30 minutes then either centrifuged immediately (1000 g for 30 minutes at 4°C) for separation of plasma or incubated at 4°C for a further 30 minutes before centrifugation for serum (3500 g for five minutes at room temperature). Plasma and serum were collected and stored under liquid nitrogen. Antioxidant assays were performed on 20 µl of 1/10 diluted serum or plasma.

Artificial (substitute) GCF was created by diluting serum 1/5 with 0.9% saline to provide a solution with a protein concentration approximating that of GCF (16–20 mg/ml).

GCF collection and preparation for HPLC analysis
GCF samples from six sites in patients and age/sex matched periodontally healthy controls were collected, volumes measured, eluted into 3.5% (vol/vol) perchloric acid transport medium, and stored under liquid nitrogen as described previously. GSH and oxidised glutathione (GSSG) were measured by HPLC using a fluorimetric detector after derivatisation with dansyl chloride. The dansylated samples were applied to a Waters HPLC system with a LiChropher® 100NH2 (5 µm) column, connected to a compatible pre-column (Merck, Lutterworth, Leicestershire, UK) and fluorescence detector (Jasco FP-920). Each sample contained an internal standard (γ-glut-glu) to verify sample delivery onto the column and derivatisation. Data were collected and processed by Turbochrom 4 (Perkin Elmer, Beaconsfield, Buckinghamshire, UK); concentrations were then determined according to standard curves acquired from a parallel measurement of external standards.

PMNL preparation and stimulation with FMLP
Neutrophils were prepared by density gradient centrifugation of peripheral blood samples (50 ml; lithium/heparin) from five healthy volunteers. Briefly, 5 ml aliquots of blood were layered over 1 ml histopaque 1.077 and 3 ml mono-poly resolving medium (ICN Biochemicals, Thame, Oxfordshire, UK) and centrifuged at 500 g for 30 minutes. Cell viability (> 99%) was confirmed by trypan blue dye exclusion. Cells were resuspended in RPMI 1640 (8 x 10^6 cells/ml) and incubated with cytochalasin B (50 µg/ml; 22 µl added to 200 µl PMNL preparation) anaerobically under 90% N<sub>2</sub>, 10% CO<sub>2</sub> for 10 minutes at 37°C. The cells were then stimulated aerobically and anaerobically using F-Met-Leu-Phe (2.2 µl 10µM FMLP in 1MS 99 carrier; final concentration 100nM) for 15 minutes at 37°C. Activated PMNLs were ultrasonicated and centrifuged at 8000 xg. The supernatant was removed and the pellet resuspended in 2 ml of RPMI 1640. All samples (5–20 µl) were assayed for ECL antioxidant activity in the presence and absence of 20% serum (5 µl).

Collection of oral epithelial cells
Crevicular epithelial cells were collected from periodontally healthy controls by simple curettage. A Gracey 1/2 “mini five”
curette (LM Dental, supplied by JS Davis, Potters Bar, Hertfordshire, UK) was used to curette the crevice epithelium from 25 sites/volunteer. Samples were initially pooled into 500 µl of PBS buffer. Cells were then washed three times with PBS, resuspended in 0.5 ml of assay buffer, and ultrasonicated. After centrifugation at 8000 × g, the supernatant was removed and the pellet resuspended in 0.5 ml of assay buffer. All samples were stored at 4°C before being assayed for ECL antioxidant activity (5–20 µl aliquots assayed after the addition of 5 µl 20% serum).

ECL assay of PMNL reducing agents and thiol compounds
Titration of various PMNL reducing agents was performed to determine the minimum concentration that eliminated light emission from the assay by > 80%. Taurine (1–30nM), hypotaurine (0.24nM), cysteamine (0.1nM to 1.0 µM), L-cysteine (1–5nM), oxidised and reduced glutathione (1nM), β NADPH (0.33nM), and β NADP (0.66nM) were then assayed alone and (with the exception of taurine and hypotaurine) after the addition to 5 µl of 1/5 diluted serum. Assays of cysteine and GSH were performed with and without preincubation with 20nM N-ethylmaleimide (five minutes).

