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Effect of cannabidiol on human gingival fibroblast extracellular matrix metabolism: MMP production and activity, and production of fibronectin and transforming growth factor β

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Background and Objective: Marijuana (*Cannabis sativa*) use may be associated with gingival enlargement, resembling that caused by phenytoin. Cannabidiol (CBD), a nonpsychotropic *Cannabis* derivative, is structurally similar to phenytoin. While there are many reports on effects of phenytoin on human gingival fibroblasts, there is no information on effects of *Cannabis* components on these cells. The objective of this study was to determine effects of CBD on human gingival fibroblast fibrogenic and matrix-degrading activities.

Material and Methods: Fibroblasts were incubated with CBD in serum-free medium for 1–6 d. The effect of CBD on cell viability was determined by measuring activity of a mitochondrial enzyme. The fibrogenic molecule transforming growth factor β and the extracellular matrix molecule fibronectin were measured by ELISA. Pro-MMP-1 and total MMP-2 were measured by ELISA. Activity of MMP-2 was determined via a colorimetric assay in which a detection enzyme is activated by active MMP-2. Data were analysed using ANOVA and Scheffe's F procedure for *post hoc* comparisons.

Results: Cannabidiol had little or no significant effect on cell viability. Low CBD concentrations increased transforming growth factor β production by as much as 40% ($p < 0.001$), while higher concentrations decreased it by as much as 40% ($p < 0.0001$). Cannabidiol increased fibronectin production by as much as approximately 100% ($p < 0.001$). Lower CBD concentrations increased MMP production, but the highest concentrations decreased production of both MMPs ($p < 0.05$) and decreased MMP-2 activity ($p < 0.02$).

Conclusion: The data suggest that the CBD may promote fibrotic gingival enlargement by increasing gingival fibroblast production of transforming growth factor β and fibronectin, while decreasing MMP production and activity.

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Marijuana (*Cannabis sativa*) is the most widely used illicit drug in the USA. Its use is associated with many adverse psychosocial and health effects, including impairment of cognitive function, cardiovascular and pulmonary effects, cancer risk, and progression of liver fibrosis in patients with chronic hepatitis C (1–3). However, cannabinoids, the collective term for *Cannabis* active compounds, also can have therapeutic effects, such as analgesia, anti-inflammatory effects, lowering of intraocular pressure, and can act as antineoplastic and antiepileptic agents (4,5).

Several reports have documented oral effects of marijuana smoking, such as xerostomia, leukoplakia, papilloma, candidiasis, head and neck cancer, and periodontal complications, including gingivitis as well as gingival enlargement (6–12). The association between chronic marijuana use and gingival enlargement was first noted by Layman and colleagues (8,11). The reported cases of marijuana-associated gingival enlargement are summarized in Table 1. We previously found oral changes, including gingival erythema and enlargement, and loss of periodontal attachment, in two patients with a history of smoking marijuana (S. Y. Rawal , D. N. Tatakis, D. A. Tipton, unpublished observations). Of particular interest in these patients was the fibrotic appearance of the gingiva, including a distinct nodular or pebbly appearance.

Cannabidiol (CBD) is a major nonpsychotropic constituent of *Cannabis*, composing as much as 40% of *Cannabis* extracts (13). It exhibits pharmacological effects, including antiepileptic properties that are comparable to those of phenytoin, and both drugs fulfil the stereochemical requirements for antiepileptic drug action (14). Cannabidiol also enhances the antiepileptic potency of both phenytoin and phenobarbital (15). Considering the similar structure and properties of CBD and phenytoin, the gingival enlargement seen in marijuana users may be caused by mechanisms similar to those of phenytoin-induced gingival enlargement (16).

Alterations in fibronectin (FN) synthesis and its increased deposition in the extracellular matrix (ECM) are characteristic of hereditary gingival fibromatosis (HGF) and phenytoin-induced gingival enlargement (17–20). Transforming growth factor β (TGFβ) is an important fibrogenic molecule, which stimulates the synthesis of FN and other ECM molecules, and its expression is increased in gingival tissue and gingival crevicular fluid of patients with HGF and drug-induced gingival overgrowth (21–27). In fibrotic conditions, there may also be impaired breakdown of ECM molecules by proteases, such as the MMPs. The balance between matrix production and degradation by MMPs is crucial in fibrosis, and the expression of some MMPs may be downregulated in pathological fibroses (23). MMP-1 and MMP-2 are produced by human gingival fibroblasts and are important in physiological and pathological gingival remodeling. Expression of both of these MMPs is decreased in HGF (24).

Currently, there is little information on the effects of CBD on gingival fibroblast metabolism and its ability to promote gingival fibrosis observed in some *Cannabis* users. Therefore, this study has determined the effects of CBD on aspects of ECM production (TGFβ and FN synthesis) and breakdown (MMP production and activity) by human gingival fibroblasts *in vitro*.

Material and methods

Cannabidiol

Cannabidiol (CBD; molecular formula $C_{21}H_{30}O_2$; molecular weight 314.5 Da) was obtained from Sigma-Aldrich (St Louis, MO, USA). Cannabidiol was supplied in solution at a concentration of 1.0 mg/mL in methanol (MeOH).

Human gingival fibroblasts

Normal human gingival fibroblasts were established from an explant of noninflamed gingival tissue from a healthy individual using standard techniques (28). The fibroblasts were routinely cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% (v/v) newborn calf serum (Invitrogen) and 100 μg/mL gentamicin (Sigma-Aldrich) (complete medium). Cells between passages five and 10 were used in the experiments.

Cytotoxic effects of CBD

Cytotoxicity of CBD (and its solvent MeOH) was assessed by measuring the

Table 1. Gingival enlargement associated with marijuana use

Reference	No. of subjects	Age, sex	Marijuana use	Gingival enlargement	Other periodontal characteristics
Layman 1978 (11)	12	17–24 years, male	2–7 years, unknown quantity	Generalized	Fiery-red gingiva; some resembled phenytoin-induced gingival enlargement
Baddour *et al.* 1984 (8)	1	24 years, male	4 years, daily, large but unknown quantity	Generalized, diffuse	Alveolar bone loss, severe mobility of mandibular molars
S. Y. Rawal, D. N. Tatakis, D. A. Tipton, unpublished observations	2	23 years, male	2 years, 3 or more times per week	Generalized	Nodular (pebbly) appearance
		42 years, male	16 years, 5 times daily	Generalized	Nodular (pebbly) appearance

