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Laboratory Investigations

Cannabinoids Stimulate Fibroblastic Colony Formation by Bone Marrow Cells Indirectly via CB_2 Receptors

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Abstract. Recently, the cannabinoid receptors CB_1 and CB_2 were shown to modulate bone formation and resorption *in vivo*, although little is known of the mechanisms underlying this. The effects of cannabinoids on mesenchymal stem cell (MSC) recruitment in whole bone marrow were investigated using either the fibroblastic colony-forming unit (CFU-f) assay or high-density cultures of whole bone marrow. Levels of the CB_1 and CB_2 receptors were assessed by flow cytometry. Treatment of CFU-f cultures with the endocannabinoid 2-arachidonylglycerol (2-AG) dose-dependently increased fibroblastic and differentiated colony formation along with colony size. The nonspecific agonists CP 55,940 and WIN 55,212 both increased colony numbers, as did the CB_2 agonists BML190 and JWH015. The CB_1-specific agonist ACEA had no effect, whereas the CB_2 antagonist AM630 blocked the effect of the natural cannabinoid tetrahydrocannabivarin, confirming mediation via the CB_2 receptor. Treatment of primary bone marrow cultures with 2-AG stimulated proliferation and collagen accumulation, whereas treatment of subcultures of MSC had no effect, suggesting that the target cell is not the MSC but an accessory cell present in bone marrow. Subcultures of MSCs were negative for CB_1 and CB_2 receptors as shown by flow cytometry, whereas whole bone marrow contained a small population of cells positive for both receptors. These data suggest that cannabinoids may stimulate the recruitment of MSCs from the bone marrow indirectly via an accessory cell and mediated via the CB_2 receptor. This recruitment may be one mechanism responsible for the increased bone formation seen after cannabinoid treatment *in vivo*.

Key words: Cannabinoid — CB_2 receptor — Colony-forming unit

The pharmacology, medicinal potential, and chemistry of cannabis are currently under intense investigation; and recent findings have been well reviewed by Pertwee [1] and Elsohly and Slade [2]. At least two cannabinoid receptors so far (CB_1 and CB_2) have been identified, with pharmacological evidence implicating the existence of others [3]. Δ^9-Tetrahydrocannabinol (Δ^9THC, or THC), the main psychoactive constituent of cannabis, mediates many of its activities through signaling via CB_1 receptors, which are expressed throughout the central nervous system (CNS) and many other tissues [4–7]. The CB_1 receptor is the most abundant G protein-coupled receptor in the CNS [5]. A second, peripheral cannabinoid receptor, CB_2, has been cloned [7] and is predominantly expressed in cells of the immune system, such as macrophages, B cells, natural killer cells, monocytes, neutrophils, and T cells [8, 9]. CB_2 receptors are not generally found in the normal CNS but have been shown to be overexpressed in neuritic plaque-associated glia in Alzheimer's disease brains [10]. Certain cannabinoids are also known to act at noncannabinoid receptors, such as vanilloid TRPV1 receptors [11] and the orphan G protein-coupled receptor GPR55 [12]. The physiological roles of the CB_2 receptor are still largely unknown, although its endogenous ligand, 2-arachidonoylglycerol (2-AG), has been shown to induce the migration of HL-60 cells differentiated into macrophage-like cells [13]. A similar effect was observed using human peripheral blood monocytes, which suggests that 2-AG, which is generated from stimulated inflammatory cells, plays an important role in immune responses and inflammatory reactions via a cannabinoid CB_2 receptor-dependent mechanism. Δ^9THC, a weak partial agonist at the CB_2 receptor, is known to suppress inflammation and immune responses *in vivo* [14], which suggests that blockade of the action of 2-AG induces inflammatory reactions and immune responses [13], indicating a role for agonists of the CB_2 receptor as anti-inflammatory agents.

CB_2 receptors have been found in normal skin, keratinocytes, and skin tumors [15] and now in bone

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tissue, specifically in osteoclasts [16, 17], where they are thought to play a role in regulating bone mass in humans [18]. However, recent work has demonstrated that the CB_1 receptor also plays a role in the regulation of bone mass and that cannabinoid receptor antagonists inhibit the effect of osteoclasts [17], which mediate bone resorption, thus preserving bone mineral density (BMD). Bab et al. [16] recently analyzed CB_1 expression in bone, comparing the skeletons of sexually mature C57($CB_1^{-/-}$) and CD1($CB_1^{-/-}$) mice. In addition to their low bone mass, both male and female C57($CB_1^{-/-}$) mice exhibited decreased bone formation rate and increased osteoclast number, but the skeletal phenotype of the CD1($CB_1^{-/-}$) mice showed a gender disparity. Females had normal trabecular bone with a slight cortical expansion, whereas male CD1($CB_1^{-/-}$) animals displayed a high bone mass phenotype, although bone formation and resorption were within normal limits. These findings also support an important role for CB_1 signaling in the regulation of bone remodeling and bone mass and suggest the possibility that CB_1 receptor agonists (including Δ^9THC) may accelerate osteoporosis [19].

