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High concentrations of dexamethasone suppress the proliferation but not the differentiation or further maturation of human osteoblast precursors *in vitro*: relevance to glucocorticoid-induced osteoporosis

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Abstract

Objective. The use of glucocorticoids (GCs) in the treatment of RA is a frequent cause of bone loss. *In vitro*, however, this same class of steroids has been shown to promote the recruitment and/or maturation of primitive osteogenic precursors present in the colony forming unit-fibroblastic (CFU-F) fraction of human bone and marrow. In an effort to reconcile these conflicting observations, we investigated the effects of the synthetic GC dexamethasone (Dx) on parameters of growth and osteogenic differentiation in cultures of bone marrow stromal cells derived from a large cohort of adult human donors ($n = 30$).

Methods. Marrow suspensions were cultured in the absence and presence of Dx at concentrations between 10 pM and 1 μM. After 28 days we determined the number and diameter of colonies formed, the total number of cells, the surface expression of receptors for selected growth factors and extracellular matrix proteins and, based on the expression of the developmental markers alkaline phosphatase (AP) and the antigen recognized by the STRO-1 monoclonal antibody, the proportion of cells undergoing osteogenic differentiation and their extent of maturation.

Results. At a physiologically equivalent concentration, Dx had no effect on the adhesion of CFU-F or on their subsequent proliferation, but did promote their osteogenic differentiation and further maturation. These effects were independent of changes in the expression of the receptors for fibroblast growth factors, insulin-like growth factor 1, nerve growth factor, platelet-derived growth factors and parathyroid hormone/parathyroid hormone-related protein, but were associated with changes in the number of cells expressing the α_2 and α_4, but not β_1, integrin subunits. At supraphysiological concentrations, the effects of Dx on the osteogenic recruitment and maturation of CFU-F and their progeny were maintained but at the expense of a decrease in cell number.

Conclusions. A decrease in the proliferation of osteogenic precursors, but not in their differentiation or maturation, is likely to be a key factor in the genesis of GC-induced bone loss.

KEY WORDS: Glucocorticoid-induced osteoporosis, Osteoblasts, Bone marrow stromal cells, Dexamethasone, Colony-forming unit, STRO-1, Alkaline phosphatase, Flow cytometry, Growth factor receptors, Integrins.

The adverse skeletal manifestations of glucocorticoid (GC) excess have long been recognized. Although these were first described in patients with endogenous hypersecretion (Cushing's syndrome), it is the iatrogenic form of GC excess that is seen most commonly today [1]. Particularly at risk are patients requiring GCs for the treatment of pulmonary, rheumatological,

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immunological or gastrointestinal disease or to prevent transplant rejection. Indeed, GC excess is now the third most frequent cause of osteoporosis after sex-steroid deficiency and ageing [1–3].

The elucidation of the cellular and molecular mechanisms that lead to GC-induced osteoporosis and the development of improved means of identifying those at risk remain important challenges [1, 4]. In human subjects receiving long-term treatment, bone loss is associated with decreases in the overall formation rate and in the mean thickness of the walls of newly synthesized trabecular packets [5, 6], a pattern of loss consistent with a decrease in the number and/or activity of osteoblasts rather than an increase in the number and/or activity of osteoclasts [6, 7]. Testing this hypothesis further has been complicated by the existence of marked species and/or age-related differences in the effects of GCs in animals [1, 3, 4], and by disease heterogeneity and the use of different treatment regimens in patients. Paradoxically, the results of several studies using cells derived from human bone have shown that treatment with GCs promotes the differentiation of cells of the osteoblast lineage [8–14], and it has been proposed that they be used as a standard supplement for the *ex vivo* expansion of cells with osteogenic potential for use in tissue reconstruction and repair [15].

In this investigation we studied the effect of a synthetic GC on the recruitment, proliferation and maturation of human cells of the osteoblast lineage at concentrations that span the physiological and supraphysiological ranges. The cells studied were obtained from the bone marrow, as this is known to serve as a repository for primitive cells with osteogenic potential in the adult and because it is trabecular bone in contact with the marrow that is most severely affected in GC-induced osteoporosis [5, 16–18]. The extent to which the effects of dexamethasone (Dx) in this cell culture system are associated with changes in the expression of the receptors for locally produced growth factors and extracellular matrix proteins was also investigated.

Patients and methods

Subjects

Adult human bone marrow was flushed from segments of rib obtained from 30 patients (17 males and 13 females) with no history of bone disease who were undergoing thoracic surgery. Their mean age ($\pm$ S.E.M.) was 59 ± 3 years (range 33–77 yr). Samples were obtained according to procedures approved by the local ethics committee. Patients with a history of GC treatment were excluded from the study.

Antibody reagents

The monoclonal antibody (mAb)-producing hybridoma cell line STRO-1 (IgM subclass) was obtained from Dr Paul Simmons, Hanson Centre for Cancer Research, IMVS, Adelaide, South Australia [19]. STRO-1 was used in the form of an undiluted tissue culture supernatant [20]. Hybridomas B4-78 and HB8737 (both IgG1 subclass), which recognize the bone/liver/kidney (B/L/K) isoenzyme of alkaline phosphatase (AP) [21] and the low-affinity nerve growth factor receptor (NGFR) respectively, were obtained from the Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, Iowa, USA. Antibody from these hybridomas was purified from culture supernatant by protein G chromatography (Pharmacia Biotech, St Albans, UK).