Analysis of data
Data were analysed using Minitab (version 9.0). Significant differences between medians were determined by the Mann Whitney U test.

RESULTS
Results from antioxidant assays of the samples from the cross sectional study showed that although mean (SD) plasma concentrations of antioxidant capacity were higher in controls (577.9 (99.8) µM Teq/litre) than patients (501.8 (123) µM Teq/litre), the difference was not significant (p > 0.1). In contrast, GCF samples from controls (mean, 1352.5; SD, 861 µM Teq/litre) contained significantly higher amounts of antioxidant activity (p < 0.015) than those from patients.
However, when the antioxidant capacity of GCF was calculated for each 30 second sample, the difference between control (mean, 0.2; SD, 0.1 nM/sample) and patient groups (mean, 0.15; SD, 0.06 nM/sample) was not significant (p > 0.15; fig 1). This finding reflected the higher GCF volumes obtained from patients with advanced periodontal disease (mean, 0.27; SD, 0.11 µl) compared with periodontally healthy controls (mean, 0.16; SD, 0.03 µl; p < 0.012).

In addition to these quantitative differences in antioxidant capacity between control and patient GCF samples, qualitative differences in the chemiluminescent light recovery curve were seen between GCF and plasma samples (fig 2). Thus, plasma samples invariably gave a smooth recovery profile when all antioxidant activity had been exhausted (fig 2A). In contrast, GCF samples, particularly those from control subjects, were often characterised by a more complex, stepped, recovery curve (fig 2C). This response could not be reproduced by running highly diluted plasma samples (fig 2B) or Trolox standards (data not shown), suggesting that it was not caused by small sample sizes or low absolute values of antioxidant activity.

These findings indicated that GCF contains a component, or components, with antioxidant properties not detectable in plasma. The most likely source for this local component would be neutrophils and/or epithelial cells. Therefore, a series of experiments was performed to investigate the effects of neutrophil products and intracellular components on the assay (table 1). Initial experiments on stimulated PMNLs revealed that measurable antioxidant activity and a stepped recovery curve were only detectable in the cytosolic fraction of anaerobically stimulated PMNLs. Furthermore, the stepped recovery was only detectable when 20% serum was added to the sample, mimicking the situation in GCF. A similar response was seen with the cytosolic fraction of GECs when assayed under the same conditions.

<table>
<thead>
<tr>
<th>Component tested</th>
<th>Quantity, dilution, and/or volume</th>
<th>Measurable AO activity (% loss caused by NEM)</th>
<th>Recovery curve profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMLP stimulated PMNL*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aerobic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant</td>
<td>5–20 µl</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Pellet</td>
<td>5–20 µl</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Anaerobic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant</td>
<td>5–20 µl</td>
<td>+</td>
<td>Stepped</td>
</tr>
<tr>
<td>Pellet</td>
<td>5–20 µl</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Intracellular reducing agents</td>
<td></td>
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</tr>
<tr>
<td>Taurine</td>
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<td>–†</td>
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</tr>
<tr>
<td>Hypotaurine</td>
<td>0.24 nM</td>
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<td>2 nM</td>
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<td>Stepped</td>
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<tr>
<td>+NEM</td>
<td>2 nM</td>
<td>+ (80%)</td>
<td>Smooth</td>
</tr>
<tr>
<td>L cysteine*</td>
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<td>+</td>
<td>Stepped</td>
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<tr>
<td>+NEM</td>
<td>1 nM</td>
<td>+ (76%)</td>
<td>Smooth</td>
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<tr>
<td>Oxidised glutathione*</td>
<td>1 nM</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Reduced glutathione*</td>
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<td>+</td>
<td>Stepped</td>
</tr>
<tr>
<td>+NEM</td>
<td>1 nM</td>
<td>+ (80%)</td>
<td>Smooth</td>
</tr>
<tr>
<td>Gingival epithelial cells*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant</td>
<td>5–20 µl</td>
<td>+</td>
<td>Stepped</td>
</tr>
<tr>
<td>Pellet</td>
<td>5–20 µl</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>GCF filtrate</td>
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<tr>
<td>&lt;30 kDa</td>
<td>100 µl</td>
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<td>Smooth</td>
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<tr>
<td>Serum</td>
<td>1/5–1/500 (20 µl)</td>
<td>+</td>
<td>Smooth</td>
</tr>
</tbody>
</table>