All of these studies have focused on the effects of cannabinoids on bone resorption and osteoclast activity. In contrast, comparatively little is known regarding the effects of cannabinoids on osteoblasts and bone formation, and indeed on musculoskeletal tissues in general. A solitary study has shown that cannabinoid agonists can inhibit nitric oxide production by chondrocytes, suggesting a possible protective effect in cartilage [20]. However, to the authors' knowledge, no other studies have investigated the effects of cannabinoids on bone-forming cells or mesenchymal cells in general.

It is now widely accepted that mesenchymal stem cells (MSCs) play a central role in bone physiology and pathology. MSCs are a class of multipotent cells originally identified by Friedenstein and coworkers some three decades ago, who demonstrated *in vivo* that these cells could adopt osteoblastic, chondrocytic, and adipocytic phenotypes [21, 22]. These findings have subsequently been confirmed in many laboratories [23–25], and it has also been shown that MSCs can be transformed into other cell types including cardiomyocytes [26], muscle cells [27], and neural cells [28]. The physiological function of MSCs is still unclear; however, it is possible that they are involved in tissue healing processes such as muscle regeneration [29] and fracture healing [30]. It has also been suggested that MSCs are involved in the response to bone anabolic drugs such as prostaglandin E_2 [31], fibroblast growth factor-2 [32], 1,25-dihydroxyvitamin D_3 [33], and parathyroid hormone [34]. Furthermore, MSC numbers have been shown to decrease with age [35–37] and their differentiation to be inhibited by unloading [38, 39], suggesting a role in bone wasting. Because of their obvious involvement in bone formation and their possible involvement in the physiology and pathology of other mesenchymal tissues, we investigated the effect of cannabinoids on MSC activity *in vitro*.

Materials and Methods

Materials

Natural cannabinoids were a kind gift from THC Pharm (Frankfurt, Germany). Endocannabinoids and cannabinoid analogues were obtained from Tocris (Bristol, UK). Anti-CB_1 and -CB_2 were obtained from Abcam (Cambridge, UK), and all other antibodies were from Serotec (Oxford, UK). Serum supreme, culture media, and ultraglutamine were obtained from Biowhittaker (Wokingham, UK). Tissue culture flasks were obtained from Scientific Laboratory Supplies (Nottingham, UK). All other chemicals and culture consumables were obtained from Sigma (Poole, UK) and used as supplied.

Bone Marrow Cell Preparation

Bone marrow cells (BMCs) were obtained centrifugally from tibiae and femora of male 200 g Wistar rats according to the method of Dobson et al. [40]. Briefly, the tibiae and femora were removed aseptically and all soft tissue removed. The proximal ends of the femora and the distal ends of the tibiae were removed; the bones were then placed in microfuge tubes supported by plastic inserts and centrifuged at 2,000 rpm for 2 minutes. Each of the marrow pellets, which were deposited in the microfuge tubes, was then resuspended in 10 mL culture medium by repeated pipetting and the final cell density adjusted to 4×10^6 mononuclear cells/mL.

Fibroblastic Colony Forming Unit Cultures

Fibroblastic colony-forming unit (CFU-f) cultures were performed as described previously [40]. Cells (2×10^6 mononuclear BMCs suspended in 0.5 mL culture medium) were plated out in 55 cm^2 Petri dishes in 10 mL Dulbecco's modified Eagle medium (DMEM) containing 10% serum supreme, 10^{-8} M dexamethasone, and 50 μg/mL ascorbic acid (cell culture medium) in the presence or absence of the appropriate can-anabinoid. The cell culture medium was replaced with fresh drug-free medium after 5 days and thereafter twice weekly. The cultures were maintained for 15 days, after which the cells were washed with phosphate-buffered saline and fixed by the addition of cold ethanol. After fixation, the cultures were sequentially stained for alkaline phosphatase (ALP), calcium- and collagen-positive colonies, and total colonies and analyzed as described below.

Analysis of CFU-f Cultures

ALP-positive colonies were stained histochemically by incubating the fixed cultures in a solution of naphthol phosphate (0.05 mg/mL) and fast red bb (1 mg/mL) in Tris 0.08 M (pH 7.5) for 30 minutes at 20°C. Calcium-positive colonies were stained with alizarin red (pH 6.5) and collagen-positive colonies were identified using sirius red (1 mg/mL) in saturated picric acid. To identify total colonies, the cultures were washed with borate buffer (10 mM, pH 8.8), stained with methylene blue (1 mg/mL) in borate buffer for 30 minutes, and then rewashed three times with borate buffer. After staining, the cultures were photographed with a digital camera and the images analyzed using Bioimage Intelligent Quantifier software (Bioimage Systems Inc., Jackson MI), as previously described [41].

