**In vitro** expression of matrix metalloproteinase-1, tissue inhibitor of metalloproteinase-1 and transforming growth factor-β1 in human periodontal ligament fibroblasts

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**SUMMARY** Extracellular matrix remodelling is mediated via matrix metalloproteinases (MMPs) and their regulatory factors such as tissue inhibitors of metalloproteinases (TIMPs) and transforming growth factor-β (TGF-β). The regulation of MMPs is thought to be associated with cytoskeletal changes. In this study, cytoskeletal changes in human periodontal ligament fibroblasts (PDfs) were induced using cytochalasin B (CB) which reorganizes actin microfilaments reversibly, and colchicine which disrupts microtubules irreversibly. The levels of MMP-1, TIMP-1 and TGF-β secreted by the CB- or colchicine-treated PDfs were measured using an enzyme-linked immunosorbent assay. Differences between experimental and control groups were tested using analysis of variance (ANOVA) and Scheffé’s ad hoc test.

Although CB treatment did not significantly increase MMP-1 expression over the controls, colchicine treatment significantly increased the expression of MMP-1 (P < 0.01) in a time-dependent manner compared with the controls. Both CB and colchicine showed a time-dependent increase in TIMP-1 and TGF-β1 expression and a dose-dependent increase in TIMP-1 and TGF-β1 expression until threshold compared with the controls (P < 0.05, P < 0.01 and P < 0.001). In addition, CB treatment produced significantly increased TGF-β1 expression over the controls (P < 0.05 and P < 0.001) from lower doses, with this effect occurring at earlier time points compared with colchicine treatment.

**Introduction**

Extracellular matrix (ECM) remodelling is a critical step in many biological processes such as wound healing (Adzick *et al*., 1985), tissue remodelling (Woessner, 1991) and orthodontic tooth movement (Sandy *et al*., 1993). ECM not only provides the microenvironment for intercellular activities, but also actively participates in ECM cell interactions (Whitby and Ferguson, 1991). During orthodontic tooth movement there must be a delicate and well-balanced ECM remodelling process in the adjacent periodontium in order to allow tooth movement while maintaining periodontal functional integrity. The maintenance of gingival and periodontal tissue involves cell ECM interactions (Redlich *et al*., 1999). The most abundant cells in the periodontium are periodontal ligament fibroblasts (PDFs). These cells produce matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) and appear to play a pivotal role in maintaining the functional integrity of the periodontal ECM (Wu *et al*., 1999).

MMPs are a family of zinc-dependent matrix-degrading enzymes (Birkedal-Hansen, 1988) that have been implicated in disease-induced ECM turnover (Woessner, 1991), in the remodelling of bone and connective tissue (Birkedal-Hansen, 1993) and in tooth eruption (Wu *et al*., 1999). MMP-1 is the most abundant member of the MMPs and is produced by fibroblasts in a variety of connective tissues. This enzyme characteristically cleaves native interstitial collagen at neutral pH (Birkedal-Hansen, 1993) and is naturally inhibited by TIMP-1, forming a 1:1 stoichiometry complex (Cawston, 1986). MMPs and TIMPs are not pre-assembled but synthesized and secreted upon stimulation (Woessner, 1991). ECM remodelling is a delicately balanced process involving the local interactions of MMPs and TIMPs (Murphy *et al*., 1985). This balance is the key to maintaining the functional integrity of the periodontium.

The expression of MMP-1 and TIMP-1 can be regulated by a large variety of cytokines, growth factors and hormones (Thomson *et al*., 1989; Circolo *et al*., 1991; Meikle *et al*., 1991; Bigg and Cawston, 1996). Transforming growth factor-β (TGF-β), in particular, is known to be a potent stimulator of pro-MMP-1 (Ignotz and Massague, 1986) and TIMP-1 (Edwards *et al*., 1987; Wright *et al*., 1991) production. However, it has also been shown that the induction of MMPs by other cytokines is blocked by TGF-β (Matrisian *et al*., 1992). Thus, TGF-β exerts a selective effect on ECM deposition by directly and indirectly modulating the action of other factors on MMP and TIMP-1 expression (Edwards *et al*., 1987; Ohji *et al*., 1993). It is reasonable to think
that there are dynamic interactions among MMP-1, TIMP-1 and TGF-β1 in periodontal tissues during ECM remodelling.

