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In Vitro Effects of Chemotherapeutic Agents on Human Osteoblast-like Cells

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Abstract. Osteopenia is a complicating problem that may occur during and after treatment for childhood malignancy. Clinical studies suggest that chemotherapeutic agents directly affect osteoblasts *in vivo*. Since combinations of agents are used for treatment, we individually investigated the chemosensitivity of human osteoblast-like cells to 11 of the chemotherapeutic agents used. The relative chemosensitivity of osteoblast-like cells representing different stages of cell differentiation was also examined. Cell numbers were evaluated following culture of an established human osteoblast-like cell line (MG63) for 3 days with clinically relevant concentrations of the chemotherapeutic agents. The chemosensitivity of MG63 cells was compared to that of a human osteoprogenitor cell line (HCC1) and primary osteoblast-like (HOB) cells derived from pediatric bone. Cell numbers were reduced by all agents in all cell types, although there was a varied response between agents at equimolar concentrations. In MG63 cells the lowest concentration of agent significantly reducing cell numbers varied between agents, for example, methotrexate (10^{-7} M), vincristine (10^{-9} M), and etoposide (10^{-7} M) (all $P < 0.01$). The less differentiated osteoblast phenotypes were significantly more chemosensitive at equimolar concentrations of methotrexate, vincristine, asparaginase, and dexamethasone than more differentiated phenotypes (all $P < 0.01$). Furthermore, four agents significantly increased alkaline phosphatase (AP) activity in HOB cells. We conclude that individual chemotherapeutic agents added to osteoblast cell cultures reduce cell numbers, with osteoblast precursor cells being preferentially depleted. These results suggest that most of the agents may contribute to osteopenia in childhood malignancy by direct effects on cell numbers.

Key words: Malignancy — children — osteopenia — chemotherapy — osteoblasts

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Children treated for malignancy have a predisposition to osteopenia both during and after therapy [1–7]. The etiology of this complication is multifactorial and includes the administration of chemotherapy. Recent studies have observed that during such treatment serum markers of bone formations are, suppressed, indicating direct adverse effects of chemotherapeutic agents on osteoblasts *in vivo* [8–10]. Children are, however, given combinations of chemotherapeutic agents during treatment, and it is thus impossible to determine from clinical studies the relative contribution of each agent administered to the resulting osteopenia. It is, therefore, surprising that there have been few *in vitro* investigations examining the local effects of these individual agents on osteoblasts.

To our knowledge, the only *in vitro* studies of the effects of individual chemotherapeutic agents on osteoblasts have been with methotrexate, dexamethasone, and prednisolone [11, 12]. The mechanisms of action of all of the chemotherapeutic agents have, of course, been studied in other cell types however there have been conflicting results. For example, one recent study [13] failed to observe any effects of methotrexate on the proliferative responses of chondrocytes, whereas another [11] demonstrated that similar concentrations of methotrexate reduced the number of osteoblasts in culture by approximately 30%. Differences such as this, between the effects of chemotherapeutic agents on different cell types, further highlight the need to study the effects of all the individual chemotherapeutic agents on osteoblasts. Such studies might lead to a better understanding of the etiology of the osteopenia observed in children treated for malignancy.

A regular supply of osteoblasts, which are derived from osteoprogenitor cells, is required for normal bone formation [14]. Some think that a reduction in osteoblast and/or osteoprogenitor cell numbers may underlie some bone disease states [15–17], although this is currently an area of intense debate. We have postulated

that chemotherapeutic agents may contribute to osteopenia by directly affecting osteoblast cell numbers. Using an *in vitro* cell culture system, the effects of 11 individual chemotherapeutic agents on osteoblast cell numbers were investigated. Furthermore, to determine whether chemotherapeutic agents preferentially affect one osteoblast phenotype, we have compared the chemosensitivity of two human osteoblast-like cell lines, MG63 osteosarcoma cells [18] and osteoprogenitor HCC1 cells [19], with more highly differentiated primary human osteoblast-like (HOB) cells derived from bone explants from children.