Culture of human bone marrow stromal cells

Cultures of bone marrow mononuclear cells were established as described previously [20, 22]. A single batch of fetal calf serum was used throughout these studies (batch number 2740; GlobePharm, Esher, UK). Adequate ascorbate nutrition was ensured by use of the long-acting ascorbate analogue L-ascorbic acid 2-phosphate (Asc 2-P, 100 μM; Alpha Laboratories, Eastleigh, UK) [23]. For cultures that were to be treated with Dx, the Dx (Sigma Chemical, Poole, UK) was added to the cells at the time of explantation and replaced with each change of medium. The non-adherent fraction was removed at 7 days and the adherent fraction, hereinafter referred to as bone marrow stromal cells (BMSC), was refed with a complete change of medium every 3–4 days. Occasionally, and irrespective of the presence or absence of Dx, cultures were found to contain large numbers of small spherical and/or ovoid mononuclear cells that remained loosely adherent but did not spread. As a matter of routine, these cultures were discarded. Under the conditions of culture employed in this investigation cells with the morphological characteristics of adipocytes were never observed.

Determination of colony-forming efficiency and the number of colonies expressing AP

After 28 days, cultures were washed with phosphate-buffered saline (PBS), fixed for 5 min at room temperature and then allowed to air-dry. The number of colonies formed, their diameter, reflecting changes in cell shape and density of packing as well as in cell number, and the proportion expressing AP were determined as described previously [24] using an Olympus SZ-CTV microscope ($15 \times$ objective) and measuring graticule. Colonies were photographed using a Nikon FM camera body with a 60 mm macro lens.

Cell counting and determination of AP activity

After 28 days, BMSC were washed in serum-free medium and incubated with 25 U/ml typeVII collagenase (Sigma) containing an additional 2 mM Ca^{2+} for 1 h at 37°C in serum-free medium, followed by trypsinization for 5 min at 37°C using 0.02% trypsin–EDTA (Gibco, Paisley Scotland) [23]. Cell number was determined electronically using a Coulter counter (Coulter Electronics, Luton, UK).

The AP activity of cell lysates was determined in triplicate as described previously [9]. The results were averaged, normalized for cell number and expressed as nanomoles of product cleaved per 10^6 cells per hour.

Immunolabelling of BMSC for STRO-1 and AP expression for flow cytometry

Dual staining of BMSC for the expression of the developmental markers STRO-1 and AP was done as described previously [20]. Cells stained with isotype-matched but irrelevant antibodies were used to assess background staining and compensation parameters were set using cells stained singly for STRO-1 and AP. We [20] and others [25] have shown independently that this approach permits the identification of cells of the osteoblast lineage at different stages of maturation: stromal precursors ($STRO\text{-}1^+/AP^-$), osteoprogenitors ($STRO\text{-}1^+/AP^+$) and maturing osteoblasts ($STRO\text{-}1^-/AP^+$).

Immunolabelling for the expression of integrins α2, α4 and β1, the low-affinity NGFR and transforming growth factor-β receptors III and IV

BMSC (5×10^5cells/tube) were harvested and incubated with PBS containing 5% (v/v) fetal calf serum (washing buffer) and 10% human AB serum (IBRGL Research Products, Elstree, UK; blocking buffer) on ice for 30 min. The cells were then incubated for 1 h at 4°C in a saturating concentration (20 μg/ml) of antibodies reactive with α2 (mouse IgG1, CD49b; Immunotech, Marseille, France), α4 (mouse IgG1, CD49d, Immunotech) and β1 integrins (mouse IgG1, CD29; Coulter Immunology, Hialeah, Florida, USA), NGFR (IgG1) and transforming growth factor-β receptor (TGFβR) III (betaglycan; IgG1; Alexis Corporation, Nottingham, UK) and TGFβR IV (endoglin; CD 105; IgG1; Ancell Immunology Research Products, Bayport, Minneapolis USA). After a wash step in washing buffer, the cells were incubated for 1 h at 4°C in the optimally diluted secondary antibody, R-phycoerythrin (RPE)-conjugated anti-mouse IgG1 (FL-2H; AMS Biotechnology, Oxfordshire, UK). The cells were then washed, resuspended in 2% (w/v) *p*-formaldehyde and then stored at 4°C until analysed (usually within 24 h) [22].