ability of the fibroblasts, via mitochondrial succinate dehydrogenase, to cleave the tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; MTT) to a formazan dye (Boehringer Mannheim Corp., Indianapolis, IN, USA). Toxic effects of MeOH were first determined. Individual wells of 96-well microtiter tissue culture plates [Becton, Dickinson and Co. (BD), Franklin Lakes, NJ, USA] were seeded with 2.5×10^4 cells in 0.2 mL of complete medium. The cells were incubated overnight at 37°C, and the medium was removed and replaced with DMEM supplemented with 100 μg/mL gentamicin (DMEM-gent) containing MeOH at concentrations of 0.001–10%. [Ethanol concentrations ≤ 1% ethanol are not toxic to these cells (29)]. After exposure times of 1–6 d, MTT was added to the cells at a final concentration of 0.5 mg/mL and incubated for 4 h at 37°C. Purple formazan crystals produced from the MTT by metabolically active cells were solubilized overnight at 37°C. Absorbance was measured at 540 nm using a microtiter plate spectrophotometer. Results were expressed as a percentage of the control value ($A_{540\,nm}$ in cells exposed to DMEM-gent only). To determine toxic effects of CBD, concentrated solutions of CBD in MeOH were made such that 1× solutions contained MeOH at a nontoxic level, as determined above. The final concentrations of CBD to which the cells were exposed after dilution in DMEM-gent were 0.01–30 μM. Toxicity of CBD solutions was determined as described above.

Effect of CBD on TGFβ production

The synthesis and release of TGFβ1 (a major TGFβ isoform produced by human cells) into fibroblast conditioned media was measured by ELISA using the human DuoSet ELISA Development Systems (R&D Systems, Minneapolis, MN, USA). The fibroblasts were seeded at 3.5×10^5 cells per well of six-well tissue culture plates (BD) in complete DMEM medium, and incubated overnight at 37°C. {As bovine serum in complete DMEM contains significant levels of TGFβ1, the following protocol was used to remove it from the system, according to the manufacturer's instructions and our previous work (29). After culturing overnight, the complete medium was removed from the wells, and DMEM containing 100 μg/mL gentamicin and 200 μg/mL bovine serum albumin (Cohn V fraction; Sigma-Aldrich) was added. This medium was replaced 4× over 24 h, and finally removed and replaced with serum-free DMEM-gent with or without CBD (0.01–30 μM; final [MeoH] = 1%)}. The cells were then incubated for 1–6 d. Control medium was serum-free DMEM-gent containing 1% MeOH. The media were harvested, and latent TGFβ1 was processed to form immunoreactive TGFβ1 by treatment with 0.167 N HCl for 10 min at room temperature, followed by neutralization with 1.2 N NaOH and 0.5 M HEPES, according to the manufacturer's instructions. The samples were assayed for TGFβ1 according to the manufacturer's directions, and the results were expressed as picograms of TGFβ1 per milligram of protein [determined by the method of Lowry *et al.* (30)], and then converted to a percentage of the control value (amount made by cells exposed to DMEM-gent–1% MeOH only).

Effect of CBD on fibronectin production

Fibronectin secreted by the fibroblasts into culture supernatants was measured by ELISA (Bender MedSystems, Burlingame, CA, USA). Fibroblasts (3.5×10^5) were seeded into each well of a six-well tissue culture plate in complete medium and cultured overnight at 37°C. The medium was then removed, the wells washed with phosphate-buffered saline, and DMEM-gent containing CBD (0.025–30 μM; final [MeoH] = 1%) was added. Control cells were exposed to serum-free DMEM-gent containing 1% MeOH. The supernatants were collected for assay at 6 d. [Fibronectin can exist as a soluble form in fluids, and in previous work we were unable to detect FN in the cell matrix *in vitro* (17)]. The ELISA was performed according to the manufacturer's instructions, and the results were expressed as nanograms of fibronectin per milligram of protein (30), and then converted to a percentage of the control value (amount made by cells exposed to DMEM-gent–1% MeOH only).

Effect of CBD on MMP production

Levels of MMP-1 and MMP-2 proteins were measured in fibroblast-conditioned media by ELISA (R&D Systems). The MMP-1 assay detects human pro-MMP-1, and the MMP-2 assay detects total MMP-2 (pro- and active MMP-2). To obtain conditioned media for the assays, the cells were seeded at 5×10^4 cells per well in 24-well plates (Corning Inc., Corning, NY, USA) in complete medium and cultured overnight at 37°C. This medium was removed, the wells were washed with PBS, and DMEM-gent containing CBD (0.05–2 μM; final [MeoH] = 1%) was added. Control cells were exposed to serum-free DMEM-gent containing 1% MeOH. The media were harvested at 6 d, and the samples were assayed. Absorbance values were read using a microtiter plate spectrophotometer, and the results were expressed as the amount of MMP [nanograms per milligram of protein (30)], and then converted to a percentage of the control value (amount made by cells exposed to DMEM-gent–1% MeOH only).

Effect of CBD on MMP-2 activity

Activity of MMP-2 was measured using the MMP-2 Biotrak™ Activity Assay System (GE Healthcare, Piscataway, NJ, USA). Conditioned media used in this assay were the same as used for the detection of MMP-1 and MMP-2 protein (above). The assay utilizes the pro form of a detection enzyme that can be activated by captured active MMP-2, into an active detection enzyme, through a single proteolytic event. The natural activation sequence in the pro detection enzyme has been replaced, using protein engineering, with an artificial sequence recognized by specific MMPs. The MMP-activated detection enzyme can then be measured using a specific

chromogenic peptide substrate. Standards and samples were incubated in microplate wells precoated with anti-MMP-2 antibody. Any MMP-2 present bound to the anti-MMP-2 antibody in the wells, other components of the sample being removed by washing and aspiration. Either the endogenous levels of active MMP-2, or the total levels of MMP-2 in a sample, were detected. In order to measure the total MMP-2 content (endogenous active + pro-MMP-2), any bound MMP-2 in its pro form was activated using *p*-aminophenylmercuric acetate. Endogenous active MMP-2 was detected in samples without *p*-aminophenylmercuric acetate treatment. The level of pro-MMP-2 activity was determined by subtraction (total activity minus endogenous activity). Color development was read at 405 nm in a microplate spectrophotometer. As MMP-2 activity is directly proportional to the generation of color through the cleavage of the substrate used in this kit, it can be represented by the rate of change of absorbance at 405 nm, i.e. $\delta Abs_{405}/h^2 \times 1000$, where h is the incubation time in hours. (The final data are multiplied by 1000 so as to be able to plot whole numbers on the graph.) These data were then converted to a percentage of the control value.