Table 1. Expression of CB_1 and CB_2 receptors by 1° BMCs and secondary BMSCs

	Percent expression	
	Primary BMCs	Secondary BMSCs
Isotype control	3.1	3.8
CD31	12.8	8.8
CD44	21.3	60.3
CD45	65.1	12.1
CD61	7.7	3.9
CB_1	13.8	4.8
CB_2	12.1	3.9

To investigate the expression of the receptors further, 1° BMCs and MSCs were analyzed for CB_1 and CB_2 expression by flow cytometry. Consistent with the data shown in Figure 6, cultures enriched for MSCs did not express significant levels of either CB_1 or CB_2 receptor, whereas a significant fraction of 1° BMCs expressed both receptors

High-Density BMC Cultures

High-density BMC cultures were established as described previously but with modifications [42]. BMCs (10^5) were plated out in 2 cm^2 wells in 0.75 mL DMEM containing 10% fetal calf serum, 10^{-8} M dexamethasone, and 50 μg/mL ascorbic acid in the presence or absence of the appropriate cananabinoid. The medium was first changed for fresh cannabinoid-free cell culture medium after 5 days and thereafter three times weekly. The cultures were maintained for 14 days, after which the cells were washed with phosphate-buffered saline, fixed by the addition of cold ethanol, and analyzed. Cell number was assessed using the alamar blue assay, ALP activity by the hydrolysis of *p*-nitrophenyl phosphate, and calcium and collagen by staining with alizarin red or sirius red, respectively. Alternatively, cultures of bone marrow stromal cells (BMSCs) were prepared by growing whole BMCs in nonosteogenic medium (DMEM, 10% serum supreme, penicillin, and streptomycin) and allowing the cells to come to confluence. Nonadherent cells were removed and the cells passaged once; at this point, virtually all hematopoietic cells are removed from the cultures (e.g., see Table 1). The cells were then plated out at 10,000/well in 24-well plates and treated identically to the whole bone marrow cultures.

Cell Filtration

Single cells from aggregates of bone marrow cells were separated as described previously [43]. Briefly, 10 mL bone marrow cell suspension, prepared as described above, was passed through a 30 μM cell sieve and then washed with a further 10 mL cell culture medium to remove any remaining single cells. The filtrate was then centrifuged and resuspended in 10 mL culture medium. The aggregates were then recovered from the retentate fraction by flushing with 10 mL cell culture medium in the reverse direction. For CFU-f cultures, 0.5 mL of either the retentate or filtrate was used corresponding to the aggregates or single cells present in a suspension of 2×10^6 BMCs, respectively.

Flow Cytometry

Primary BMCs and secondary subcultures of BMSCs were analyzed with a Guava personal cytometry system (Guava Technologies, Hayword, CA). The percentages of cells positive for CD31, CD44, CD45, CD61, CB_1, and CB_2 were determined and analyzed with Guava Express protein analysis software.

Data Handling and Statistical Analyses

Data are presented as group mean ± standard deviation (SD). At least three replicates of each experiment were performed, and the results presented in the figures and tables are representative of these. For each variable, effects across treatment groups were compared with one-way analysis of variance (ANOVA). If the overall difference was significant, multiple comparisons were performed between groups with Tukey's test. Nonparametric data (colony sizes) were analyzed using Kruskal-Wallis one-way ANOVA by ranks. Differences were considered significant at $P < 0.05$ on a two-tailed test.

Results

Treatment of CFU-f cultures with a variety of naturally occurring cannabinoids, including cannabidivarine (CBDV), cannabigerol (CBG), cannabinol (CBN), cannabidiol (CBD), THC, and tetrahydrocannabivarin (THCV), all at 10 μM, produced a stimulation of colony formation in all cases. This varied from a relatively small stimulation of around 20% in the case of CBG to as much as ~100% after treatment with CBDV or THCV. This increase in colony number was also reflected by a parallel increase in the number of colonies that were positive for both ALP expression and collagen synthesis (Figs. 1 and 2). Furthermore, visual inspection of the plates revealed that cultures treated with cannabinoids showed increased numbers of large colonies relative to control cultures (Fig. 1). Subsequent analysis of the images confirmed this and showed that while the median colony size did not differ greatly, the number of large colonies with a staining index (colony area in pixels × staining intensity) of 70 or more (corresponding to ~14,000 cells/colony [41]) was significantly increased (Fig. 3).

The dose dependence of this effect was investigated using the endogenous cannabinoid 2-AG. 2-AG produced a similar dose-dependent increase in the total number of colonies, with statistically significant effects being seen at concentrations as low as 1 nM and reaching a peak at 1 μM. As well as total colonies, there was a parallel and proportional increase in the number of colonies adopting an osteoblastic phenotype, with increases in the number of ALP- and collagen-positive colonies being seen (Fig. 4a). There was also a dose-related increase in the median colony size, reaching a maximum at 10 μM 2-AG, which did achieve statistical significance. Image analysis revealed that this increase was due to the presence of a relatively small number of particularly large colonies similar to those seen in the cannabinoid-treated plates shown in Figure 1 (Fig. 4b).