Immunolabelling for the expression of the receptors for fibroblast growth factor receptors 1 and 2, TGFβRs I and II, insulin-like growth factor receptor type-I α and β, platelet-derived growth factor receptors α and β and parathyroid hormone/parathyroid hormone-related protein

The antibody reagents recognizing fibroblast growth factor receptors (FGFR) 1 and 2, TGFβRs I and II, insulin-like growth factor receptor type-I (IGF-IR) α and β, platelet-derived growth factor receptors (PDGFR) α and β (all from Insight Biotechnology, Wembley, UK) and the receptor for parathyroid hormone/parathyroid hormone-related protein (PTH/PTHrP) (mAb 3D1.1; a kind gift from Dr Larry Suva of SmithKline Beecham, Philadelphia, USA) all recognized intracellular epitopes, which made it necessary that the cells be permeabilized by a combination of fixation (15 min at 4°C in PBS containing 2% w/v *p*-formaldehyde) and the inclusion of 0.1% (w/v) saponin in the washing and blocking buffers. After blocking, the cells were incubated for 1 h at 4°C in the presence of antibodies reactive with FGFRs 1 and 2, TGFβRs I and II, IGF-IRs α and β, PDGFRs α and β (1 μg/ml) or mAb 3D1.1 (10 μg/ml), incubated for 1 h at 4°C with fluorescein isothiocyanate-conjugated anti-rabbit IgGs (FL-1H; Sigma) or anti-mouse IgG1, and then treated as described previously. Cells stained with rabbit IgG (Sigma; FGFRs, TGFβRs, IGF-IR, PDGFR) and mouse IgG1 (Sigma; PTH/PTHrP receptor) were used to set 5% confidence limits for analysis, and the specific binding of the anti-receptor antibodies was assessed using cells stained with the anti-receptor antibodies in the presence of a 20-fold excess of the relevant carboxy-terminus peptide (typically >90% inhibition of binding).

FACS acquisition and analysis

Flow cytometric analysis was performed using a Vantage FACS (fluorescence-activated cell sorter) (Becton-Dickinson, Oxfordshire, UK; 488 nm; 180 mW power) using fluorescence emission wavelengths of 530 ± 30 and 575 ± 26 nm for FL-1 and FL-2 respectively. For all analyses, 10 000 events were collected. Data acquisition and statistical analysis (Kolmogorov–Smirnov test) were done using the Lysis II and CellQuest software packages (Becton-Dickinson).

Statistical analysis

Unless stated otherwise, all values are quoted as the median, median absolute deviation and interquartile range of determinations made in quadruplicate. The Mann–Whitney *U*-test was used to analyse differences between Dx-treated and non-treated values for each individual donor. Non-parametric two-way analysis of variance (Friedman test) was used to test for differences between the treatment groups using the pooled data from all donors. Spearman's rank correlation coefficient (Spearman's ρ) was used to analyse the relationship between the expression of STRO-1 and that of AP. All statistical analyses were performed using the Statview programme (v. 4.5; Abacus Concepts, Berkeley, California, USA).

Results

Colony-forming efficiency, colony size and cell number

There was a wide variation in the colony-forming efficiency (CFE) of bone marrow cell suspensions prepared from different donors (Fig. 1a). In a subset of the donors studied [eight males and six females, mean age ($\pm$ S.E.M.) 62 ± 4 yr, range 42–73 yr], the median CFE was 29 ± 11 colonies/10^5 mononuclear cells (~1 in 3500; range ~1 in 33 000 to 1 in 1800). Over a wide concentration range (10 pM to 1 μM), treatment with Dx had no discernible effect on the CFE of marrow cell suspensions (Figs 2a and Fig 3). Experiments performed using cells from a subgroup of donors ($n = 14$) in the presence of Dx at 10 nM, the concentration used most frequently to induce the differentiation of human marrow stromal cells [8–13, 15, 26–28] and which

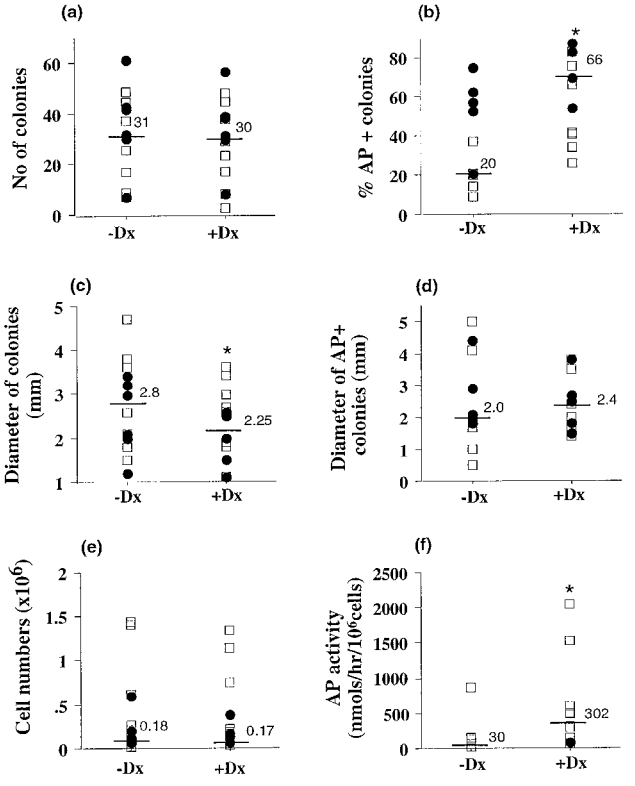

FIG. 1. The effect of 10 nM Dx on the growth and differentiation of cultures of adult human BMSC. Each point represents a single Dx-treated or untreated individual. Female donors are shown as closed circles and male donors as open squares. The median responses for each group are also indicated; responses significantly different from those of non-Dx treated cultures are marked with an asterisk ($P < 0.001$, Friedman test). (a–d) Fourteen donors (nine males, five females; mean age 62 ± 4 yr). (e) Sixteen donors (10 males, six females; mean age 62 ± 3 yr. (f) Ten donors (nine males, one female; mean age 58 ± 4 yr).