Statistical analysis

All experiments were performed with triplicate samples. The data were expressed as means ± SD and were analysed using a one-way ANOVA and Scheffe's F procedure for *post hoc* comparisons, using StatView® software (SAS Institute, Cary, NC, USA).

Results

Cytotoxicity

Cytotoxic effects of MeOH, the CBD diluent, were first determined. Concentrations of MeOH ≤ 1% had no significant effect on cell viability after 1–6 d of exposure (data not shown). There were significant decreases in cell viability caused by 10% MeOH after 3 d exposure (approximately 60%) and 6 d exposure (> 80%) (data not shown). Therefore, in subsequent work, stock solutions of CBD were made so that final MeOH concentration to which the cells were exposed was 1%. To determine cytotoxic effects of CBD, the fibroblasts were exposed to CBD (0.01–30 μM; final [MeoH] = 1%). No significant differences in cell viability compared with the control (DMEM-gent–1% MeOH) were noted at any of the above concentrations, at any time point ($p > 0.05$) (data not shown).

Effect of CBD on TGFβ and fibronectin production

After 24 h exposure, low CBD concentrations (0.01–0.05 μM) increased TGFβ production by as much as 40% over control, while higher concentrations (4–30 μM) decreased it by as much as 40% (Fig. 1). After longer periods of exposure to CBD (3 or 6 d), there was less or no increase in TGFβ production at lower concentrations (generally not statistically significant). However, decreased production at higher concentrations remained evident (approximately 20–50% decrease; $p < 0.04$ at some concentrations) at these longer time periods (data not shown).

Figure 2 shows that CBD, at all concentrations tested, increased FN production. At ≤ 0.1 μM, CBD increased FN levels by approximately 50–75%, but these increases were not statistically significant. Cannabidiol at ≥ 0.5 μM significantly increased FN production by approximately 100–150% over control ($p < 0.04$).

Effect of CBD on pro-MMP-1 and total MMP-2 production

There was a biphasic effect of CBD on pro-MMP-1 production (Fig. 3). Cannabidiol concentrations ≤ 1 μM generally increased production of pro-MMP-1 by as much as 50–100% [significant at 0.1 ($p < 0.005$) and 0.5 μM ($p < 0.05$)]. Within this range of CBD concentrations (0.05–1 μM), stimulation reached a maximum at 0.1 μM CBD, which then generally declined as the CBD concentration increased. However, the highest CBD concentration tested (2 μM) decreased pro-MMP-1 production by approximately 30% ($p < 0.05$). Likewise, there was also a biphasic effect of CBD on total MMP-2 production, shown in Figure 3. As CBD concentrations increased, MMP-2 levels increased, reaching a maximum of approximately 150% increase at 0.5 μM CBD ($p < 0.005$). MMP-2 was stimulated to a lesser extent (approximately 70%) by CBD at 1 μM ($p < 0.05$). At 2 μM, the highest concentration of CBD tested, total MMP-2 production decreased by > 30% ($p = 0.001$).

Effect of CBD on MMP-2 activity

Figure 4 shows constitutive levels of total MMP-2 activity, and the effects of CBD. Cannabidiol concentrations of 0.05 and 0.5 μM had no significant effect on total (endogenous active + *p*-aminophenylmercuric acetate-activated pro-MMP-2) MMP-2 activity. Cannabidiol at 1 μM decreased activity by approximately 30% compared with control ($p < 0.02$), while 2 μM CBD decreased activity by approximately 60% ($p < 0.0001$). Most of these changes in total activity were due to alterations in pro-MMP-2 activated by *p*-aminophenylmercuric acetate. Changes in endogenous active MMP-2 contributed little (generally 1% or less) to this effect (data not shown).

Discussion

In addition to its antiepileptic, anti-inflammatory and anti-tumor cell activities (31,32), the effects of CBD on human gingival fibroblast ECM metabolism presented here suggest that it could also contribute to gingival fibrosis. Toxic effects of CBD were first determined before measuring its effects on cellular activities. The concentration range of CBD used for initial cytotoxicity studies (0.01–30 μM) was based on other *in vitro* studies, which used CBD at concentrations of 0.01–40 μM (33–36). Appropriate levels of CBD for *in vitro* experimentation can differ with different types of cells. As there was no cytotoxicity at any of the CBD concentrations tested, these levels were used for further experimentation in the

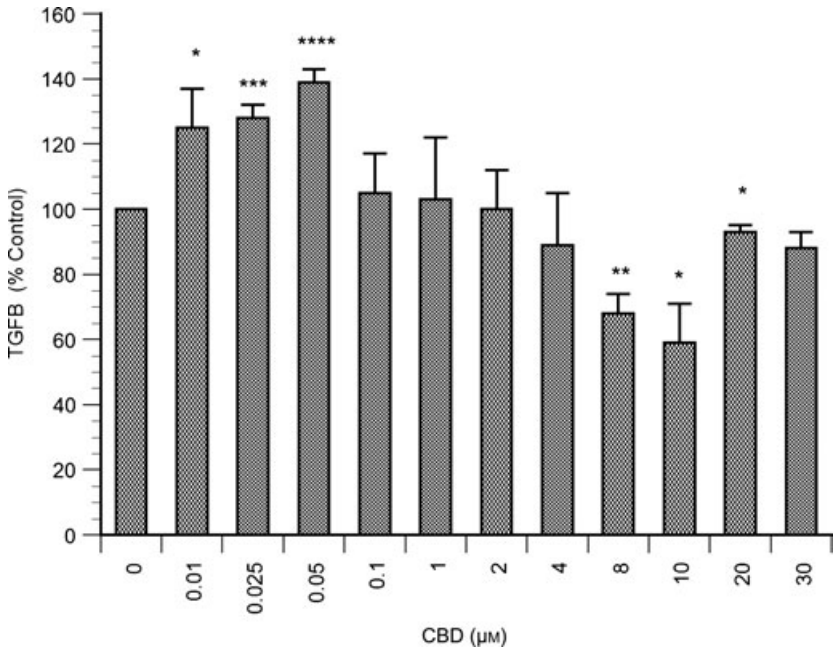

Fig. 1. Effect of cannabidiol (CBD) on production of transforming growth factor β (TGFβ). Fibroblasts (3.5×10^5) were seeded in complete medium in six-well plates and cultured overnight at 37°C. The medium was removed, the cells were washed with phosphate-buffered saline, and then Dulbecco's modified Eagle's medium supplemented with 100 μg/mL gentamicin (DMEM-gent) containing CBD (0.01–30 μM; final [MeOH] = 1%) was added. After 1–6 d incubation, levels of TGFβ were measured in the cell supernatants by ELISA. The mean values and standard deviations of triplicate measurements in multiple experiments were expressed as picograms per milligram of protein in the cell supernatants, and then converted to a percent of the control value (amount made by cells exposed to DMEM-gent–1% MeOH only, set at 100%). Significance levels vs. control: $*p < 0.02$; $**p = 0.001$; $***p = 0.0003$; and $****p = 0.0001$.