In order to elucidate which of the two cannabinoid receptors was mediating these effects, the cultures were treated with a range of cannabinoid agonists of known specificity. The nonspecific cannabinoid receptor agonists CP 55,940 and WIN 55,212 both produced statisti-

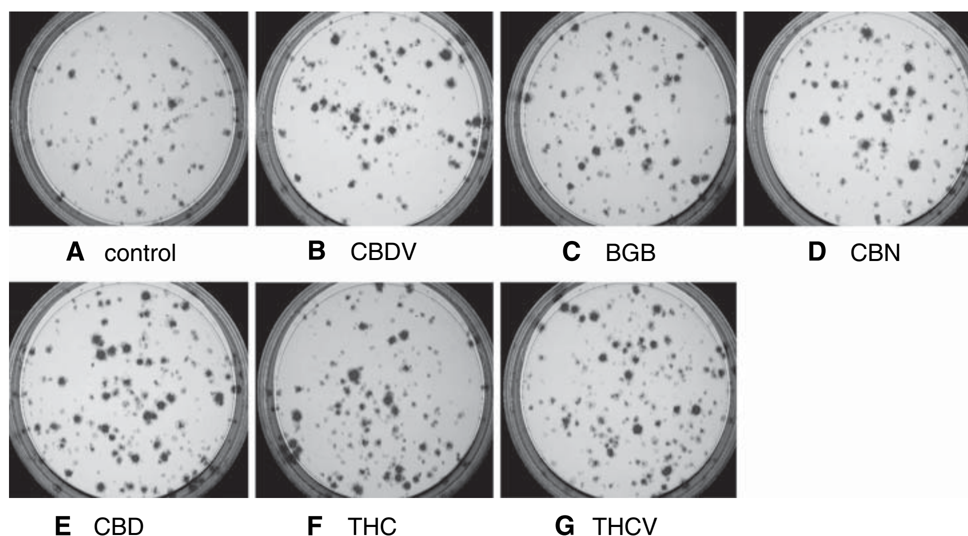

Fig. 1. The effect of cannabinoids on fibroblastic colony formation by BMCs *in vitro*. Whole BMCs (2×10^6) were plated out in Petri dishes as described in the text and treated with (**a**) vehicle, (**b**) CBDV, (**c**) CBG, (**d**) CBN, (**e**) CBD, (**f**) THC, or (**g**) THCV, all at a concentration of 10 μmol. Medium was changed for fresh cannabinoid-free medium after 5 days and then twice weekly. After 14 days, cultures were stopped and fixed by addition of 100% ethanol, stained, and photographed.

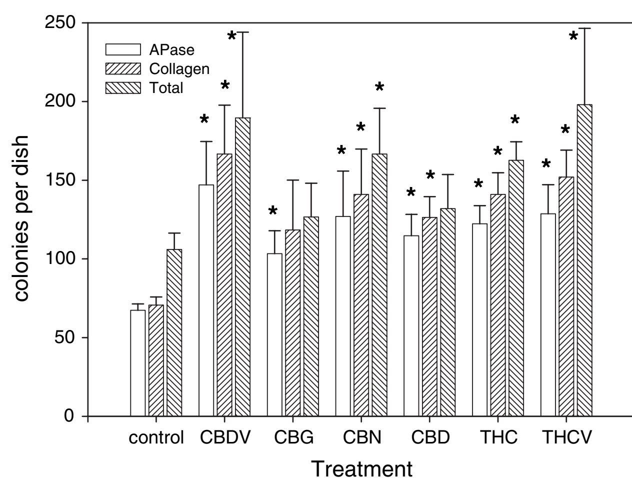

Fig. 2. The effect of cannabinoids on fibroblastic colony formation by BMCs *in vitro*. Whole bone marrow cells were cultured as described in Figure 1. After fixation, cultures were sequentially stained for ALP (naphthol phosphate/fast red), collagen (sirius red), and total colonies (methylene blue). After each staining, a digital image of each culture was acquired, and the number and size of the colonies were determined by image analysis. Data are presented as means ± SD (n = 3). *Statistical significance from corresponding control cultures, $P < 0.05$, as determined by ANOVA.

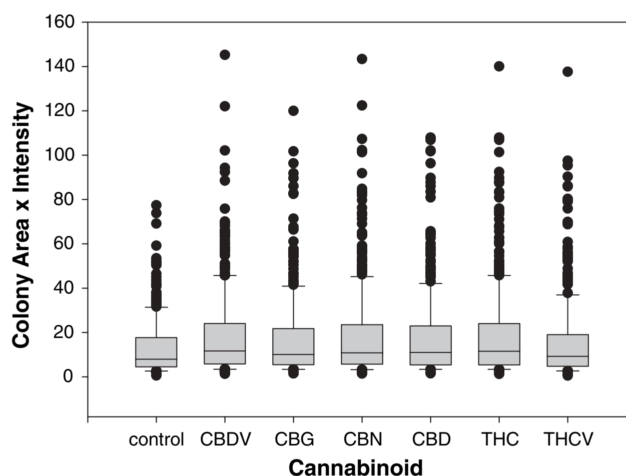

Fig. 3. The effect of cannabinoids on bone marrow-derived fibroblastic colony size *in vitro*. BMCs were cultured as described in Figures 1 and 2. After image acquisition, colony size was determined by image analysis (colony area multiplied by staining intensity), and the data are presented here as box plots where the *box* represents the 25th to 75th percentiles, the *whiskers* the 10th to 90th percentiles, the *dots* the remaining outliers, and the *line in the box* the median colony size. All cannabinoid-treated cultures were found to differ significantly from controls as determined by Kruskal-Wallis one-way ANOVA by ranks, $P < 0.05$.