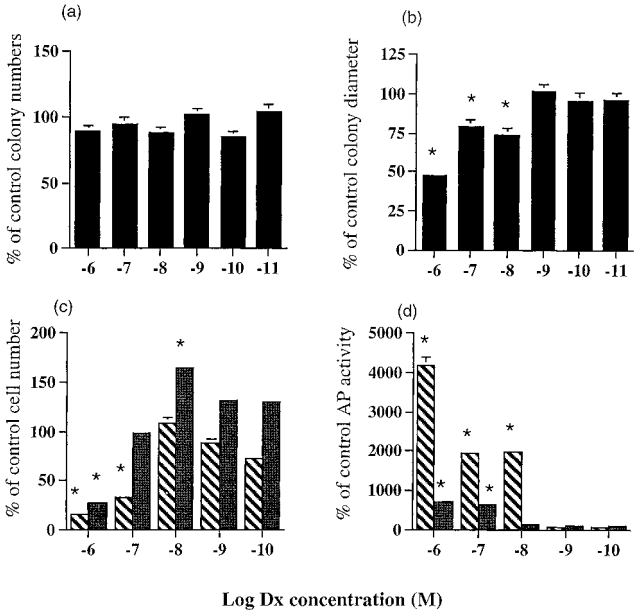

FIG. 2. Dose–response relationships for the effects of Dx. (a) Colony formation. Treatment with Dx did not alter the number of colonies that formed in cultures derived from any of the donors investigated (one male and two females; mean age 65 yr). The data shown are from a female donor aged 68 yr. (b) Colony diameter. Treatment with Dx ≥ 10 nM was associated consistently with a decrease in the median diameter of colonies that formed. (c) Cell proliferation. The data shown are for two male donors aged 63 yr (hatched bars) and 65 yr (shaded bars). The median number of cells harvested from the control cultures was 0.2 and 0.26×10^6 cells per flask for these two donors respectively. (d) Activity of AP. Patient details are the same as in (c). Basal AP activity was 103 and 876 nmol/10^6 cells per h for the 63- and 65-yr-old patients respectively. *$P < 0.02$ (Mann–Whitney U-test) compared with untreated cultures.

approximates to a physiological concentration of GC [2], confirmed that this steroid does not alter CFE in this cell culture system (Fig. 1a).

At concentrations of 10 nM and above, treatment with Dx was associated with a decrease in the median diameter of the colonies that formed (Figs 2b and 3). Analysis of the pooled data for cultures derived from a subgroup of 11 donors confirmed that at 10 nM the effect of Dx on the median diameter of colonies that formed, although statistically significant ($P < 0.001$), was minimal (median decrease $\sim 20\%$; Fig. 1c). On individual analysis, however, it is apparent that a decrease in mean colony diameter occurred in cultures derived from only eight of the 11 donors studied, and in only five of these was the decrease of statistical significance.

At concentrations of 100 nM and above, the effect of Dx on the number of cells harvested after 28 days in primary culture was consistently inhibitory (Fig. 2c). At lower concentrations its effects were markedly donor-dependent. Thus, in cultures derived from a total of 16 donors, treatment with Dx at 10 nM decreased, increased or had no effect on cell number (8, 4 and 4 donors respectively). These changes, however, were of small magnitude and analysis of the pooled data did not reveal a statistically significant effect of treatment with Dx at this concentration on cell number (Fig. 1e). The variation in the response of the cultured cells to treatment with Dx could not be explained by differences in the age and/or gender of the donors studied (data not shown).

Expression of the developmental markers STRO-1 and AP

Colonies positive for AP were present in cultures derived from 12 of the 14 donors studied. The median number of AP-positive colonies formed was 7.7 ± 6.5 per 10^5 mononuclear cells seeded (range 1–44). Between donors, there was a wide variation in the number of AP-positive colonies that formed, possibly reflecting the observed donor-dependent differences in CFE (see above). Expression of the data as a percentage of the total number of colonies formed reduced, but did not eliminate, this donor-dependent variation (median number of colonies $20 \pm 17.5\%$ of the total, range

Table 1 Mechanism of action of the effect of 10 nM Dx on AP expression of BMSC

	Without Dx		With Dx	
Donor	Percentage of AP$^+$ cells	Expression level per cell (MFI[a])	Percentage of AP$^+$ cells	Expression level per cell (MFI[a])
J	50	18.5	95*	471*
K	39	51	78*	100*
T	89	54.5	95*	471*
U	52	44	80*	195*
W	40	34.5	72*	79*
X	84	111	93*	122*
Median ± MAD[b]	51.0 ± 11.5	47.5 ± 10.0	86.5 ± 8.5	158.5 ± 69

BMSC from six donors (mean age 51 yr, range 32–70 yr, four males, one female, one gender-unknown) were cultured with or without 10 nM Dx for 28 days, harvested and stained for the expression of AP using the mAb B4-78, and the cells were analysed by flow cytometry. The results are the means of duplicate samples. *$P < 0.001$ (Kolmogorov–Smirnov test) compared with untreated cultures.
[a]Arbitrary units of mean fluorescence intensity.
[b]Median absolute deviation.