Quantity refers to nM added to assay; for FMLP stimulated PMNL and gingival epithelial cells see materials and methods for details of original cell numbers, etc.; 100 µl of GCF eluate is equivalent to an entire single GCF sample; GCF filtrates were produced by passage through 30, 10, or 3 kDa Microcon concentrating filters. *Assayed with the addition of 5 µl of 20% serum; †complete light suppression without recovery. AO, antioxidant; GCF, gingival crevicular fluid; FMLP, F-Met-Leu-Phe; NEM, N-ethylmaleimide; PMNL, polymorphonuclear leucocytes.

In an attempt to identify the nature of the component responsible for the stepped recovery, several intracellular PMNL reducing agents were individually tested for their ability to produce an ECL curve similar to that found in GCF (table 1). On their own, none of the reducing agents produced an antioxidant response showing a stepped recovery. Both taurine and hypotaurine caused irreversible light suppression in a concentration dependent manner. However, β NADPH, cysteamine, cysteine, and GSH, when assayed in the presence of 20% serum produced antioxidant effects similar to those seen in clinical GCF samples. Except for β NADPH, preincubation of these compounds with N-ethylmaleimide caused a large reduction in their antioxidant activity and complete removal of the stepped recovery curve (table 1).

To demonstrate biological relevance, N-ethylmaleimide blocking studies were performed on pooled GCF samples (fig 2C, D). These demonstrated that preincubation with N-ethylmaleimide completely removed the stepped recovery curve.
profile, indicating that thiol groups were involved in this effect. The effect on T10% values (and therefore antioxidant capacity) was a reduction of between 75% and 80% (T10% is the time taken for the light signal to recover to 10% of its initial peak value (once all antioxidants have been exhausted)).

Filtration studies using Microcon microconcentrators showed that the thiol component within GCF was of low molecular weight (< 3 kDa; table 1; fig 2E) and, of the molecules tested, could be cysteamine, cysteine, or GSH.

HPLC analysis of GCF samples from patients with periodontitis and controls demonstrated mean GSH concentrations in the millimolar range (fig 3). GSH concentrations were significantly lower in patients with periodontal disease (mean, 1183.1; SD, 580.3μM) relative to control subjects (mean, 1899.8; SD, 494.4μM; p < 0.022). The concentrations of GSSG in the GCF of both patients and controls were significantly lower than those of GSH (p = 0.0002). Furthermore, GCF concentrations of both GSSG and total glutathione were significantly lower in patients (mean, 150.1; SD, 44.9μM GSSG; mean, 1483; SD, 650μM total) compared with control subjects (mean, 256.8; SD, 152.4μM GSSG; p < 0.026; mean, 2431; SD, 627μM total; p < 0.015).

To confirm that the concentrations of GSH detected in GCF would produce changes in the antioxidant profile seen, GSH was added to 20% human plasma in saline to give a final concentration equivalent to the mean values detected by HPLC in GCF from healthy subjects. The results (fig 4A–C) demonstrated antioxidant profiles that were both qualitatively and quantitatively similar to clinical GCF samples. As with GCF samples, prior blocking with N-ethylmaleimide eliminated the stepped recovery profile.