cally significant increases in colony number, as did the CB_2-specific agonists BML190 and JWH015. In contrast, the CB_1-specific agonist ACEA had no significant effect on colony number (Fig. 5a), suggesting that the increased colony number caused by cannabinoid treatment is mediated via the CB_2 receptor. Consistent with this, the increase in colony number seen after treatment with THCV could be reduced by treatment with the CB_2-specific antagonist AM630 (Fig. 5b).

It was expected that MSCs would be the target cells for the effects of the cannabinoids. However, treatment of secondary subcultures of BMSCs, which would be expected to be enriched for MSCs, with 2-AG had no effect on either cell number or collagen accumulation (Fig. 6b). In contrast, treatment of primary cultures of whole BMCs with 2-AG stimulated both of these parameters (Fig. 6a). This would suggest that the target cell is not the MSC but rather an accessory cell present in the bone marrow. Consistent with this, secondary subcultures of MSCs were negative for the presence of either the CB_1 or CB_2 receptor as shown by flow-cytometric analysis. On the other hand, flow-cy-

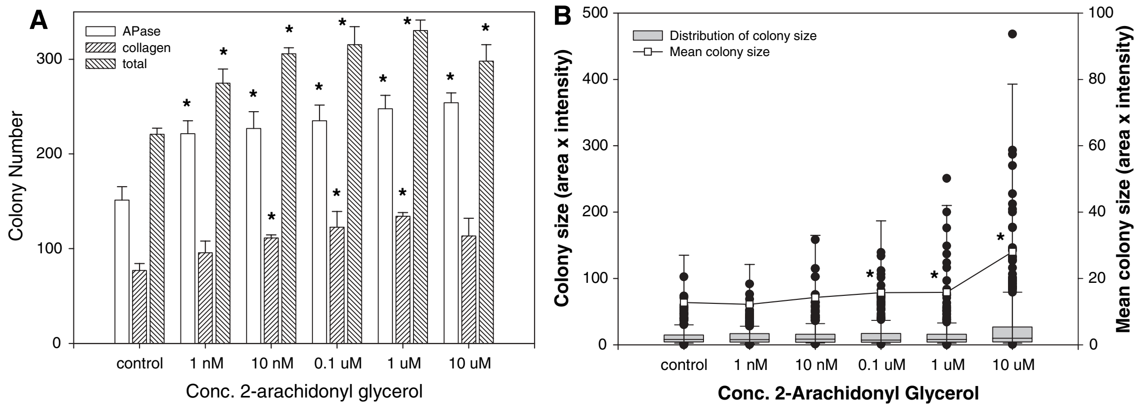

Fig. 4. Effect of 2-AG on fibroblastic colony formation by BMCs. BMCs were cultured and analyzed as described in Figures 1–3 after challenge with 1 nmol to 10 μM arachidonylglycerol. 2-AG produced a dose-related increase in total and differentiated colony formation (**a**) along with an increase in average colony size (**b**). In particular, there was a noticeable dose-related increase in the number of large colonies. Data in (**a**) are presented as means ± SD ($n = 3$). *Statistical significance from corresponding control cultures, $P < 0.05$, as determined by ANOVA. Data in (**b**) are presented as box and whisker plots as described in Figure 3. *Statistical significance from control cultures, $P < 0.05$, as determined by Kruskal-Wallis one-way ANOVA by ranks.