Materials and Methods

MG63 Cells

MG63 cells are derived from an osteogenic sarcoma of a 14-year-old male [18]. These cells are considered to show a number of features typical of an undifferentiated osteoblast phenotype including expression of types I and III collagen and low basal expression of alkaline phosphatase (AP) [20, 21]. MG63 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies Ltd, Paisley, UK) containing penicillin (100 U/ml), streptomycin (100 μg/ml), and fungizone (250 μg/ml) with 5% FCS (Life Technologies Ltd, Paisley, UK) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

HCC1 Cells

HCC1 cells were kindly donated by Dr. B. Ashton, Department of Rheumatology, Robert Jones and Agnes Hunt, Orthopaedic Hospital, Oswestry, UK. They were originally established following transfection of an adenovirus containing an SV40 large T construct into primary, adherent human bone marrow cell cultures [19]. When these cells are cultured in the presence of Minimum Essential Medium (MEM) supplemented with 10% FCS they establish an osteoblastic phenotype expressing AP activity, osteocalcin peptide, and mRNA, which are all up-regulated with 1,25-dihdroxyvitamin D_3 [19]. Furthermore, AP activity is known to increase in these cells following incubation with 10^{-7}M dexamethasone [19]. In our study cells were maintained in MEM (Life Technologies Ltd) containing penicillin (100 U/ml), streptomycin (100 μg/ml); and fungizone (250 μg/ml) with 5% FCS at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Primary Human Osteoblast-like (HOB) Cells

Ethical approval for collection of clinical samples was granted by Bro Taf Local Research Ethics Committee, Cardiff. Specimens of bone were obtained from children undergoing elective orthopedic procedures. These children were free from malignancy and other diseases with systemic effects likely to have any adverse effects on bone physiology. HOB cells were isolated and cultured from bone explants as described previously [22, 23]. Bone explants were washed extensively in phosphate buffered saline (PBS) to remove bone marrow cells. Bone was dissected into 0.2–0.5 cm pieces, placed in 24-well plates, and cultured in alpha MEM (αMEM) (Life Technologies Ltd) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and fungizone (250 μg/ml) containing 10% FCS and HEPES (25 mM), at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The culture medium was changed twice weekly and the cells were subcultured by trypsinization. Only cells from passage 3 or less were used for analysis.

Characterization of Primary Human Osteoblast-like (HOB) Cells

Alkaline phosphatase (AP) as a marker for osteoblast phenotype and differentiation was assessed biochemically and cytochemically. AP activity was quantified biochemically by incubation of HOB cells in 96-well plates with para-nitrophenyl phosphate and an alkaline buffer, which together comprise the 104-LL Sigma Diagnostic Kit (Sigma-Aldrich Co Ltd, Dorset, UK), and by measuring the absorbance change of the developing yellow color over 1 hour at 405 nm using a SpectraCount™ Microplate Photometer (Canberra-Packard Ltd, Berks, UK). The measured AP activity was corrected for cell numbers. Biochemical AP activity was evaluated in MG63 cells for comparison.

AP was detected cytochemically by incubating cells for 16 hours with bromochloroindolyl phosphate and nitroblue tetrazolium in a Tris buffer (pH 9.5) [24]. The number of highly positively stained cells was counted in random microscopic fields using an image analyzer (Image proplus 3) interfaced with a color camera (JVC TK1070E) mounted on a microscope (Olympus BH2). The number of AP stained cells was expressed as a percentage of total cell number.

Type I collagen was quantified in the culture medium collected from 3 days of cell culture using a specific ELISA (PROLAGEN-C) (Metra Biosystems, Oxon, UK) and measuring the absorbance at 405 nm using a SpectraCount™ Microplate Photometer (Canberra-Packard Ltd, Berks, UK). Type I collagen synthesis was also assessed in MG63 cells for comparison.

Total cellular RNA was prepared from HOB cell lines using the Purescript RNA isolation kit (Flowgen, Leicestershire, UK). Reverse transcription-PCR (RT-PCR) was then carried out using standard techniques and specific primers for the human osteocalcin [25] and CBFA1 genes [26].

Cell population doubling times were assessed by seeding cells in 96-well plates and measuring the change in cell numbers over 14 days using a CellTiter96® Non-Radioactive Cell Proliferation assay (Promega, Southampton, UK).

Chemotherapeutic Agents

Methotrexate, thioguanine, mercaptopurine, and cytarabine are classed as antimetabolites and inhibit DNA synthesis of leukemic cells by acting as false analogs of purines and pyrimidines. Vincristine inhibits leukemic cell division by interfering with microtubule assembly and induces metaphase arrest. Etoposide (epipodophyllotoxin), daunorubicin (anthracycline), and mafosfamide (alkylating agent) directly damage DNA by respectively inducing strand breaks, intercalating with DNA, and alkylating adjacent base pairs. Asparaginase reduces the amount of asparagine available to leukemic cells thereby inhibiting cell proliferation. Dexamethasone and prednisolone (glucocorticoids) induce leukemic cell lysis. Chemotherapeutic agents were freshly prepared for *in vitro* experiments by serial dilution in tissue culture medium. Stock solutions of dexamethasone and prednisolone (Sigma-Aldrich Co Ltd, Dorset, UK) in 99.5% ethanol were stored at −20°C. For each drug, a concentration range was used which encompassed plasma levels achieved *in vivo* [27–31].