9–75%; Fig. 1b). Culture in the presence of 10 nM Dx increased the median number of AP-positive colonies formed to 18.0 ± 11.0 per 10^5 mononuclear cells seeded (range 1–55), corresponding to 66 ± 18% of the total (range 26–88%; Fig. 1b). In four of the 12 donors, treatment with Dx significantly increased the median diameter of AP-positive colonies that formed ($P < 0.05$, data not shown). For the population as a whole, however, the difference between the sizes of the AP-positive colonies that formed in control and Dx-treated cultures was not significant.

The activity of AP in lysates prepared from cultures derived from 10 donors varied by more than two orders of magnitude (73 ± 32 nmol/h per 10^6 cells; range 0.5–875 nmol/h per 10^6 cells; Fig. 1f). Treatment with Dx (100 pM to 1 μM) increased AP activity in a concentration-dependent manner in cultures derived from all donors, although to markedly differing extents (Fig. 2d). In cultures derived from the majority of donors, an increase in AP activity was observed at concentrations of 10 nM and above (Figs 1f and 2d) and at this concentration the median increase was 10 ± 6 ($P < 0.05$; 10 donors). The observed increases ranged from 2.5- to 180-fold, depending on the donor, and were statistically significant in every case ($P \leq 0.05$; Fig. 2d).

The mechanism(s) by which treatment with Dx influences the expression of AP in cultures of human BMSC was investigated by flow cytometry using the mAb B4-78. Under the conditions of culture employed here, AP-positive cells constituted 51 ± 11% of the total cell population and their mean fluorescence intensity (MFI, arbitrary units) was 48 ± 10 (Table 1). Culture in the presence of 10 nM Dx increased the proportion of cells to 86 ± 9% and their MFI to 159 ± 69 ($P < 0.05$ in each case; Table 1), indicating that treatment with this GC increases the number of cells expressing AP and the level at which it is expressed.

The Dx-induced increase in AP expression was associated with a decrease in the expression of the STRO-1 antigen, a marker of more primitive human marrow stromal cells, including those with osteogenic potential [19, 29]. Analysis of the pooled data from a total of 14 donors (13 males and one female, mean age 49 ± 6 yr; data not shown) revealed the existence of a statistically significant, inverse association between the expression of STRO-1 and AP in this cell culture system, which was strengthened considerably in the presence of 10 nM Dx (Spearman's $\rho = -0.569$, $P < 0.05$ and $\rho = -0.934$, $P < 0.001$, for control and Dx-treated cultures respectively; Fig. 4a–c). Further support for the existence of an inverse association between the

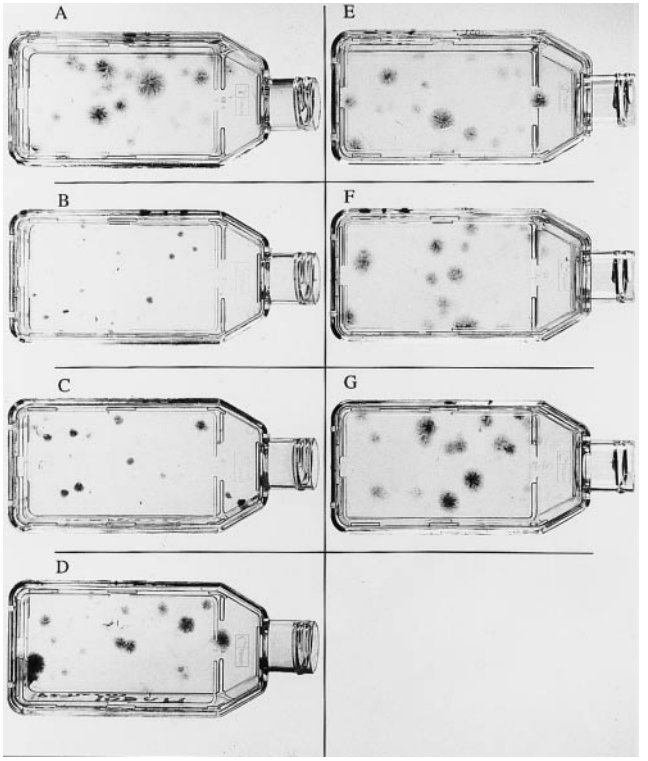

Fig. 3. Treatment with Dx influences the size but not the number of colonies that form in culture of adult human BMSC. BMSC were cultured for 28 days without Dx (A) or in the presence of Dx at 10^{-6} M (B), 10^{-7} M (C), 10^{-8} M (D), 10^{-9} M (E), 10^{-10} M (F) and 10^{-11} M (G); the colonies were then fixed and stained with methylene blue.