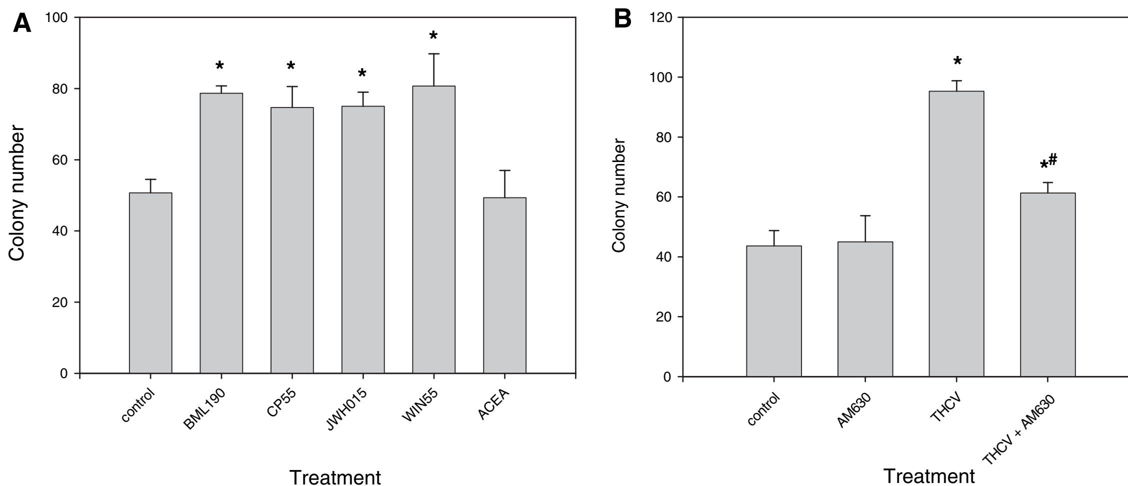

Fig. 5. Effective cannabinoid receptor agonists and antagonists on fibroblastic colony formation by BMCs. CFU-f cultures were established as described above and then treated with a series of agonists with varying specificity for the CB_1 and CB_2 receptors. It was found that the nonspecific agonists BML190 and CP 55,940 both stimulated fibroblastic colony formation, as did the CB_2-specific agonists JWH015 and WIN 55,212. In contrast, the CB_1- specific agonist ACEA had no effect on colony formation (**a**). The stimulation of colony formation induced by THCV was blocked by coincubation with the CB_2-specific antagonist AM630, thus confirming mediation by the CB_2 receptor (**b**). Data in (**a**) are presented as means ± SD ($n = 3$). Statistical significance *from corresponding control cultures and #from THCV-treated cultures, $P < 0.05$, as determined by ANOVA.

tometric analysis of whole bone marrow demonstrated the presence of a small population of cells positive for both the CB_1 and CB_2 receptors (13.8 and 12.1, respectively, Table 1). In a previous publication, we demonstrated the importance of cell aggregates in MSC proliferation, showing that the aggregates had an enhanced colony-forming capacity as well as ALP expression and response to prostaglandin E_2 [43]. This was also found to be the case for the endogenous cannabinoid 2-AG. As before, whole bone marrow responded to 0.1 μM 2-AG with an increase in total and ALP-positive colonies, as did cellular aggregates isolated using a 30 μm filter (30 μm retentate, Fig. 7). In contrast, the 30 μm filtrate and dissociated retentate did not produce any differentiated colonies as found previously [43] and did not respond to treatment with 2-AG (Fig. 7). As MSCs do not express significant levels of CB_1 or CB_2, this would in turn suggest that direct cell-cell contact is required for communication between the accessory cells and the MSCs.

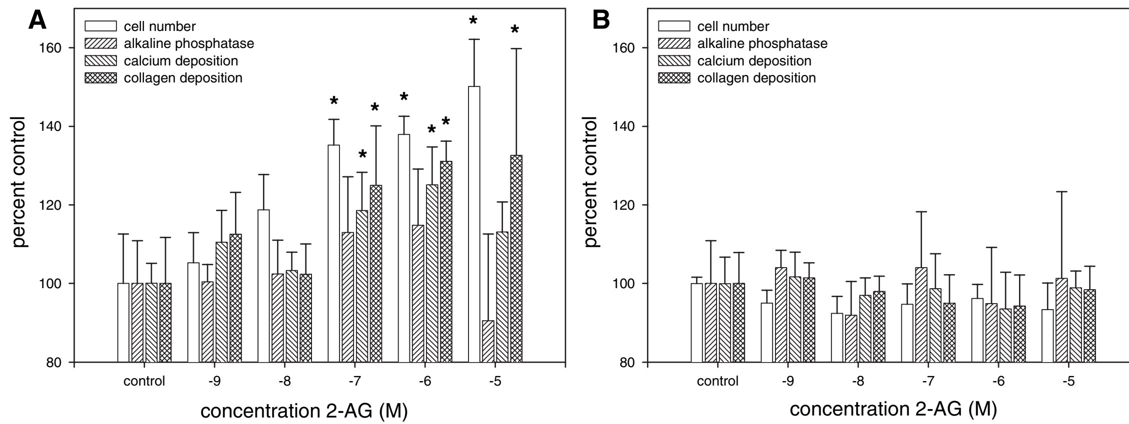

Fig. 6. Effect of 2-AG on high-density BMC cultures. To determine whether the cannabinoids were having a direct or an indirect effect on MSCs, high-density cultures containing either primary BMCs or secondary subcultures of MSCs were treated with 2-AG and assessed for ALP expression, collagen and calcium deposition and total cell number. 2- AG produced no significant effect on secondary subcultures of MSCs any of the parameters measured (**B**), whereas primary BMCs responded with a dose-related increase for all parameters (**A**), suggesting an indirect mechanism. Data are presented as means ± SD (n = 4). *Statistical significance from corresponding control cultures, $P < 0.05$, as determined by ANOVA.