**DISCUSSION**

This is the first reported investigation of possible differences in GCF antioxidant capacity between periodontal health and disease. The results from this cross sectional study show that local, GCF total antioxidant capacity is significantly decreased in patients with periodontal disease compared with periodontally healthy controls. Furthermore, this local decrease was reflected systemically by lower mean antioxidant capacity in plasma from patients with periodontitis. That alterations in antioxidant capacity might be detectable both locally and systemically is supported by recent studies indicating that chronic periodontal disease is associated with peripheral neutrophils that are hyper-reactive with respect to the production of ROS in response to Fcγ stimulation. Although these data suggest that lowered antioxidant defence is associated with periodontal disease, they do not indicate whether this compromised antioxidant capacity is important in pathogenesis, or is essentially a consequence of hyper-reactive neutrophils and/or ROS mediated tissue damage within the periodontal tissues.

These studies have confirmed previous observations that the antioxidant light recovery profile obtained with GCF, irrespective of patient group, was qualitatively different from that seen with plasma, and was often characterised by a stepped response. This suggested that GCF contained a locally derived antioxidant not present in plasma. The three most likely local sources of such a component were plaque bacteria, neutrophils, and junctional/crevicular epithelium. Preliminary
studies of the antioxidant profiles of washed plaque samples and periodontal bacteria (P. gingivalis, T. forsythensis, and S. anginosus) indicated that the flora within the gingival crevice were not the source of this novel component (data not shown). By contrast, cysteine extracts of crevicular epithelial cells and anaerobically stimulated neutrophils elicited the stepped recovery curve characteristic of GCF. This response was also mimicked by cysteamine, and has also been reported in epithelial curetage samples from the uterine cervix.

Data from the HPLC analyses indicate that GSH is the local component within GCF responsible for the stepped antioxidant response. Thus, GSH was detected in GCF at concentrations (range, 0.5–2.5 mM) that were 1000 fold higher than that normally found within extracellular tissue compartments,1 and that could qualitatively and quantitatively reproduce the stepped recovery in in vitro experiments. Importantly, GCF concentrations of GSH were lower in subjects with periodontitis than in healthy controls, reflecting differences in total antioxidant values between the two groups. These findings are similar to those obtained in studies of GSH in inflammatory diseases of the lung. Alveolar epithelial lining fluid (ELF) contains high concentrations of GSH (0.4 mM),2 with values being raised in smokers and deficient in patients suffering from pulmonary fibrosis3 and acute respiratory distress syndrome.4

The junctional epithelium of the gingival crevice is similar in its relative location and function to the alveolar epithelium in the lungs, where ELF rather than GCF bathes and protects the lung epithelium. The implications of the reported findings relate to the potential role of GSH within secretions/exudates that bathe epithelial lined surfaces exposed to the external environment. Both the lung and periodontium are subject to neutrophilic inflammation in response to microbial insult, within an environment high in activating cytokines produced by the lining epithelium and underlying fibroblasts and inflammatory cells. Normally, intracellular concentrations of GSH are high (0.5–10 mM) but extracellular fluid values are low (0.5–5 μM in human plasma). The high concentrations of GSH found in GCF and ELF are likely to influence the regulation of proinflammatory cytokines, involved in the processes that lead to host tissue damage.5 This is of potential importance in the pathobiology of such inflammatory disease processes. There is evidence that increasing cystolic cysteine (and thus GSH) concentrations within monocytes and macrophages (using the synthetic form of cysteine, N-acetylcysteine) block ROS mediated activation of the transcription factor nuclear factor κB (NF-κB) and the subsequent upregulation of proinflammatory cytokine production.6

"Data from the high performance liquid chromatography analyses indicate that reduced glutathione is the local component within gingival crevicular fluid responsible for the stepped antioxidant response"7