TABLE 2 Effect of 10 nM Dx on the expression on a range of markers present on BMSC [median (interquartile range)]

Antigen	Without Dx		With Dx	
	Percentage of cells positive for marker	Expression level per cell (MFI)	Percentage of cells positive for marker	Expression level per cell (MFI)
[a]α2	31 (11)	25 (9)	*13 (9)	22 (4)
[a]α4	32 (15)	20 (15)	*17 (13)	21 (8)
[a]β1	94 (1.0)	65 (14)	90 (3)	72 (7)
[a]NGFR	18 (8)	14 (10)	21 (8)	22 (6)
[c]FGFR 1	84 (9)	44 (36)	79 (12)	49 (32)
[c]FGFR 2	58 (37)	15 (12)	59 (38)	13 (11)
[d]TGFβR I	49 (29)	13 (3)	52 (12)	12 (2)
[d]TGFβR II	82 (9)	30 (8)	71 (15)	40 (10)
[a]TGFβR III (betaglycan)	88 (9)	35 (15)	92 (9)	34 (14)
[a]TGFβR IV (endoglin)	92 (13)	57 (23)	89 (13)	36 (12)
[a]PDGFR α	48 (25)	36 (33)	38 (15)	34 (16)
[a]PDGFR β	43 (12)	48 (46)	42 (29)	49 (44)
[b]PTH/PTHrP receptor	64 (50)	20 (15)	75 (30)	16 (26)
[a]IGF-IR α	65 (21)	10 (4)	72 (20)	12 (11)
[a]IGF-IR β	70 (29)	11 (0)	68 (29)	11 (4)

BMSC were cultured with or without 10 nM Dx for 28 days and stained for reactivity with mAbs specific for a range of antigen markers, and the cells were analysed by flow cytometry. The percentage of cells positive for each marker and their geometric mean fluorescence intensity (MFI; arbitrary units) was based on the corresponding isotype-matched control antibody using BMSC derived from at least three donors ([a]3 donors; [b]5 donors; [c]9 donors; [d]10 donors).
*$P < 0.02$ (Friedman test) compared with untreated cultures.

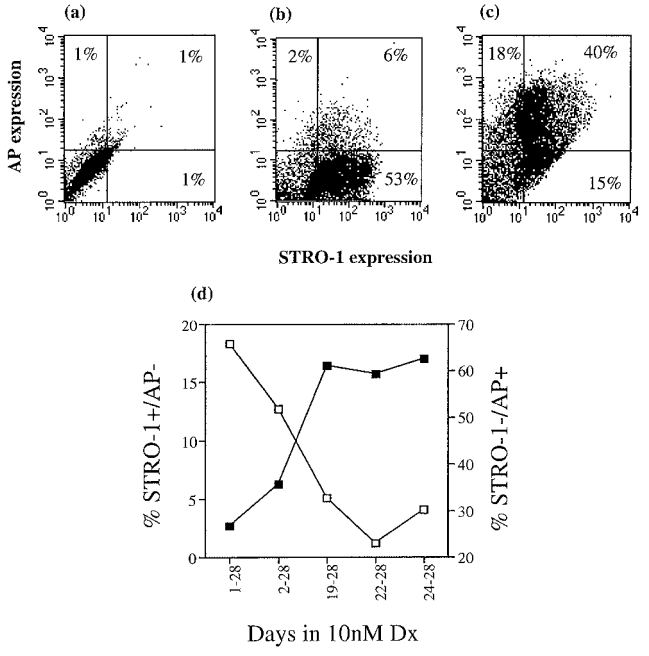

FIG. 4. Treatment with Dx influences the expression of STRO-1 and AP in cultures of adult human BMSC in a time and concentration-dependent manner. BMSC derived from donor Y (female aged 70 yr) were cultured continuously for 28 days in the absence (b) or presence of 10 nM Dx (c) or cultured for various lengths of time in 10 nM Dx (d, donor L; male aged 70 yr). The cells were immunolabelled for STRO-1 and AP expression. Quadrants defining the STRO-$1^+/AP^-$, STRO-$1^+/AP^+$ and STRO-$1^-/AP^+$ populations were set using isotype-matched control antibodies as shown in (a), and were used to enumerate the percentage of positive cells in each quadrant. (d) There was an inverse reciprocal relationship between the percentage of cells expressing the STRO-$1^+/AP^-$ (solid squares) and STRO-$1^-/AP^+$ (open squares) phenotypes.

expression of these developmental markers was obtained from experiments in which the proportion of cells present in the STRO-$1^+/AP^-$ (stromal precursor) and STRO-$1^-/AP^+$ (maturing osteoblast) fractions was determined at increasing intervals after the addition of Dx (Fig. 4d).