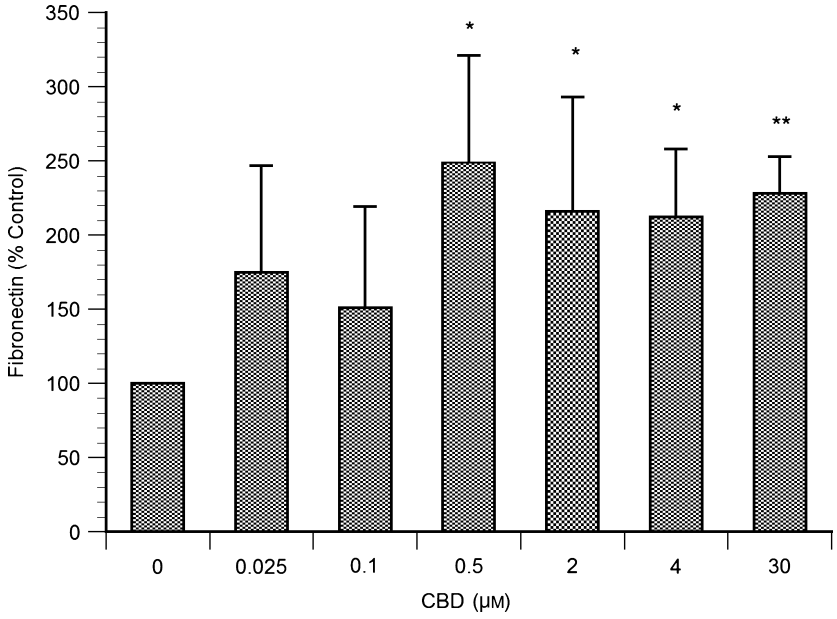

Fig. 2. Effect of cannabidiol (CBD) on fibronectin (FN) production. Fibroblasts (3.5×10^5) were seeded in complete medium in six-well plates and cultured overnight at 37°C. The medium was removed, the cells were washed with phosphate-buffered saline, and then DMEM-gent containing CBD (0.025–30 μM; final [MeoH] = 1%) was added. After 6 d incubation, levels of FN were measured in the cell supernatants by ELISA. The mean values and standard deviations of triplicate measurements in multiple experiments are indicated and expressed as nanograms per milligram of protein in the cell supernatants, and then converted to a percentage of the control value (amount made by cells exposed to DMEM-gent–1% MeOH only, set at 100%). Significance levels vs. control: $*p < 0.04$; and $**p = 0.0001$.

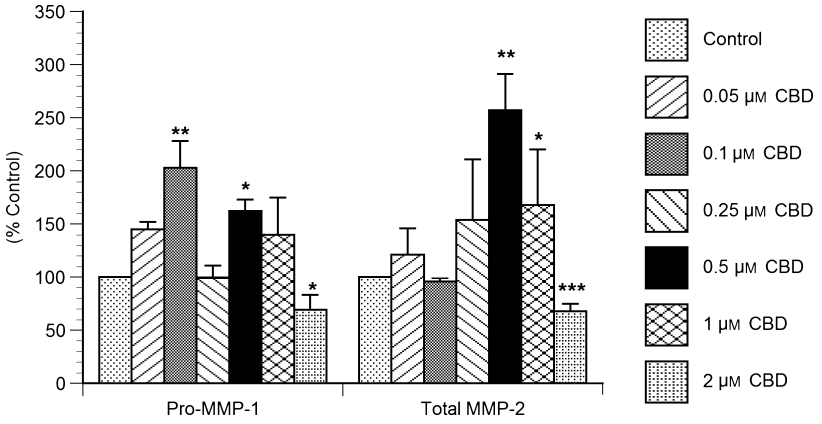

Fig. 3. Effect of cannabidiol (CBD) on MMP-1 and MMP-2 production. Fibroblasts (5×10^4) were seeded in complete medium in 24-well plates and cultured overnight at 37°C. The medium was removed, the cells were washed with phosphate-buffered saline, and then DMEM-gent containing CBD (0.05–2 μM) was added. After 6 d incubation, levels of pro-MMP-1 or total MMP-2 were measured in the cell supernatants by ELISA. The mean values and standard deviations of triplicate measurements in multiple experiments are indicated and expressed as nanograms per milligram of protein in the cell supernatants, and then converted to a percentage of the control value (amount made by cells exposed to DMEM-gent–1% MeOH only, set at 100%). Significance levels vs. control: $*p < 0.05$; $**p < 0.005$; and $***p = 0.001$.

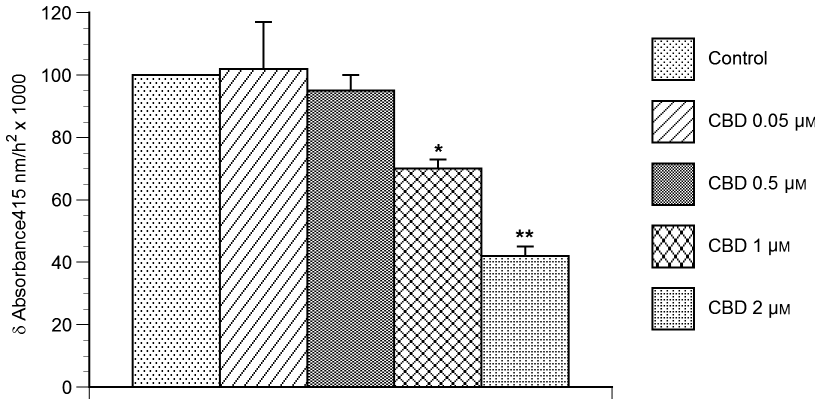

Fig. 4. Effect of cannabidiol (CBD) on MMP-2 activity. Fibroblasts (5×10^4) were seeded in complete medium in 24-well plates and cultured overnight at 37°C. The medium was removed, the cells were washed with phosphate-buffered saline, and then DMEM-gent containing CBD (0.05–2 μM) was added. After 6 d incubation, total MMP-2 activity was determined. The mean values and standard deviations of triplicate measurements in multiple experiments are indicated and expressed as $\delta Abs_{405}/h^2 \times 1000$, where h is the incubation time in hours, converted to a percentage of the control value. Significance levels vs. control: $*p < 0.02$; and $**p < 0.0001$.

present study. Others have shown that 10 μM CBD had no significant cytotoxic effect on human colon adenocarcinoma cells after 24 h, and < 15 μM CBD was not cytotoxic to human glioma cells (33,34). However, Mato *et al.* (37) showed that rat oligodendrocytes were somewhat more sensitive to CBD, with concentrations > 0.1 μM causing cell death.