Drug Sensitivity Measurements

Cells were seeded in 96-well plates at a density of 3×10^3 per well in 150 μl of medium. After 24 hours chemotherapeutic agents were added over the concentration range 10^{-10} M to 10^{-5} M. Cells were incubated with each agent for 3 days. For experiments with glucocorticoids, charcoal stripped serum was used and the volume of ethanol added to test cultures was also added to control cultures.

In experiments where HCC1 cells were preincubated with 10^{-7} M dexamethasone, cells were treated with dexamethasone

in 25 cm^2 flasks for 4 or 7 days before seeding into 96-well plates and chemotherapeutic agents and dexamethasone were added for a further 3 days as described above. At the same time, cells were seeded onto coverslips for staining of AP as described previously. In these experiments only one concentration of each agent was used (methotrexate 10^{-7} M, vincristine 10^{-7} M, etoposide 10^{-5} M, aspariginase 0.5 U/ml).

Cell numbers were measured using a CellTiter96® Non-Radioactive Cell Proliferation assay (Promega, Southampton, UK) [32, 33]. This method compares favorably with [^{3}H]-thymidine incorporation and direct cell counting for quantification of cell numbers [34, 35]. After washing in PBS, cells were incubated for 2 hours at 37°C with 120 µl of a 2 mg/ml solution of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium and phenazine methosulfate in phenol-red-free DMEM (Life Technologies Ltd, Paisley, UK) according to the manufacturer's instructions. The resulting optical density (OD) was measured at 490 nm on a SpectraCount™ Microplate Photometer (Canberra Packard Ltd, Berks, UK). Because evaporation was observed from the outer wells of a 96-well plate, these wells were not included in the final analyses. Initial assays were carried out to determine the relationship between MG63 cell number and absorbance at 490 nm using this assay. In any one experiment, there were 12 replicates for each treatment, and each experiment was performed on two separate occasions. Results are expressed as a percentage of control cell numbers on day 3.

Effect of Chemotherapeutic Agents on AP Activity in HOB Cells

HOB cells were seeded in 96-well plates at a density of 3×10^3 per well in 150 µl of medium. After 24 hours chemotherapeutic agents were added at the following concentrations (methotrexate 10^{-7} M, vincristine 10^{-7} M, etoposide 10^{-5} M, aspariginase 0.5 U/ml). Cells were incubated with each agent for 3 days. After 3 days AP activity was quantified biochemically as described above.

Statistical Analysis

The results presented were from a minimum of 24 replicates, and determined by comparing means of cell populations using the Student's unpaired *t*-test. Differences were considered to be significant when $P \leqslant 0.05$. Data are presented as the mean ± SEM.

Results

Characterization of Primary Human Osteoblast-like Cells

Using the culture conditions described, the cells derived from bone samples displayed characteristics typical of primary HOB cells. These cell populations expressed AP (Fig. 1a,b) consistent with that observed by others [36] and expressed high levels of type I collagen (Fig. 1c). Furthermore, both cell lines used expressed osteocalcin and CBFA1 mRNA (Fig. 2). Even though multiple bands are present in these gels, the two bands marked with arrows were demonstrated to be CBFA1 and osteocalcin, respectively, by extraction of the cDNA followed by automated sequencing. It is possible that the other bands are splice variants of the full-length transcripts as previously shown by Gronthos et al. [26] for CBFA1. Population doubling times were longest with