There are several lines of evidence showing that cytoskeletal changes are associated with the expression of MMPs and their regulatory factors (Werb et al., 1986; Unemori and Werb, 1986; Varedi et al., 1995, 1997). Actin and tubulin of tendon cells can be altered in vitro in response to cyclic compression (Banes et al., 1985, 1995), suggesting that mechanical force can alter the cytoskeleton. Pender and McCulloch (1991) reported that the microfilament system of PDFs exhibits a rapid dynamic response to mechanical deformation prior to cell shape changes.

Chemicals affecting cell morphology have been used to control the expression of MMPs and TIMPs (Cooper, 1987; Miyajima et al., 1994; Saito et al., 1994). Cytochalasin B (CB) reorganizes the microfilaments of actin reversibly (Werb et al., 1986; Cooper, 1987; Berman, 1994) and colchicine disrupts microtubules irreversibly (Bauer and Valle, 1982; Unemori and Werb, 1986). In addition, CB stimulates the secretion of MMP-1 in corneal fibroblasts in vitro, albeit to different extents in different cultures (Berman, 1994). Colchicine affects the microtubular system and the secretion of a variety of proteins including MMP-1 and TIMP-1 (Murphy et al., 1985). Because these chemicals can induce changes in cell shape and the secretion of enzymes (Sandy et al., 1993) they can be used to study the regulation of ECM remodelling through the expression of MMP-1, TIMP-1 and TGF-β1 via cytoskeletal changes. Therefore, the hypothesis tested in this study was that CB and colchicine induce cytoskeletal change in PDFs in vitro and increase the expression of MMP-1, TIMP-1 and TGF-β1.

Materials and methods

Human PDF primary culture and treatments

Human PDFs were obtained from the lower half of the periodontium of first premolars extracted for orthodontic reasons. The PDFs were pooled and cultured in α-minimum essential medium (α-MEM; Gibco, Grand Island, New York, USA) supplemented with 10 per cent foetal bovine serum (FBS; Gibco) at 5 per cent CO₂ and 37°C. The cells used for these experiments were taken between the fourth and sixth passages. Equal numbers of cells were seeded into each well of six-well plates and cultivated until they reached confluence. To ensure that the fibroblast populations in every well had a similar level of protein synthesis, the total protein in the supernatant of each well was measured using the BCA protein assay reagent kit (Pierce, Rockford, Illinois, USA) before the cells were used in the experimental protocol. These cells were then subjected to CB or colchicine (Sigma, St. Louis, Missouri, USA) pulse treatments for dose- and time-dependent studies.

In the dose-dependent study, confluent cells in six-well plates were treated with either CB (at 0.1, 0.5, 1.0 or 2.0 µg/ml) or colchicine (at 0.1, 0.2, 0.5 or 1.0 µg/ml) for 3 hours (Werb et al., 1986). After 3 hours the cells were rinsed and further cultivated in α-MEM containing 1 per cent FBS for 24 hours.

In the time-dependent investigation, confluent cells were treated with CB 1.0 µg/ml or colchicine 1.0 µg/ml for 3 hours. The cells were then rinsed and incubated in α-MEM containing 1 per cent FBS for a further 6, 12, 24, 48 or 72 hours.