Cannabidiol was tested for effects on TGFβ and FN production, and on MMPs (production of MMP-1 and MMP-2, and MMP-2 activity). Transforming growth factor β increases matrix protein synthesis and decreases degradation, which can result in clinical fibrosis (38). Low CBD concentrations increased TGFβ levels by as much as 40% at 24 h, suggesting that CBD may promote fibrosis in this way. At higher CBD concentrations, however, TGFβ levels decreased. Transforming growth factor β can stimulate human gingival fibroblast production of connective tissue growth factor (CTGF), which may be involved in the onset and progression of fibrosis in many human tissues (39). In a study of dermal fibrosis, *CTGF* gene expression was continuously detected, while TGFβ was found only in the earliest stage (40). Uzel *et al.* (41) found increased CTGF and TGFβ in phenytoin-induced gingival overgrowth tissues and suggested, based on their findings, that some types of fibroses may exhibit transient TGFβ expression, with longer lasting expression of CTGF. Although CBD and phenytoin have structural similarities (16), it is not known whether CBD increases CTGF in gingival tissue. In the present study, the fibroblasts were not assayed for production of CTGF. However, our observation of increased TGFβ expression at 24 h but not at longer periods of exposure to CBD may be

consistent with the model proposed by Uzel *et al*., suggesting short-term increase in TGFβ expression coupled with persistent CTGF expression. This awaits further investigation.

Others have demonstrated similar biphasic effects of cannabinoid drugs on a variety of functions in different cell types (42–45). Statins and nonsteroidal anti-inflammatory drugs can also have biphasic effects on processes such as angiogenesis and prostaglandin production (46,47). Exposure times > 24 h caused less or no increase in TGFβ at lower CBD concentrations. However, the decreased production at higher concentrations (≥ 4 μM) observed at >24 h remained evident. In mice with streptozotocin-induced diabetes, Rajesh *et al*. (48) also found that CBD decreased elevated cardiac TGFβ production. Similar to other drugs, CBD effects may differ depending on many factors, including species, tissue/organ, dose and time of exposure.

Cannabidiol increased FN production, reaching statistical significance ≥ 5 μM. Transforming growth factor β can increase production of FN by fibroblasts. However, in the present study, while 6 d exposure to CBD did not increase TGFβ, it did increase FN production. Connective tissue growth factor also stimulates fibroblast production of FN (41). If, in response to CBD, CTGF is continuously elevated and TGFβ is only transiently elevated, as discussed earlier, this might contribute to the elevated FN production observed after 6 d exposure to CBD. While fibroblast growth factor (FGF) also increases FN production by fibroblasts (49), it is unknown whether CBD increased FGF production by the fibroblasts used in this study, and whether this could contribute to increased FN production via autocrine/paracrine stimulation. In both instances, there may be an increase in the rate of FN gene transcription, or stabilization of FN mRNA. However, all of these possibilities need further investigation to determine the mechanism of increased FN production by gingival fibroblasts in response to CBD. Overdeposition of FN is characteristic of fibroses of gingiva and other tissues (17–20,50). Fibronectin mediates fibroblast attachment to collagen and other ECM molecules and is a fibroblast chemoattractant, and may also contribute to gingival overgrowth directly by adding to the tissue bulk.

Similar to effects on TGFβ production, CBD had a biphasic effect on pro-MMP-1 and total MMP-2, generally increasing levels with maxima at 0.1–0.5 μM, and decreasing levels at a relatively high concentration. There was a similar decrease in MMP-2 activity at higher CBD concentrations. Expression of MMP-1 and MMP-2 is also decreased in other types of gingival fibroses (24,51–53). While CBD increased MMP production in our study at some lower concentrations, its lack of significant effects at others and inhibition of production of both MMPs and MMP-2 activity at the highest concentration(s) are consistent with findings of other related studies. For example, a cannabidiol hydroquinone affected expression of the MMP-1 gene in vascular endothelial cells, and other cannabinoids decreased MMP-2 expression and activity in rat tumors (54,55). Furthermore, CBD had no effect on MMP-2 in normal mice and decreased elevated cardiac MMP-2 production in mice with streptozotocin-induced diabetes (48). Cannabidiol concentrations as high as 10 μM had no effect on MMP-2 production in human cancer cell lines, but tetrahydrocannabinol downregulated MMP-2 expression in these cells (56,57). Finally, a study by Massi found that CBD inhibited MMP-2 release and activity in human glioma cells (58). In our study, despite increased total MMP-2 production at some lower CBD concentrations, there was no parallel increase in total MMP-2 activity *in vitro*. This effect needs further investigation, but may be due to several factors, including CBD-induced alterations of endogenous inhibitors, or direct effects on MMP-2 activation or conformation. For example, Bonniaud *et al*. (59) found that bleomycin and CTGF resulted in a fibrotic response in mice associated with a strong increase in *TIMP-1* gene expression.

Transforming growth factor β promotes fibrosis by generally increasing ECM and protease inhibitor production, and reducing protease synthesis (60). In the present study, the effects of CBD on TGFβ varied with dose and time of exposure, and although difficult to correlate, elevated TGFβ production at some CBD concentrations may contribute, among other factors, to higher FN levels. Our earlier study suggested that autocrine stimulation of gingival fibroblasts in HGF by TGFβ contributed to their increased FN production (27). In addition, TGFβ inhibited gingival fibroblast MMP-1 expression, but in contrast to its effects on many proteases, it modestly increased MMP-2 expression in a study by Overall *et al*. (61). Autocrine TGFβ stimulation may also be involved in alteration of expression of both MMP-1 and MMP-2 in HGF (24). Clearly, regulation of MMP expression and activity is a complex process that is affected by many factors, including TGFβ.