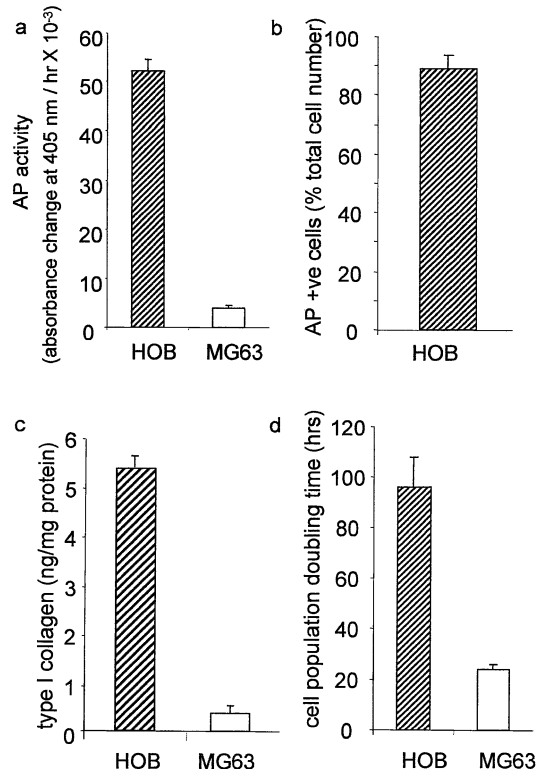


Fig. 1. Characterization of HOB cells (with MG63 cells for comparison), (a) AP activity was evaluated biochemically by the change in units of absorbance at 405 nm/hour following incubation with paranitrophenyl phosphate and then correction for cell numbers. (b) The percent of cells highly positive for AP (AP +ve) in the HOB cell population was evaluated. (c) Type I collagen was quantified from media collected after 3 days of cell culture, (d) Cell numbers were evaluated at time points over 7 days using the Cell Titer96® Non-Radioactive Cell Proliferation assay.

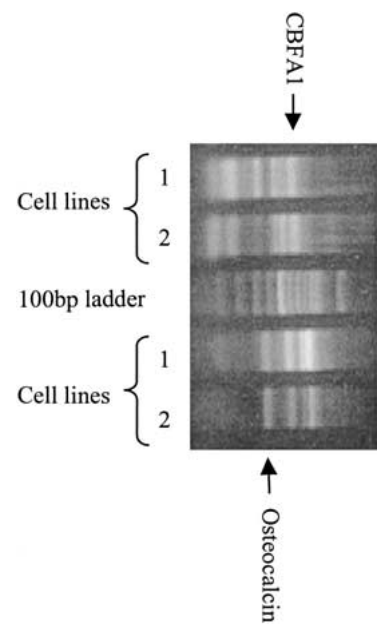


Fig. 2. Agarose gel of RT-PCR products obtained with RNA isolated from two HOB cell lines and specific primers for the osteocalcin and CBFA1 genes.

HOB cells (96 hours) and shortest with MG63 cells (23 hours) (Fig. 1d). The population doubling time for HCC1 was 26 hours.

Relationship Between Cell Number and OD

A linear relationship was observed between cell numbers quantified by the CellTiter96® Non-Radioactive Cell Proliferation assay and absorbance ($r = 0.98$) (data not shown). We have also demonstrated that this method of determining the number of viable cells correlates well with direct cell counting using a Coulter counter (data not shown).

Influence of Chemotherapeutic Agents on MG63 Cell Numbers

Cell numbers were reduced by some concentrations of all chemotherapeutic agents tested (Fig. 3). Drugs within the same class of antineoplastic agent demonstrated variation in their ability to diminish MG63 cell numbers and this variation occurred at equimolar concentrations of each agent. For example, methotrexate and cytarabine, both antimetabolites when present at a concentration of 10^{-7} M demonstrated a 71% and 19% reduction in cell numbers compared with control, respectively (Fig. 3a,d). With the nonglucocorticoid chemotherapeutic agents tested, the greatest reduction in cell numbers occurred with daunorubicin 10^{-5} M, whereas mercaptopurine 10^{-5} M was the least effective (Fig. 3g,b) with reduction in cell numbers compared with control of 90% and 18%, respectively. The lowest concentration at which each of these agents reduced cell numbers also varied between the different drugs. Vincristine depleted cell numbers at a concentration as low as 10^{-9} M (Fig. 3e). By contrast, mercaptopurine and thioguanine (antimetabolites) depleted cell numbers only at 10^{-5} M (Fig, 3b,c). Furthermore, dexamethasone reduced cell numbers at 10^{-8} M and above, whereas prednisolone reduced cell numbers at 10^{-6} M or higher (Fig. 3j,i).

A variety of dose-response curves were observed with the chemotherapeutic agents tested. For example, increasing concentrations of daunorubicin reduced cell numbers in a linear fashion (Fig. 3g), whereas no further reductions in cell numbers occurred at concentrations of methotrexate greater than 10^{-7} M (Fig. 3a).