In each study, the conditioned media at the end of each time interval were collected to determine the levels of MMP-1, TIMP-1 and TGF-β1 using an enzyme-linked immunosorbent assay (ELISA). MMP-1 and TIMP-1 are commonly assayed using zymography, an approach that relies on the enzyme’s biological activity (Otsuka et al., 1984; Overall et al., 1989; Cawston et al., 1998). The major disadvantage of using this approach for this study is that it requires the MMP-1 to be activated and it cannot distinguish between MMP-1 and MMP-8 and the inhibitors TIMP-1, -2, -3 and α2-macroglobulin. In addition, MMP-1 is often assayed in a sample containing inhibitors, resulting in the measurement of net enzyme activity rather than the quantification of specific enzyme levels (Plumpton et al., 1995). As the purpose of this study was to evaluate the effects of cytoskeletal changes on MMP-1, TIMP-1 and TGF-β1 expression but not the net activity of these three molecules, the ELISA method was used to quantify enzyme levels.

MMP-1 and TIMP-1 assays

The levels of MMP-1 or TIMP-1 in the conditioned media were measured in triplicate using the Biotrak ELISA system for human MMP-1 or TIMP-1 (Amersham, Arlington Heights, Illinois, USA). As claimed by the manufacturer, the MMP-1 assay detects total human MMP-1 (free and/or bound to TIMP-1) and the TIMP-1 assay detects total human TIMP-1 (free and complexed with MMPs). The MMP-1 assay does not detect MMP-1 bound to the non-specific protease inhibitor, α₂-macroglobulin or show significant cross-reactivity with or interference from MMP-2, -3, -9 and TIMP-1. The TIMP-1 assay does not cross-react with TIMP-2.

The assay was performed as recommended by the manufacturer. Briefly, 1 ml of conditioned media was incubated for 2 hours in the microtitre wells pre-coated with anti-MMP-1 or anti-TIMP-1 antibody to immobilize any MMP-1 or TIMP-1 present in the conditioned media. The immobilized MMP-1 or TIMP-1 was detected by incubating with a primary antibody to MMP-1 or TIMP-1 for 2 hours followed by a horseradish peroxidase-conjugated secondary antibody for 1 hour and the
substrate tetramethylbenzidine (TMB) for 30 minutes. The reaction was terminated by using 1 M sulphuric acid and the intensity of the reaction was read using a microtitre plate spectrophotometer at a wavelength of 450 nm. Known amounts of MMP-1 or TIMP-1 were used in the ELISA system under the same conditions to generate a standard curve. The levels of MMP-1 or TIMP-1 were calculated by comparing the readings of the MMP-1 or TIMP-1 in the conditioned media and the MMP-1 or TIMP-1 in the standard curve.

**TGF-β1 assay**

The levels of TGF-β1 in the conditioned media were measured in triplicate using the Biotrak ELISA system for human TGF-β1 (Amersham). One millilitre of conditioned media was pre-treated with 0.2 ml of 1 M HCl for 10 minutes and then neutralized with 0.2 ml of 1.2 M NaOH/0.5 M HEPES free acid (N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulphonic acid]). The treated conditioned media was incubated for 1 hour in the microtitre wells pre-coated with anti-TGF-β1 antibody to immobilize any TGF-β1 in the condition media. The immobilized TGF-β1 was detected by incubating with a primary antibody to TGF-β1 for 1 hour followed by a biotinylated secondary antibody for 30 minutes, streptavidine peroxidase for 30 minutes and the substrate TMB for 30 minutes. The reaction was terminated by using 1 M sulphuric acid and the intensity of the reaction was read using a microtitre plate spectrophotometer at a wavelength of 450 nm. Known amounts of TGF-β1 were used in the ELISA system under the same conditions to generate a standard curve. The levels of TGF-β1 were calculated by comparing the readings of the TGF-β1 in the conditioned media and the TGF-β1 in the standard curve.

**Statistical analysis**

Each experiment and assay was undertaken in triplicate, and the means and standard deviations were calculated and compared with those in the controls. Differences between experimental and control groups were tested by analysis of variance (ANOVA), followed by Scheffé’s *ad hoc* test to confirm the result.

**Results**

PDFs treated with CB or colchicine *in vitro* resulted in significant changes in cell shape (Figures 1 and 2). In addition to the cytoskeletal changes, there was expression of MMP-1, TIMP-1 and TGF-β1 after removal of the CB and colchicine.