Expression of other cell surface markers

It has been hypothesized that an important facet of GC action is the ability to modulate the expression of receptors for components of the extracellular matrix and for a diverse array of soluble mediators in their target cell populations [9, 11, 26, 27, 30–39]. In the case of bone, the evidence in support of this hypothesis has been obtained largely from experiments conducted using tissue or cells of animal origin [30–32, 35–39]. We sought to establish, therefore, if a similar mechanism was operative in cultures of adult human bone-derived cells. To this end, BMSC were cultured for 28 days in the absence or presence of 10 nM Dx and then analysed by flow cytometry for the expression of cell-surface receptors for factors known to be present in bone and to influence the proliferation and/or differentiation of cells of the osteoblast lineage. In cultures derived from a large number of donors there was no evidence for an effect of Dx on the expression of FGFR 1 and 2, IGF-IR α and β, PDGFR α and β, NGFR, PTH/PTHrP receptor, TGFβR III and IV and the β_1 integrin subunit (Table 2). Treatment with Dx was associated, however, with a statistically significant ($P < 0.02$) decrease in the proportion of cells expressing the α2 and α4 integrin subunits (Table 2).

Discussion

GCs exert complex effects on the skeleton that are dependent on the species under investigation and its stage of development as well as on the concentration and duration of exposure [7, 9, 14, 26, 37–43]. In humans, prolonged exposure to supraphysiological levels of GCs is clearly associated with deleterious effects on the skeleton and is a major cause of osteoporosis [1–4]. The results of *in vivo* investigations, including the recent localization of the sites of GC receptor expression in adult human bone, suggest that this is due primarily to a negative effect of GC excess on the number and/or differentiated function of cells of the osteoblast lineage [2, 7, 44, 45]. Paradoxically, the results of *in vitro* investigations using bone-derived cells of adult human origin have suggested that GCs promote the differentiation of cells of this lineage [8–13, 26]. Whether this effect of GCs can be dissociated from their frequently negative effect on cell proliferation has not always been clear. Furthermore, the results have been obtained using cells derived from only a limited number of donors, and the extent to which they are truly representative is therefore uncertain. This is of particular importance given the current widespread interest in the use of *ex vivo* expanded populations of human marrow stromal cells for tissue reconstruction and repair and in the use of GCs to promote their osteogenic differentiation [15]. These considerations, and the desire to define more fully the mechanism of action of GCs on human cells of the osteoblast lineage, provided the impetus for these studies, which used cells from a large cohort of adult human donors.

The results presented here represent a substantial and important contribution to our understanding of the mechanism of action of GCs on human cells of the osteoblast lineage. They show that treatment with Dx at concentrations that span the physiological and supraphysiological ranges does not affect the ability of clonogenic cells present in the STRO-1$^+$ fraction of human bone and marrow to adhere and to initiate colony formation. It does, however, influence the ability of their progeny to proliferate and to express two developmental markers characteristic of cells of the osteoblast lineage, STRO-1 and AP. Our results show further that there is an inverse association between the expression of these markers in this cell culture system that is strengthened by culture in the presence of Dx. The use of flow cytometry and the monoclonal antibody B4-78 has allowed us to resolve the mechanism of the effect of Dx on the expression AP in this cell culture system into two components: an increase in the number of cells that express AP and an increase in the level at which it is expressed. In contrast, the effect of Dx on the expression of STRO-1 appears to be related primarily to a decrease in the number of cells that are expressing this marker of more primitive marrow stromal cells.

Analysis of the pooled data revealed that the effects of Dx on the proliferation and differentiation of human marrow stromal cells are, to a degree, dissociable. The critical concentration in this regard is 10 nM, which approximates to a physiological amount of GC. At this concentration, effects on the expression of STRO-1 and AP, but not cell number, are clearly evident. At higher concentrations ($\sim$100 nM, which approximates to a supraphysiological amount of GC) the effect of Dx on differentiation is enhanced, but it now occurs at the expense of a marked and progressive decrease in cell number. A critical factor in allowing the effect of Dx on differentiation to be dissociated from that on proliferation may be the precise conditions of culture employed, particularly with regard to the maintenance of adequate ascorbate nutrition. The results of previous studies have shown that GCs are potent inhibitors of collagen synthesis, and that this is an important component of their inhibitory action on cell proliferation [1]. When cultured in the presence of the long-acting ascorbate analogue, however, the effects of GCs on collagen synthesis are markedly diminished, to the extent that at 10 nM Dx or 200 nM hydrocortisone they are no longer apparent [46]. We speculate that our use of Asc 2-P as a standard supplement may account for some of the discrepancies between the results presented here and those of previous investigations with regard to the effect of Dx on the proliferation of human bone-derived cells.

An important component of the action of GCs on cells of the osteoblast lineage is their ability to influence the production and/or bioavailability of a variety of osteotropic factors in the bone microenvironment [1, 26]. The extent to which GCs also regulate the expression of the receptors for these factors has been less well investigated, particularly in cells derived from human bone [47, 48]. In this study we were unable to demonstrate an effect of Dx at 10 nM on the expression (percentage of positive cells or surface density of expression) of FGFRs 1 and 2, IGF-IRs α and β, the PTH/PTHrP receptor, PDGFRs α and β, NGFR and TGFβRs I–IV. However, this does not preclude the possibility that Dx modulates signalling via these receptors by altering their affinity and/or the extent to which they are coupled to downstream components of the signal transduction pathway. Indeed, despite the fact that we were unable to detect any change in the expression of the PTH/PTHrP receptors in Dx-treated cultures, the cells' responsiveness to exogenous PTH in terms of increased production of cAMP was consistently enhanced (treated/control ratio 3.9 ± 0.7; mean $\pm$ S.E.M. for four donors).