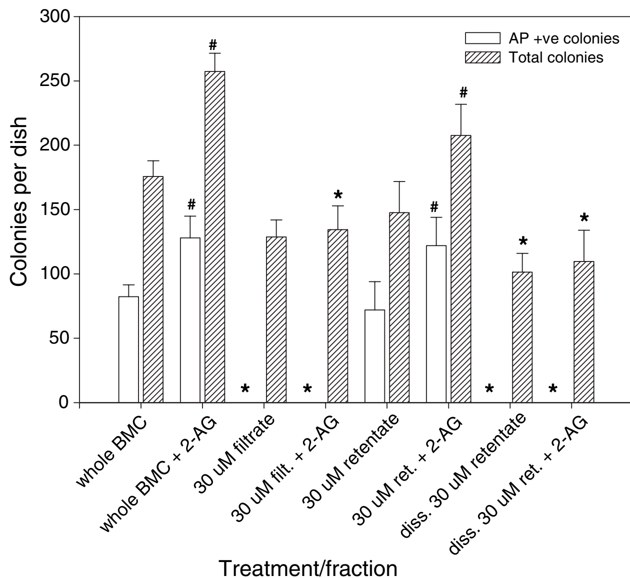

Fig. 7. Effect of disaggregation and cell sieving on the response to 2-AG. Using a 30 µm cell sieve, whole bone marrow was separated into retentate (aggregate) and filtrate (single-cell) fractions and an aliquot of the retentate was disaggregated using ethylenediaminetetraacetic acid. The resulting fractions were cultured in the CFU-f assay, in the presence or absence of 10 µM 2-AG, such that the number of cells in each culture was equivalent to the number of aggregates or single cells present in a suspension of 2×10^6 whole BMCs. Data are presented as mean colonies per Petri dish ± SD. Significant difference *from whole bone marrow and #from filtrate cultures, $P < 0.05$.

Discussion

In this study, we have demonstrated that cannabinoids can stimulate the recruitment of quiescent MSCs present in bone marrow. Furthermore, we have shown that this effect appears to be mediated via the CB_2 receptor and is indirect in nature; the target cells of the effect, MSCs, do not appear to respond directly to cannabinoids and, in their undifferentiated forms, do not express either the CB_1 or CB_2 receptor. These findings may have considerable repercussions as MSCs are now thought to play a central role in a wide range of physiological and pathophysiological processes, including bone formation [44], fracture healing [30], muscle regeneration [29], vascular damage [45], atherosclerosis [46], bone marrow fibrosis [47], as well as support of hematopoietic stem cells [48]. In addition, many therapeutic applications for MSCs have been suggested, including among many others myocardial regeneration [49]; tissue engineering of bone, cartilage [50], and vascular tissue [51]; and cell or gene therapy of genetic conditions such as osteogenesis imperfecta [52] and muscular dystrophy [53].

Two recent investigations have reported seemingly contradictory roles for the cannabinoid receptors in bone metabolism. Idris et al. in 2005 [17] reported an apparent increase in BMD in CB_1 knockout mice mediated by a decrease in osteoclastic bone resorption. This was also supported by the finding that CB_1 receptor agonists stimulated the formation of osteoclasts *in vitro*. These data naturally led to speculation that cannabis abuse may cause osteoporosis, although there are no data to support this. In contrast, Ofek et al. [54] reported in 2006 that CB_2 knockout mice developed an apparent high-turnover osteoporosis involving changes in both osteoblast and osteoclast numbers. Furthermore, treatment of wild-type mice with a CB_2-specific agonist decreased bone resorption and accelerated formation, thus preventing the bone loss associated with ovariectomy. Although these two studies report appar-

ently contradictory data, they should not be considered to be mutually exclusive. Bone metabolism is a complex multifactorial process involving cells of both hematopoietic and mesenchymal lineages. The result of this is that stimuli can give rise to anabolic or catabolic effects depending on dosage, timing, and side of application. For example, it has been known for some years that parathyroid hormone [55], prostaglandin E_2 [56, 57], and calcitriol [33, 58] can induce bone formation or bone loss according to the treatment regimen.