In the dose-dependent study (CB, 0.1, 0.5, 1.0 and 2.0 µg/ml; colchicine, 0.1, 0.2, 0.5 and 1.0 µg/ml), no dose-dependent response was observed with MMP-1 expression; rather, the pattern was biphasic with either no response or an active response at 0.5 µg/ml of CB and 0.2 µg/ml of colchicine and higher levels (Table 1). The expression of TIMP-1 significantly increased with CB and colchicine treatment in a dose-dependent manner until a threshold concentration was reached at 0.5 µg/ml of CB and 0.2 µg/ml of colchicine (Table 1). Although the expression of TGF-β1 was similar between CB and colchicine, CB showed a stronger expression of TGF-β1 at lower doses compared with colchicine (Table 1). As with TIMP-1 expression, TGF-β1 significantly increased with CB treatment in an early dose-dependent manner until a threshold concentration was reached at 1.0 µg/ml of CB. A similar trend was observed with colchicine treatment, although there was lower expression at 0.1 µg/ml of colchicine than in the control (Table 1).
In the time-dependent study (6, 12, 24, 48 and 72 hours), the onset of MMP-1 expression in both the CB and colchicine groups occurred by the 24 hour time point compared with the controls in which expression was observed by the 48 hour time point (Table 2). The production of MMP-1, TIMP-1 and TGF-β increased steadily with time (Table 2). MMP-1 expression with colchicine treatment was significantly stronger than treatment with CB or in the controls (P < 0.01) at 48 and 72 hours (Table 2). TIMP-1 expression with CB and colchicine treatment was significantly greater than in the controls during the duration of the entire experiment but CB treatment showed stronger TIMP-1 expression than colchicine treatment at 24, 48 and 72 hours (Table 2). TGF-β1 expression with CB treatment was greater than the control from 12 hours. TGF-β1 production of MMP-1, TIMP-1 and TGF-β1 increased steadily with time (Table 2). MMP-1 expression with colchicine treatment was significantly stronger than in the controls during the duration of the entire experiment but CB treatment showed stronger TIMP-1 expression than colchicine treatment at 24, 48 and 72 hours (Table 2). TGF-β1 expression with CB treatment was greater than the control from 12 hours. TGF-β1

Table 1  The dose-dependent effects of cytochalasin B and colchicine on the production of matrix metalloproteinase-1 (MMP-1), tissue inhibitor of metalloproteinase-1 (TIMP-1) and transforming growth factor-β1 (TGF-β1) in vitro in human periodontal ligament fibroblasts.

<table>
<thead>
<tr>
<th>control</th>
<th>cytochalasin B treatment (µg/ml)</th>
<th>colchicine treatment (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>MMP-1 (ng/ml)</td>
<td>Not detected</td>
<td>Not detected</td>
</tr>
<tr>
<td>TIMP-1 (ng/ml)</td>
<td>137.7 ± 16.3</td>
<td>141.3 ± 4.5</td>
</tr>
<tr>
<td>TGF-β1 (pg/ml)</td>
<td>454.9 ± 38.9</td>
<td>9740 ± 164.4*</td>
</tr>
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</table>

After treatment with either cytochalasin B or colchicine for 3 hours, the cells were rinsed and further cultivated in α-minimum essential medium containing 1 per cent foetal bovine serum for 24 hours. The conditioned media at the end of the time interval were collected for determining the levels of MMP-1, TIMP-1 and TGF-β1 using an enzyme-linked immunosorbent assay. The data represent the means and standard deviations of three replicates. Controls are without treatment with cytochalasin B and colchicine. Differences between experimental and control groups were tested by analysis of variance followed by Scheffé's ad hoc test. Significantly different from the controls: *P < 0.05; **P < 0.01; ***P < 0.001.
Table 2 The time-dependent effects of cytochalasin B and colchicine on the production of matrix metalloproteinase-1 (MMP-1), tissue inhibitor of metalloproteinase-1 (TIMP-1) and transforming growth factor-β1 (TGF-β1) in vitro in human periodontal ligament fibroblasts.