Relative Chemosensitivity of MG63, HCC1 and HOB Cells to Chemotherapeutic Agents

The chemosensitivity of MG63 cells was compared with that of HCC1 and HOB cells using a chemotherapeutic agent from each class of antineoplastic agent described above (methotrexate (anti-metabolite), vincristine (vinca alkaloid), etoposide (epipodophyllotoxin), dexamethasone (glucocorticoid), and asparaginase). Similar patterns of dose-response curves were obtained with all three cell types (Fig. 4). Furthermore, similar results

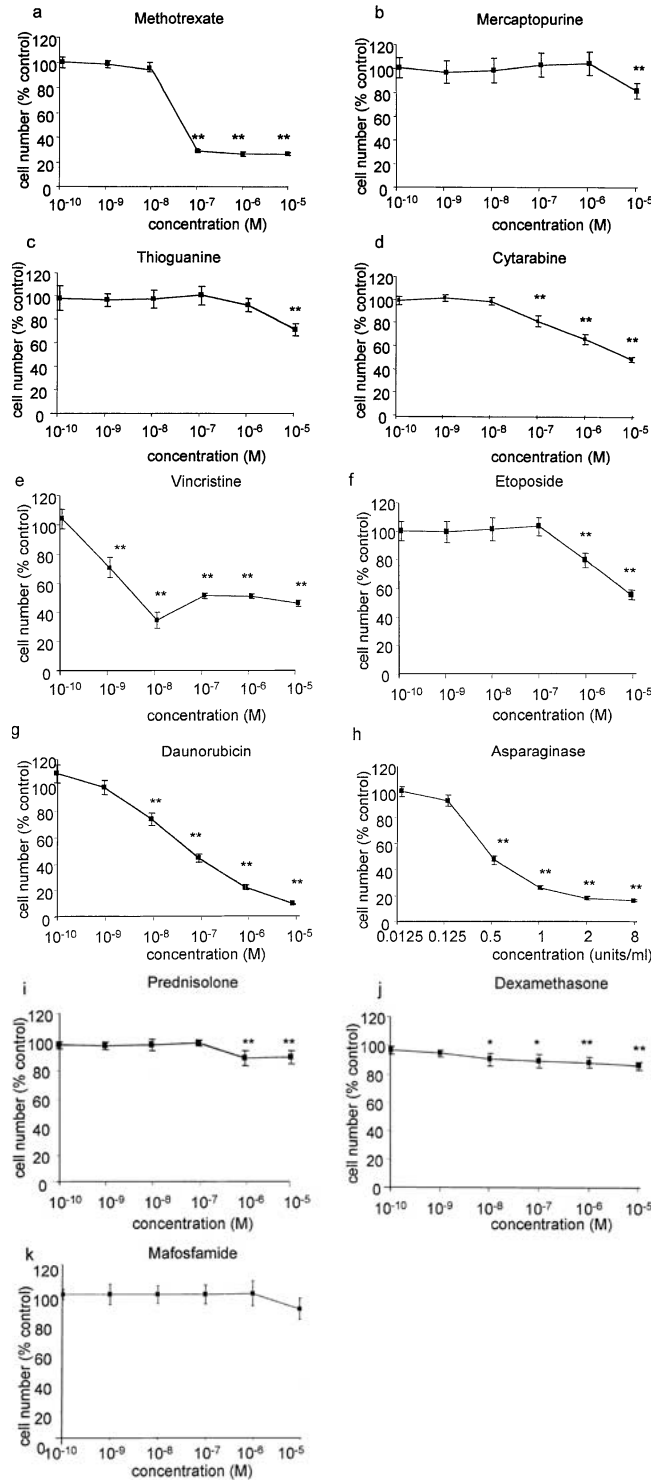


Fig. 3. The effect of chemotherapeutic agents used in the treatment of childhood malignancy on human osteoblast-like cell (MG63) numbers. Cells were incubated with each chemotherapeutic agent and cell numbers were measured at 3 days. The y-axis shows cell number expressed as a percentage of control values at day 3. Each point is the mean of 24 replicates and error bars represent ±SE. $^{*}P < 0.05$ versus control; $^{**}P < 0.01$ versus control.