In many cases, fibrosis is the result of chronic inflammation, and there may be an interaction between dental plaque, the inflammatory host response and the development of gingival overgrowth caused by drugs such as phenytoin, but this relationship is not clear (62). However, these drugs may alter the normal balance of cytokines/growth factors normally present in gingiva (because of the continual remodeling and repair that occurs in this tissue), including interleukin-6 (IL-6), interleukin-1β, platelet-derived growth factor, FGF-2, TGFβ and CTGF (63,64). Phenytoin-enlarged gingiva contains subpopulations of inflammatory cells that differ from those in healthy gingiva and which could modify the normal reparative gingival response to one that promotes fibrosis (65). Furthermore, phenytoin increases interleukin-4, and fibroblast production of IL-6 and interleukin-8, associated with fibrosis in various organs (62,66,67). Therefore, there is evidence to suggest that altered inflammatory responses may contribute to drug-induced gingival overgrowth in general, and to phenytoin-induced gingival overgrowth in particular. Given the structural similarities of phenytoin and CBD, this

may also occur in gingival fibrosis caused by *Cannabis*. Further complicating this scenario are the known anti-inflammatory effects of CBD (4,5).

Little is known of the mechanisms of CBD action (68). Two major types of cannabinoid receptors, CB_1 and CB_2, bind exogenous and endogenous cannabinoids [endocannabinoids, lipid mediators which are derived from arachidonic acid, including *N*-arachidonoyl ethanolamine (AEA or anandamide)]. The CB_1 receptor is found primarily in the central nervous system, but also in other tissues and cells (69,70). The CB_2 receptor is expressed peripherally, mainly in immune cells (71). Both CB_1 and CB_2 are expressed by human gingival fibroblasts and are upregulated in gingival inflammatory conditions (71). While CBD has very low affinity for CB_1 and CB_2 (72), it inhibits AEA degradation, and thus some CBD effects may be mediated indirectly, via increasing AEA levels (62). In human fibrotic liver, CB_1 receptors are upregulated in liver cells, and in mouse models CB_1 antagonists inhibited the progression of fibrosis, in part by lowering hepatic TGFβ1 (73). This suggests that CB_1 may be involved in development of some types of fibroses.

In vivo, CBD may increase the levels of AEA, which may promote fibrosis via CB_1 or other receptors (74), but an unindentified endogenous ligand or constitutive activation of CB_1 may also be involved. In a rat gingival wound-healing model, Kozono *et al*. (75) found that CB_1 and CB_2 were upregulated on gingival fibroblasts, that AEA was increased in crevicular fluid after periodontal surgery, and that, *in vitro*, AEA increased human gingival fibroblast proliferation. They concluded that AEA may promote periodontal wound healing. It is unknown whether the gingival fibroblasts in the present study produced AEA, although several other types of cells do produce it *in vitro* (74,76–79). A more thorough understanding of the direct and indirect effects of CBD on gingival fibroblasts could have important implications regarding treatment of gingival fibrotic diseases as well as periodontitis.

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References

1. Hall W, Solowij N. Adverse effects of cannabis. *Lancet* 1998;**352:**1611–1616.
2. Khalsa JH, Genser S, Francis H, Martin B. Clinical consequences of marijuana. *J Clin Pharmacol* 2002;**42:**7S–10S.
3. Hezode C, Roudot-Thoraval F, Nguyen S *et al*. Daily cannabis smoking as a risk factor for progression of fibrosis in chronic hepatitis C. *Hepatology* 2005;**42:** 63–71.
4. Grotenhermen F. Pharmacology of cannabinoids. *Neuro Endocrinol Lett* 2004; **25:**14–23.
5. Mechoulam R. Access to cannabinoids and marijuana for research and treatment. In: Mechoulam R ed. *Cannabinoids as Therapeutic Agents*. Boca Raton, FL: CRC Press Inc., 1986:175–176.
6. Rees TD. Oral effects of drug abuse. *Crit Rev Oral Biol Med* 1992;**3:**163–184.
7. Silverstein SJ, Noel D, Heilbron D. Social drug use/abuse and dental disease. *J Calif Dent Assoc* 1978;**6:**32–37.
8. Baddour HM, Audemorte TB, Layman FD. The occurrence of diffuse gingival hyperplasia in a patient using marijuana. *J Tenn Dent Assoc* 1984;**64:**39–43.
9. Darling MR, Arendorf TM. Review of the effects of cannabis smoking on oral health. *Int Dent J* 1992;**42:**19–22.
10. Darling MR, Arendorf TM. Effects of cannabis smoking on oral soft tissues. *Community Dent Oral Epidemiol* 1993; **21:**78–81.
11. Layman FD. Marijuana: harmful or not? *Tex Dent J* 1978;**96:**6–8.
12. Versteeg PA, Slot DE, van der Velden U, van der Weijden GA. Effect of cannabis usage on the oral environment: a review. *Int J Dent Hyg* 2008;**6:**315–320.
13. Grlie L. A comparative study on some chemical and biological characteristics of various samples of cannabis resin. *Bull Narc* 1976;**14:**37–46.
14. Tamir I, Mechoulam R, Meyer AY. Cannabidiol and phenytoin: a structural comparison. *J Med Chem* 1980;**23:**220–223.
15. Mechoulam R, Parker LA, Gallily R. Cannabidiol: an overview of some pharmacological aspects. *J Clin Pharmacol* 2002;**42:**11S–19S.
16. Seymour RA, Thomason JM, Ellis JS. The pathogenesis of drug-induced gingival overgrowth. *J Clin Periodontol* 1996;**23:** 165–175.
17. Tipton DA, Howell KJ, Dabbous MK. Increased proliferation, collagen, and fibronectin production by hereditary gingival fibromatosis fibroblasts. *J Periodontol* 1997;**68:**524–530.
18. Huang J, Ho K, Chen C *et al*. Collagen synthesis in idiopathic and dilantin-induced gingival fibromatosis. *Kaohsiung J Med Sci* 1997;**13:**141–148.
19. Kito T, Okahashi N, Ohno T, Inaba H, Kawai S, Amano A. Effect of phenytoin on collagen accumulation by human gingival fibroblasts exposed to TNFα in vitro. *Oral Dis* 2006;**12:**156–162.
20. Romanes G, Schroter-Kermani C, Hinz N, Bernimoulin J-P. Distribution of fibronectin in healthy, inflamed, and drug-induced gingival hyperplasia. *J Oral Pathol Med* 1992;**21:**256–260.
21. Dooley A, Shi-Wen X, Adeb N *et al*. Modulation of collagen type I, fibronectin, and dermal fibroblast function and activity in systemic sclerosis by the antioxidant epigallocatechin-3-gallate. *Rheumatology (Oxford)* 2010;**49:**2024–2036.
22. Kuru L, Yilmaz S, Kuro B, Kose K, Noyan U. Expression of growth factors in the gingival crevice fluid of patients with phenytoin-induced gingival enlargement. *Arch Oral Biol* 2004;**49:**945–950.
23. Saito K, Mori S, Iwakura M, Sukanoto S. Immunohistochemical localization of transforming growth factor beta, basic fibroblast growth factor, and heparin sulphate glycosaminoglycans in gingival hyperplasia induced by nifedipine and phenytoin. *J Periodontal Res* 1996;**31:** 545–555.
24. Coletta R, Almeida O, Reynolds M, Sauk J. Alteration in expression of MMP- 1 and MMP-2 but not TIMP-1 and TIMP-2 in hereditary gingival fibromatosis is mediated by TGFβ1 autocrine stimulation. *J Periodont Res* 1999;**34:**457–463.
25. Buduneli N, Kutukculer N, Aksu G, Atilla G. Evaluation of transforming growth factor-β1 level in crevicular fluid of cyclosporin A-treated patients. *J Periodontol* 2001;**72:**526–531.
26. Wright HJ, Chapple IL, Matthews JB. TGF-β isoforms and TGF-β receptors in drug-induced and hereditary gingival overgrowth. *J Oral Pathol Med* 2001;**30:** 281–289.
27. Tipton D, Dabbous M. Autocrine transforming growth factor β stimulation of extracellular matrix production by fibroblasts from fibrotic human gingiva. *J Periodontol* 1998;**69:**609–619.
28. Tipton DA, Pabst MJ, Dabbous MKh. Interleukin-1β- and tumor necrosis factor α-independent monocyte stimulation of fibroblast collagenase activity. *J Cell Biochem* 1990;**44:**253.