<table>
<thead>
<tr>
<th></th>
<th>MMP-1 (ng/ml)</th>
<th>TIMP-1 (ng/ml)</th>
<th>TGF-β1 (pg/ml)</th>
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<tbody>
<tr>
<td></td>
<td>6 hours</td>
<td>12 hours</td>
<td>24 hours</td>
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<tr>
<td>Control</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Not detected</td>
</tr>
<tr>
<td>Colchicine</td>
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<td>Not detected</td>
<td>Not detected</td>
</tr>
<tr>
<td>Colchicine</td>
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<td>Not detected</td>
<td>11.3 ± 1.1</td>
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<tr>
<td>Cytochalasin B</td>
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<td>86.3 ± 4.0</td>
<td>137.7 ± 16.3</td>
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<tr>
<td>Cytochalasin B</td>
<td>65.9 ± 5.6*</td>
<td>160.7 ± 8.3***</td>
<td>295.8 ± 16.4***</td>
</tr>
<tr>
<td>Colchicine</td>
<td>74.7 ± 2.1*</td>
<td>144.7 ± 6.2***</td>
<td>232.2 ± 11.5**</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>160.9 ± 33.4</td>
<td>318.8 ± 8.9</td>
<td>454.9 ± 38.9</td>
</tr>
<tr>
<td>Colchicine</td>
<td>228.7 ± 0.9</td>
<td>649.4 ± 43.8***</td>
<td>1578.2 ± 164.5***</td>
</tr>
<tr>
<td>Colchicine</td>
<td>151.0 ± 16.6</td>
<td>354.2 ± 53.6</td>
<td>803.1 ± 16.4***</td>
</tr>
</tbody>
</table>

After treatment with 1.0 µg/ml cytochalasin B and colchicine for 3 hours, the cells were rinsed and incubated in α-minimum essential medium containing 1 per cent foetal bovine serum media for another 6, 12, 24, 48 or 72 hours. The conditioned media at the end of each time interval were collected for determining the levels of MMP-1, TIMP-1 and TGF-β1 using an enzyme-linked immunosorbent assay. The data represent the means and standard deviations of three replicates. Controls are without treatment with cytochalasin B and colchicine. Differences between experimental and control groups were tested by analysis of variance followed by Scheffé’s ad hoc test. Significantly different from the controls: *P < 0.05; **P < 0.01; ***P < 0.001.

Discussion

CB and colchicine treatment of PDFs in vitro caused cytoskeletal changes (Figures 1 and 2) and active expression of MMP-1, TIMP-1 and TGF-β1 (Tables 1 and 2). These results are consistent with those of Murphy et al. (1985) who showed that the stimulation of TIMP secretion also induced the synthesis and secretion of MMP-1 in human fibroblasts, indicating that the proteolytic enzyme and its controlling inhibitor may be synthesized in parallel. This parallel stimulation of the synthesis of proteolytic enzymes and of their specific inhibitors could be the general paradigm for refined localized control of proteolysis.

Although CB treatment did not significantly increase MMP-1 expression over the controls, colchicine treatment significantly increased the expression of MMP-1 (P < 0.001) in a time-dependent manner compared with the controls (Tables 1 and 2). This could indicate that the expression of MMP-1 is influenced more by the disruption of microtubules than the reorganization of actin microfilaments.

The dose- and time-dependent TIMP-1 expressions of CB and colchicine treatment were similar (Tables 1 and 2). This suggests that the expression of TIMP-1 seems to be influenced by both the reorganization of actin microfilaments and the disruption of microtubules. TGF-β1 expression reached a significant level over the controls at a lower concentration and at an earlier time point with CB treatment compared with colchicine treatment (Tables 1 and 2). These findings suggest that the expression of TGF-β1 is probably more sensitive to the reorganization of actin microfilaments than to the disruption of microtubules.