The lack of effect of Dx on the expression of the receptors for TGF-β in this cell culture system is in marked contrast to the findings of previous investigations [47, 48]. In general, however, these effects were observed at concentrations of GC greater than that used here (10 nM). Additionally, these studies were conducted using cells derived from the bones of fetal or neonatal rodents, raising the possibility that this effect of GCs, in common with many other effects, is species- and/or

developmental stage-specific. Further studies will be required to confirm or refute this possibility.

Cell–matrix interactions mediated by integrins, which are known to be affected by treatment with GCs [35, 36, 49], exert an important regulatory influence on the proliferation, differentiation, maturation and survival of cells of the osteoblast lineage [49–53]. Treatment with Dx resulted in consistent down-regulation (~50%) of the number of cells expressing α2 and α4 integrin subunits, whilst expression of the β1 subunit remained unaffected. This latter result was unexpected, given that there have been reports describing an effect of GCs on the expression of this integrin subunit [35, 36]. However, these findings were obtained using bone or bone-derived cells of animal origin and, for the reasons outline above, this makes any meaningful comparison difficult. Furthermore, the results of an *in situ* investigation are consistent with the restriction of this effect of GCs to mature osteoblasts [44], a cell type not likely to be represented to any significant extent in the cultures described here.

To our knowledge, this is the first time that an effect of GCs on the expression of the α2 and α4 integrin subunits by cells of the osteoblast lineage has been reported. When heterodimerized with the β_1 integrin subunit, α2 functions as a receptor for type I collagen, and may mediate the permissive effects of this extracellular matrix protein on osteoblast maturation [50]. When similarly partnered, α4 is capable of binding osteopontin [51] and fibronectin, which may function as an osteoblast survival factor [30, 53]. Without further experimentation, the functional consequences of their down-regulation are difficult to predict. However, the results of studies conducted using the murine MC3T3-E1 cell line are consistent with cell–matrix interactions mediated by α_2-integrins having a pivotal, facilitatory role in the regulation of osteoblast differentiation [50, 54]. If the same is true in cells derived from human bone, then our demonstration that treatment with Dx down-regulates the expression of this integrin subunit provides an additional mechanism whereby GCs might exert their deleterious effects on the skeleton.

A distinguishing feature of this investigation, compared with previous studies, is the large number of donors that was employed. In consequence, the results obtained are likely to be more representative of the effects of GCs on human cells of the osteoblast lineage derived from the ageing population as a whole. By presenting data for individual donors as well as from the population as a whole, we show that there is wide variation among donors in the response of the cells to treatment with Dx. For the most part, this consisted of differences in the magnitude of the response but not in its direction. In the case of cell number, however, variations in both the magnitude and the direction of the response were observed. These differences were apparent despite our use of a rigorously controlled procedure for the isolation and culture of the cells and the use throughout of the same lot of fetal calf serum, a factor known to exert a profound influence on the proliferation and differentiation of cells of the osteoblast lineage. Because of this we speculate that, rather than representing an *in vitro* artefact, this variation reflects some aspect of the donor's physiological status at the time of surgery. What this may be remains to be established; however, a preliminary analysis suggests that it is not related simply to the age and/or sex of the donors (not shown). Nor is it restricted to the effects of GCs. In a recent report, Phinney *et al*. [55] noted the existence of similar donor-dependent variation in the developmental potential of adult human marrow stromal cells and were also unable to account for it on the basis of differences in donor age or sex. Thus, whilst its cause remains unknown, its existence cannot be ignored and should be taken account of in the design of experiments or proposed therapeutic strategies involving the use of human marrow stromal cells. We have also found substantial interdonor variation in the response of these cultured cell populations to treatment with FGF-2 [22].

In conclusion, the results of this investigation are consistent with the hypothesis that GCs at concentrations within the physiological range promote the recruitment and further maturation of human cells of the osteoblast lineage from the developmentally heterogeneous CFU-F population [8–13, 26]. By manipulating the conditions of culture it is possible to dissociate these positive effects of GCs on recruitment and maturation from their negative effect on cell proliferation. The extent to which this can achieved, however, is markedly donor-dependent. Thus if, as has been proposed, GCs are to be included as a standard supplement in media used for the *ex vivo* expansion of cells with osteogenic potential, the concentration used will have to be carefully optimized in advance for each patient. At concentrations in the supraphysiological range the effects of GCs on the recruitment and maturation of osteogenic precursors are retained but are associated with a progressive and concentration-dependent decrease in cell number. Two mechanisms, not necessarily mutually exclusive, could account for this observation. First, the proliferative lifespan of cells in the osteoprogenitor population is reduced; secondly there is developmental stage-specific elimination (apoptosis) of cells in the maturing osteoblast population [45, 56, 57]. *In vivo*, either mechanism would lead to a reduction in osteoblast number and hence could be a factor in the development of the deleterious skeletal manifestation of GC excess.

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