29. Tipton D, Lewis J. Effects of a hindered amine light stabilizer (HALS) and a UV light Absorber (UVA) used in maxillofacial elastomers on human gingival epithelial cells and fibroblasts. *J Prosthet Dent* 2008;**100:**220–231.
30. Lowry O, Rosebrough N, Farr A, Randall R. Protein measurement with the folin-phenol reagent. *J Biol Chem* 1951;**193:** 265–275.
31. Ruiz-Valdepenas L, Martinez-Orgando J, Benito C, Millan A, Tolon R, Romero J. Cannabidiol reduces lipopolysaccharide-induced vascular changes and inflammation in the mouse brain: an intravital microscopy study. *J Neuroinflammation* 2011;**8:**5–13.
32. Mechoulam R, Peters M, Murrillo-Rodriguez E, Hanus L. Cannabidiol – recent advances. *Chem Biodivers* 2007;**4:** 1678–1692.
33. Borrelli F, Aviello G, Romano B *et al*. Cannabidiol, a safe and non-psychotropic ingredient of the marijuana plant Cannabis sativa, is protective in a murine model of colitis. *J Mol Med* 2009;**87:**1111–1121.
34. Massi P, Vaccani A, Ceruti S, Colombo A, Abbracchio M, Parolaro D. Antitumor effects of cannabidiol, a non-psychotropic cannabinoid, on human glioma cell lines. *J Pharmacol Exp Ther* 2004;**308:**838–845.
35. Vaccani A, Massi P, Colombo A, Rubino T, Parolaro D. Cannabidiol inhibits glioma cell migration through a cannabinoid receptor-independent mechanism. *Br J Pharmacol* 2005;**144:**1032–1036.
36. McKallip R, Jia W, Schlomer J, Warren J, Nagarkatti P, Nagarkatti M. Cannabidiol in the regulation of $p22^{phox}$ and Nox4 expression. *Mol Pharmacol* 2006;**70:**897–908.
37. Mato S, Sanchez-Gomez M, Matute C. Cannabidiol induces intracellular calcium elevation and cytotoxicityin oligodendrocytes. *Glia* 2010;**58:**1739–1747.
38. Branton M, Kopp J. TGF-β and fibrosis. *Microbes Infect* 1999;**1:**1349–1365.
39. Black S, Trackman P. Transforming growth factor-β1 (TGFβ1) stimulates connective tissue growth factor (CCN2/CTGF) expression in human gingival fibroblasts through RhoA-independent, RAC1/Cdc42-dependent mechanism. *J Biol Chem* 2008;**283:**10835–10847.
40. Igarashi A, Nashiro K, Kikuchi K *et al*. Significant correlation between connective tissue growth factor gene expression and skin sclerosis in tissue sections from patients with systemic sclerosis. *J Invest Dermatol* 1995;**105:**280–284.
41. Uzel M, Kantarci A, Hong H- H *et al*. Connective tissue growth factor in drug induced gingival overgrowth. *J Periodontol* 2001;**72:**921–931.
42. Howlett A, Fleming R. Cannibinoid inhibition of adenylate cyclase. Pharmacology of the response in neuroblastoma cell membranes. *Mol Pharmacol* 1984;**26:**532–538.
43. Ligresti A, Moriello A, Starowicz K *et al*. Antitumor activity of plant cannabinoids with emphasis on the effect of cannabidiol on human breast carcinoma. *J Pharmacol Exp Ther* 2006;**318:**1375–1387.
44. Hart S, Fischer O, Ullrich A. Cannabinoids induce cancer cell proliferation via tumor necrosis factor α-converting enzyme (TACE/ADAM17)-mediated transactivation of the epidermal growth factor receptor. *Cancer Res* 2004;**64:**1943–1950.
45. Steffens A, Veillard N, Arnaud C *et al*. Low dose oral cannabinoid therapy reduces progression of atherscerosis in mice. *Nature* 2005;**434:**782–786.
46. Weiss M, Heeschen C, Glassford A, Cooke J. Statins have biphasic effects on angiogenesis. *Circulation* 2002;**105:**739–745.
47. Raisz L, Simmons H, Fall P. Biphasic effects of nonsteroidal anti- inflammatory drugs on prostaglandin production by cultured rat calvariae. *Prostaglandins* 1989;**37:**559–565.
48. Rajesh M, Mukhopadhyay P, Bátkai S *et al*. Cannabidiol attenuates cardiac dysfunction, oxidative stress, fibrosis, and inflammatory and cell death signaling pathways in diabetic cardiomyopathy. *J Am Coll Cardiol* 2010;**56:**2115–2125.
49. Song R, Bian H-N, Lai W, Chen H-D, Zhao K-S. Normal skin and hypertrophic scar fibroblasts differentially regulate collagen and fibronectin expression as well as mitochondrial membrane potential in response to basic fibroblast growth factor. *Braz J Med Biol Res* 2011;**44:**402–410.
50. Rukosuev VS, Nanaev AK, Milovanov AP. Participation of collagen types I, III, IV, V, and fibronectin in the formation of villi fibrosis in human term placenta. *Acta Histochem* 1990;**89:**11–16.
51. Tipton D, Dabbous M. Binding and subcellular distribution of cyclosporine in human fibroblasts. *J Cell Biochem* 1993; **51:**345–352.
52. Tipton D, Stricklin G, Dabbous M. Fibroblast heterogeneity in collagenolytic response to cyclosporine. *J Cell Biochem* 1991;**46:**152–165.
53. Thomason J, Sloan P, Seymour R. Immunolocalization of collagenase (MMP-1) and stromelysin (MMP-3) in the gingival tissues of organ transplant patients medicated with cyclosporin. *J Clin Periodontol* 1998;**25:**554–560.
54. Kogan N, Blazquez C, Alvarez L *et al*. A cannabinoid quinine inhibits angiogenesis by targeting vascular endothelial cells. *Mol Pharmacol* 2006;**70:**51–59.
55. Blazquez C, Casanova L, Planas A *et al*. Inhibition of tumor angiogenesis by cannabinoids. *FASEB J* 2003;**17:**529–531.
56. Ramer R, Merkord J, Rohde H, Hinz B. Cannabidiol inhibits cancer cell invasion via upregulation of tissue inhibitor of matrix metalloproteinases-1. *Biochem Pharmacol* 2010;**79:**955–966.
57. Ramer R, Hinz B. Inhibition of cancer cell invasion by cannabinoids via increased expression of tissue inhibitor of matrix metalloproteinases-1. *J Natl Cancer Inst* 2008;**100:**59–69.
58. Massi P. Cannabidiol, a non-psychoactive cannabinoid compound, inhibits human glioma cell migration and invasiveness. 2009; http://hdl.handle.net/2434/142503.
59. Bonniaud P, Martin G, Margetts P *et al*. Connective tissue growth factor is crucial to inducing a profibrotic environment in "fibrosis-resistant" Balb/c mouse lungs. *Am J Respir Cell Mol Biol* 2004;**31:**510–516.
60. Overall C, Wrana J, Sodek J. Independent regulation of collagenase, 72-kDa progelatinase, and metalloendoproteinase inhibitor expression in human fibroblasts by transforming growth factor-beta. *J Biol Chem* 1989;**264:**1860–1869.
61. Overall C, Wrana JL, Sodek J. Transcriptional and post-transcriptional regulation of 72-kDa gelatinase/type IV collagenase by transforming growth factor-beta1 in human fibroblasts. Comparisons with collagenase and tissue inhibitor of matrix metalloproteinase gene expression. *J Biol Chem* 1991;**266:**14064–14071.
62. Correa J, Queiroz-Junior C, Costa J, Teixera A, Silva T. Phenytoin-induced gingival overgrowth: a review of the molecular, immune, and inflammatory features. *ISRN Dent* 2011; (in press) doi:10.5402/2011/497850.
63. Riches D. Macrophage involvement in wound repair, remodeling, and fibrosis. In: Clark R, ed. *The Molecular and Cellular Biology of Wound Repair*, 2nd edition. New York: Plenum Press, 1996: 95–141.
64. Trackman P, Kantarci A. Connective tissue metabolism and gingival overgrowth. *Crit Rev Oral Biol Med* 2004;**15:**165–175.
65. Dahllof G, Modéer T, Otteskog P, Sundqvist K. Subpopulations of lymphocytes in connective tissue from phenytoin-induced gingival overgrowth. *Scand J Dent Res* 1985;**93:**507–512.
66. Modeer T, Domeij H, Anduren I, Mustafa M, Brunius G. Effect of phenytoin on the production of interleukin-6 and interleukin-8 in human gingival fibroblasts. *J Oral Pathol Med* 2000;**29:**491–499.
67. Lonnemann G, Engler-Blum G, Muller F, Dinarello C. Cytokines in human renal interstitial fibrosis. II. Intrinsic interleukin [IL]-1 synthesis and IL-1 dependent production of IL-6 and IL-8 by cultured kidney fibroblasts. *Kidney Int* 1995;**47:** 845–854.