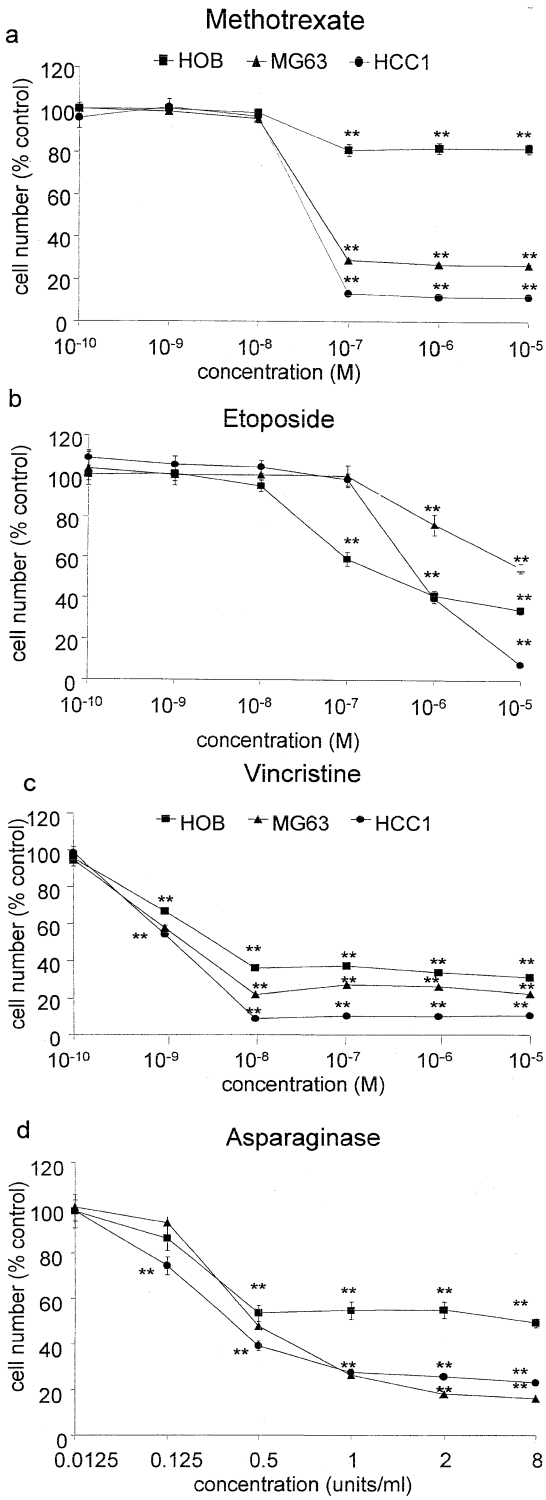
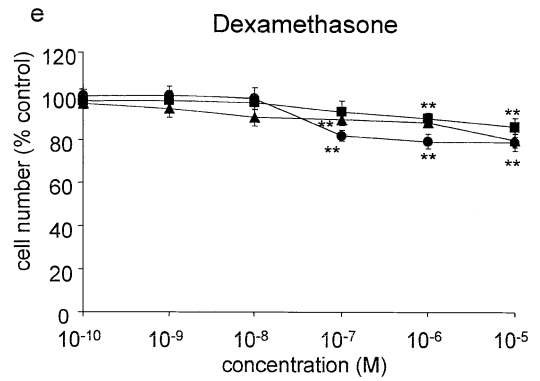


Fig. 4. The relative chemosensitivity of differing human osteoblast-like cells to chemotherapeutic agents used in the treatment of childhood malignancy. Each cell line (HOB, HCC1 and MG63) was incubated with a chemotherapeutic agent separately and cell numbers were measured at 3 days. The y-axis shows cell number expressed as a percentage of control values at day 3. Each point is the mean of 24 replicates and error bars represent ±SE. $^{*}P < 0.05$ versus control; $^{**}P < 0.01$ versus control.



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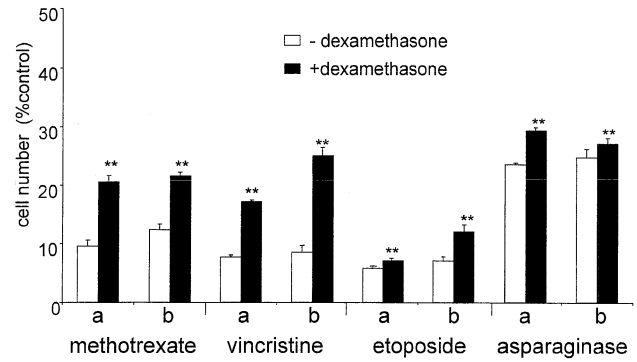