Although the source of the high concentrations of GSH (and GSSG) in GCF remains to be elucidated, it is likely that neutrophils and/or junctional/crevicular epithelium are major contributors. It is interesting to speculate that GSH can be added to the list of defence factors in the gingival crevice and lung known to be epithelial products (for example, β defensins8 and migration inhibitory factor 89). Whether glutathione release is secondary to cell death or the result of some active secretory mechanism must also form the focus of future work. Indeed, the mechanisms underlying reduced concentrations of GSH in disease states relative to health may involve an inborn defect in the γ-glutamyl pathway of GSH synthesis (normally controlled by a complex of cellular factors),9 or other endogenous or exogenous influences such as the local microbiota. It has been shown that certain putative periodontal pathogens (P. micros, F. nucleatum, F. nucleatum, and F. nucleatum) readily degrade GSH to form hydrogen sulphide.10 The formation of hydrogen sulphide within the periodontal pocket is toxic to mammalian cells by inactivating cytochrome oxidase11 and is also reported to inhibit catalase.12 The latter activity could positively feed back into NF-κB mediated transcription of proinflammatory cytokines. The degradation of cysteine or GSH by oral microbiota may also prevent the inhibition of NF-κB mediated transcription of proinflammatory cytokines in the periodontal environment, thereby increasing the risk of cytokine related tissue damage.

These results indicate that the gingival crevice is similar to the lung in that it is normally bathed in fluid containing high concentrations of GSH, which is known to have powerful anti-inflammatory (anti-proinflammatory cytokine) and anti-oxidant activity. Furthermore, both sites GSH concentrations are reduced in patients who suffer from chronic inflammatory diseases, where normal tissue homeostatic mechanisms fail to protect against host mediated tissue damage. The lower concentrations of glutathione (reduced, oxidised, and total) in the GCF of patients with periodontitis could be the result of a variety of factors leading to decreased synthesis and/or enhanced local degradation. Whatever the reason, these observations have important implications for the pathogenesis and treatment of periodontal disease. Additional work aimed at investigating the source, function, and regulation of lining fluid GSH may help determine new therapeutic strategies based upon these apparent local disease associated reductions in concentrations.

**ACKNOWLEDGEMENTS**

This work was supported in part by a grant project from Unilever Dental Research. We would also like to acknowledge the generous support of Professor S Socransky of the Forsyth Research Centre, Boston, MA, where the initial studies were performed, and the stimulating discussions with Professor S Socransky, Dr A Halfajee, and Dr S Dibartii.

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RAS regulates fluid dynamics in eyes

New pharmacological agents for treating glaucoma may be in the offing after researchers have shown that a renin-angiotensin system (RAS) regulates secretion of intracellular fluid in cells from the non-pigmented ciliary body.

The researchers showed that angiotensinogen and AT1 receptor were expressed in human non-pigmented epithelial (HNPE) cells. The cells responded to extracellular angiotensin II with increased intracellular calcium ion concentration. This activated a large conductance potassium ion (BK) channel in the cell membrane, resulting in excretion of potassium ions and intracellular fluid, and a resulting drop in cell volume.

Applying AT1 receptor antagonist losartan extracellularly inhibited this sequence, and intracellular calcium and potassium ion channel activity were unchanged.

PCR and reverse transcriptase PCR were applied to cultured HNPE cells to identify the RAS components AT1 receptor and angiotensinogen. Spectrofluorescence imaging microscopy was used to measure intracellular calcium concentration and cell volume. Single channel patch clamping and whole cell current voltage analysis were used to monitor ion channel activity.

Preliminary indications have suggested that RAS may regulate aqueous humour through its ability to regulate vascular tone in the eye, but not directly by receptor activated signal transduction and ion secretions, as shown in this study.

Glutathione in gingival crevicular fluid and its relation to local antioxidant capacity in periodontal health and disease
I L C Chapple, G Brock, C Eftimiadi and J B Matthews

*Mol Path* 2002 55: 367-373
doi: 10.1136/mp.55.6.367

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