68. Costa B, Giagnoni G, Franke C, Trovato A, Colleoni M. Vanilloid TRPV1 receptor mediates the antihyperalgesic effect of the nonpsychoactive cannabionid, cannabidiol, in a rat model of acute inflammation. *Br J Pharmacol* 2004;**143:**247–250.
69. Ryberg E, Larsson N, Sjögren S *et al*. The orphan receptor GPR55is a novel cannabinoid receptor. *Br J Pharmacol* 2007; **152:**1092–1101.
70. Howlett A. The cannabinoid receptors. *Prostaglandins Other Lipid Mediat* 2002; **69:**619–631.
71. Nakajima Y, Furuichi Y, Biswas KK *et al*. Endocannabinoid, anandamide in gingival tissue regulates the periodontal inflammation through NF- κB pathway inhibition. *FEBS Lett* 2006;**580:**613–619.
72. Pertwee R, Ross R, Craib S, Thomas A. (-)-Cannabidiol antagonizes cannabinoid receptor agonistsand noradrenaline in the mouse vas deferens. *Eur J Pharmacol* 2002;**456:**99–106.
73. Teixeira F, Julien B, Grenard P *et al*. CB1 cannabinoid receptor antagonism: a new strategy for the treatment of liver fibrosis. *Nat Med* 2006;**12:**671–676.
74. Walter L, Franklin A, Witting A, Moller T, Stella N. Astrocytes in culture produce anandamide and other acylethanolamides. *J Biol Chem* 2002;**277:**20869–20876.
75. Kozono S, Matsuyama T, Biwasa K *et al*. Involvement of the endocannabinoid system in periodontal healing. *Biochem Biophys Res Commun* 2010;**394:**928–933.
76. Deutsch D, Goligorsky M, Schmid P *et al*. Production and physiological actions of anandamide in the vasculature of therat kidney. *J Clin Invest* 1997;**100:**1538–1546.
77. Ahluwalia J, Yaqoob M, Urban L, Bevan S, Nagy I. Activation of capsaicin-sensitive primary sensory neurons induces anadamide production and release. *J Neurochem* 2003;**84:**585–591.
78. Varga K, Wagner J, Bridgen D, Kunos G. Platelet- and macrophage-derived cannabinoids are involved in endotoxin-induced hypotension. *FASEB J* 1998;**12:**1035–1044.
79. Wagner J, Varga K, Ellis E, Rzigalinski B, Martin B, Kuno G. Activation of peripheral CB1 cannabinoid receptors in haemorrhagic shock. *Nature (London)* 1997; **390:**518–521.