Fig. 5. The effect of chemotherapeutic agents on HCC1 cell numbers following incubation of cells with 10^{-7} M dexamethasone. (a) Cells were cultured with dexamethasone for 4 days before being treated (3×10^{-3} cells per well in 96-well plates) with individual chemotherapeutic agents for 3 days. (b) As above but cells were cultured with dexamethasone for 7 days before chemotherapeutic agents were added. The y-axis shows cell number expressed as a percentage of control values at day 3. Each point is the mean of 24 replicates and error bars represent ±SE. $^{*}P < 0.05$ versus control; $^{**}P < 0.01$ versus control.

were obtained with two different HOB cell lines (results not shown). Apart from etoposide, the chemosensitivity between the cell types differed such that MG63 and HCC1 cells were more chemosensitive than HOB cells (Fig. 4). For example, at a concentration of 10^{-7} M methotrexate there was a 86% reduction in HCC1 cell numbers and a 71% reduction in MG63 cell numbers compared with control, whereas there was only a 19% reduction with HOB cells (Fig. 4a). With the exception of etoposide (Fig. 4b), the lowest concentration of each agent causing any reduction in cell numbers was similar for the 3 cell types (Fig. 4).

Effect of Chemotherapeutic Agents on HCCL Cells Following Incubation for 4 or 7 Days with Dexamethasone to Induce Osteoblastic Differentiation

Following preincubation of HCC1 cells with 10^{-7} M dexamethasone for 4 or 7 days, the cells were less sensitive to all of the chemotherapeutic agents tested than untreated control cells (Fig. 5). Furthermore, cells treated with dexamethasone for 7 days were less sensitive to

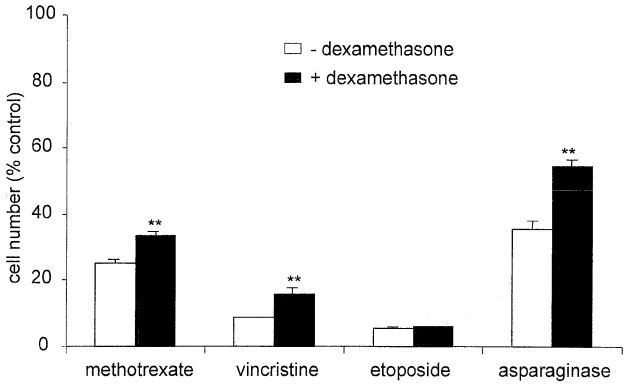

Fig. 6. The effect of chemotherapeutic agents on HCC1 cell numbers in serum-free medium following incubation of cells with 10^{-7}M dexamethasone. Cells were cultured with dexamethasone for 8 days before being treated (3×10^{-3} cells per well in 96-well plates) with individual chemotherapeutic agents for 3 days. Cells were maintained in serum-free medium for 48 hours prior to and during treatment with the individual chemotherapeutic agents. The y-axis shows cell number expressed as a percentage of control values at day 3. Each point is the mean of 24 replicates and error bars represent ±SE. $^{*}P < 0.05$ versus control; $^{**}P < 0.01$ versus control.

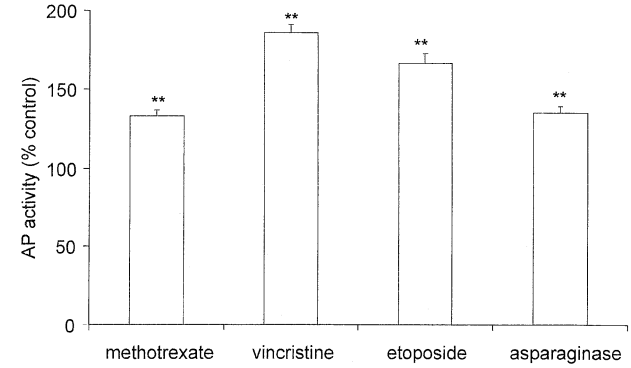

Fig. 7. The effect of chemotherapeutic agents on AP activity in HOB cells. Cells were incubated with each agent for 3 days and AP activity was evaluated using the previously described biochemical assay. The y-axis shows AP values corrected for cell numbers and expressed as percentage of control on day 3. The y-axis shows cell number expressed as a percentage of control values at day 3. Each point is the mean of 24 replicates and error bars represent ±SE. $^{*}P < 0.05$ versus control; $^{**}P < 0.01$ versus control.

vincristine and etoposide than those that had been treated for 4 days only ($P < 0.01$). Such a difference between cells treated for 4 and 7 days was not detected with methotrexate or asparaginase (Fig. 5). Furthermore, AP staining techniques demonstrated that AP activity increased significantly following treatment of HCC1 cells for 4 or 7 days with 10^{-7} M dexamethasone (results not shown), indicating that dexamethasone induced these cells to differentiate along the osteoblastic pathway.