The data in this study are largely supportive of those previously published by Ofek et al. [54]. In particular, the increase in bone formation seen by these authors was accompanied by an increase in the recruitment of bone marrow-derived MSCs. The recruitment of MSCs has been suggested as a possible mechanism the effects of a number of bone anabolic drugs including prostaglandin E_2 [31], parathyroid hormone [34], and calcitriol [33]. It is also consistent with the findings of this study whereby the direct application of natural and endogenous cannabinoids to CFU-f cultures resulted in increased formation of fibroblastic colonies. As with previous studies investigating the effects of bone anabolic drugs on CFU-f cultures, a proportion of the colonies adopted an osteoblastic phenotype in the presence of dexamethasone and ascorbate [42, 59–61]. In contrast to the findings of Ofek et al. [54], however, no mitogenic effect was seen after treatment of enriched cultures of BMSCs with cannabinoids. This lack of effect may be due to the stage of differentiation of the cells in these cultures. Ofek et al. [54] did not find any CB_2 expression by primary BMSCs until they had been cultured in osteogenic medium for 20 days, at which point they were expressing an osteoblastic phenotype (ALP- and parathyroid hormone receptor-positive). Consistent with this, in our hands, MSCs grown under nondifferentiating conditions did not express either the CB_1 or CB_2 receptor and did not respond to challenge with cannabinoids. Primary BMC cultures, however, did respond with an increase in total cell number, ALP, calcium, and collagen deposition. Flow-cytometric analysis of whole BMCs revealed a sizeable subpopulation of cells expressing both CB_1 and CB_2 receptors. This would suggest, therefore, that the cannabinoids are not acting directly on the MSCs but rather indirectly via either hematopoietic or endothelial cells, both of which are known to express these receptors [62, 63]. Furthermore, the effect of 2-AG was only seen in cultures containing aggregates of BMCs. We have shown previously that BMC aggregates show enhanced colony formation and ALP expression [43], and in this study only whole bone marrow and isolated aggregates responded to 2-AG with an increase in colony formation. This is consistent with the suggestion that the cannabinoids act indirectly via direct cell contact and that the lack of effect in single-cell suspensions appears to rule out the involvement of humoral factors. However, due to the complexities of the bone marrow population, we cannot rule out other possibilities; in particular, due to the lack of a good *in vivo* marker for MSCs, we cannot be sure that the MSCs do not simply lose CB_1 or CB_2 expression in culture. Nevertheless, similar interactions have been demonstrated previously between megakaryocytes and MSCs [64, 65] and endothelial cells and MSCs [66, 67]. Furthermore, although the vasodilatory effects of cannabinoids are well known [68], cannabinoid receptors have not been identified on vascular smooth muscle cells or pericytes (apart from one report demonstrating expression of CB_1 on a smooth muscle cell line [69]). Because of this, similar indirect mechanisms for cannabinoid-mediated vasodilation have been suggested [63].

The data shown suggest that these effects are mediated via the CB_2 receptor. Colony formation was stimulated by the CB_2-specific agonists JWH015 and WIN 55,212 and blocked by the CB_2-specific antagonist AM630. Furthermore, the CB_1 agonist ACEA had no effect on colony formation. Taken together these data suggest that cannabinoids can indirectly stimulate the recruitment of MSCs via the CB_2 receptor, thus facilitating an adequate supply of osteoprogenitor cells for bone formation and may represent one cellular mechanism underlying the stimulation of bone formation by cannabinoids.

The importance of further investigation into the long-term effects of cannabinoids on basic metabolic processes, including those concerned with skeletal tissue, is illustrated by the proposed usage of therapeutic cannabis extracts, isolated cannabinoids, and cannabinoid antagonists. In general, they are being investigated for use in chronic rather than acute conditions, e.g., multiple sclerosis, rheumatoid arthritis, Parkinson's disease, and other degenerative diseases; but there are many new therapeutic targets being identified (for review, see Pertwee [70]). In elderly patients, osteoporosis may already be a preexisting condition and one implication of the work of Idris et al. [17] is that the use of CB_1 agonists may exacerbate bone loss. This scenario would apply to cannabis itself, whether used medicinally or illicitly. Conversely, the CB_1 receptor antagonist rimonabant, which has recently been approved for sale in the United Kingdom following clinical trials for the treatment of obesity and as an aid to smoking cessation [71, 72], may afford some protection against osteoporosis.

CB_2 receptors are peripherally expressed, and recent reports suggest that the selective activation of cannabinoid CB_2 receptors produces antinociception without traditional cannabinergic side effects; thus, selective cannabinoid CB_2 receptor agonists might be useful in the management of pain [73]. According to our results, which support the work of Ofek et al. [54], the use of CB_2 agonists may actually have a beneficial effect on bone mineralization and may represent a new direction

for the investigation of osteoporosis mechanisms as well as for drug development. The postulated bone anabolic effect of CB_2 agonists would apply to cannabis and its major constituent tetrahydrocannabinol, which also acts at the CB_2 receptor; and it is therefore possible that the overall effect of cannabis is neither bone anabolism nor catabolism, but this remains to be investigated. It also raises the intriguing possibility that the endocannabinoid system is an important regulatory mechanism for bone mass regulation, involving a balance between the activity of CB_1 and CB_2 receptors. In general toxicity terms, cannabis is considered to be fairly safe except in patients predisposed to some psychiatric conditions and with the usual caveats applied to any drug which can impair mental alertness and consciousness. However, our results showed that these anabolic effects on bone are independent of the CB_1 receptor and were confirmed by the observation that nonpsychoactive cannabinoids were as effective as THC. In fact, all cannabinoids tested showed activities of similar orders of magnitude, indicating that it should be possible to develop cannabinoid drugs to target osteoporosis without this particular disadvantage, from both a medical and a legal viewpoint.

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