In the time-dependent experiments, the onset of MMP-1 expression appeared by the 24 hour time point in the presence of CB or colchicine (Table 2), at an earlier time than the controls which expressed MMP-1 by the 48 hour time point. As DNA translation and cytoplasmic mRNA are associated with the state of the cytoskeleton (Patcher, 1992) the earlier appearance of MMP-1 could be related to influences on the timing of translation in response to cytoskeletal reorganization (Werb et al., 1986). MMP-1 expression with CB treatment was similar to the control groups at the 48 and 72 hour time points.
(Table 2). However, during the same period, MMP-1 expression with colchicine treatment was significantly increased relative to the control groups (Table 2). These differences suggest that the effect of CB may only occur during the first 24 hours. It is possible that CB facilitates the expression of MMP-1 via actin microfilament reorganization (Bauer and Valle, 1982; Unemori and Werb, 1986; Cooper, 1987; Berman, 1994; Varedi et al., 1995). According to Werb et al. (1986) the effects of CB are rapidly reversible so that the cells flatten and spread out within 30–60 minutes after CB treatment and washing. The minimum period of CB treatment that caused the cells to subsequently synthesize collagenase and stromelysin was 3 hours. Therefore, the CB-induced increase in MMP-1 expression was only temporary and returned to levels similar to the controls as soon as the actin microfilaments returned to their original organization. By contrast, the colchicine-induced increase in MMP-1 expression was sustained, possibly due to the irreversible effect of colchicine.

Unlike MMP-1, expression patterns of TIMP-1 in the CB- and colchicine-treated groups were similar during the experimental time points (Table 2). This suggests that once the fibroblasts commit to producing TIMP-1 via the reorganization of actin microfilaments or the disruption of microtubules, the expression of TIMP-1 remains active independent of the restoration of actin organization or permanent disruption. The time-dependent study also showed that TGF-β1 expression increases with time. With CB treatment, the levels of TGF-β1 increased significantly from 12 to 72 hours. With colchicine treatment, a similar trend was observed, although differences were statistically significant only at 24 and 72 hours. This suggests that TGF-β1 production of PDF is facilitated more by the reorganization of actin microfilaments than the disruption of microtubules and once the commitment is made the expression of TGF-β1 remains active even if the actin microfilaments return to their original state.

MMP-1 degrades the ECM, whereas TIMP-1 and TGF-β1 inhibit MMP-1 directly and indirectly. TGF-β1 is secreted by fibroblasts in a latent form and can be activated by acidification or exposure to protease such as plasmin (Lyons et al., 1988). This suggests that the extracellular microenvironment may have an influence on the activation of TGF-β1 (Park and Kim, 1988). It is possible that the TGF-β1 secreted by the PDFs in this study was in a latent form. Activated TGF-β1 down-regulates the expression of MMP-1 (Meikle et al., 1991; Wright et al., 1991) and upgrades the expression of TIMP-1 (Overall et al., 1989; Meikle et al., 1991; Wright et al., 1991; Slavin et al., 1994). If the TGF-β1 in this study was in a latent form, it may not have had a direct influence on the secretions of MMP-1 and TIMP-1 as there were insufficient amounts of TGF-β1 activation factor in this tissue culture microenvironment. Thus, the MMP-1 and TIMP-1 detected in this study were most likely due to the direct effects of cytoskeletal changes rather than the influence of the TGF-β1 present in the conditioned media.

MMP-1 is expressed somewhat later than TIMP-1 and TGF-β1 in response to cytoskeletal changes, but once initiated by the cytoskeletal changes, the earlier and continuous expression of TIMP-1 and TGF-β1 may be a safeguard mechanism so that ECM remodelling can be controlled.

Conclusion

CB induces relatively stronger expression of TIMP-1 and TGF-β1 than colchicine in PDFs in vitro. Both agents increase TGF-β1 and TIMP-1 expression relative to controls. However, their effects are not identical, possibly due to the different actions of these agents. In addition, MMP-1 expression appears in vitro earlier in the presence of these agents than in the controls. These data and the observations made by other groups suggest that cytoskeletal changes in PDFs could modulate remodelling of the periodontium by triggering the expression of MMP-1, TIMP-1 and TGF-β1.

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