Other studies in which cells had been treated with dexamethasone for 8 days, but maintained in serum free medium for 2 days prior to, and during treatment with individual chemotherapeutic agents, provided similar results (Fig. 6). There was, however, no difference between dexamethasone treated and untreated cell numbers following incubation with etoposide, although it should be noted that only 5% of control cell numbers remained in these cultures.

Effect of Chemotherapeutic Agents on AP Activity in HOB Cells

Following incubation of HOB cells for 3 days with all agents tested, AP activity was significantly increased ($P < 0.001$) (Fig. 7). The effect was greatest with vincristine, with AP values 185.9 ± 5.3% of control values, whereas methotrexate increased AP values to 133.1 ± 3.6% of control.

Discussion

The adverse effects of chemotherapeutic agents on bone have largely been attributed to steroid therapy and the administration of methotrexate. In addition, recent clinical studies have demonstrated that serum markers of bone formation (e.g., type I collagen and bone AP) are suppressed during the administration of combinations of chemotherapeutic agents, with recovery of these markers during less intensive therapy [8–10]. This may, however, be a consequence of agents other than methotrexate and steroids interfering directly with osteoblast numbers or function. Chemotherapy may reduce cell numbers by mechanisms such as apoptosis, cytostasis, or by causing loss of proliferative ability [37]. In our study we observed that osteoblast-like cell numbers are reduced *in vitro* by numerous agents over a range of clinically relevant concentrations.

The concentration of chemotherapeutic agent reaching bone (and therefore mature osteoblasts) and bone marrow (containing osteoprogenitor cells) will be variable even after doses are adjusted for body surface area or weight, as there is considerable pharmacokinetic variability among children receiving chemotherapy [38, 39]. Methotrexate has previously been demonstrated to cause a dose-dependent reduction in human osteoblast-like cell numbers with no effect on AP expression [11]. We also observed dose-dependent reductions of human osteoblast-like cell numbers by other chemotherapeutic agents. These reductions in cell numbers were comparable to those seen in leukemic cells derived from children when the same agents were used with similar concentrations [40]. Furthermore, equimolar concentrations of chemotherapeutic agents reduced cell numbers by differing degrees. Thus, the concentration of a drug in the microenvironment of osteoblasts *in vivo* may be critical in determining the degree by which cell numbers are reduced and is drug dependent.

Glucocorticoid-induced osteoporosis is a complicating feature of prolonged glucocorticoid use [7]. We

observed greater reductions in cell numbers with dexamethasone than with prednisolone following incubations at equimolar concentrations for 3 days. This effect was more pronounced following a longer culture period (data not shown). This is consistent with the finding that dexamethasone exerts greater inhibition of DNA synthesis in human osteoblast-like cells compared with prednisolone at equivalent concentrations [41]. Interestingly, we found that nonsteroid chemotherapeutic agents depleted cell numbers by a greater extent than glucocorticoids. However, glucocorticoids adversely effect bone metabolism by a number of other mechanisms including decreased calcium absorption from the intestine and hypercalciuria [7]. Moreover, glucocorticoids modulate the response of osteoblasts to parathyroid hormone and vitamin D and may also inhibit type 1 collagen synthesis [7]. It should also be noted that we observed reductions in cell numbers with a concentration of dexamethasone 100-fold weaker (10^{-8} M) than the concentration of prednisolone producing the same effect (10^{-6} M). This relative potency of dexamethasone compared with prednisolone in suppressing cell numbers is considerably greater than the oft quoted antiinflammatory potency of these steroids when used in clinical practice [42].

It is possible that age-related bone loss may be due to an inadequate supply of osteoblast precursor cells with a resultant decline in osteoblast numbers [14]. We have postulated that the mechanism of chemotherapy-induced osteopenia is similar. Although the use of established cell lines such as MG63 and HCC1 has potential limitations, they have the advantage of less phenotypic diversity than would be observed in osteoblast precursors derived from bone marrow. Apart from with etoposide, we observed that MG63 and HCC1 cells were relatively more chemosensitive to equivalent concentrations of chemotherapeutic agents than HOB cells. Increased sensitivity to etoposide has been related to the increased expression of topoisomerase II in other cell types [43]. It is therefore possible that our results are due to higher levels of this enzyme in HOB cells when compared with the other cell types studied.

Consistent with our findings as described above, we observed that HCC1 cells became more chemoresistant following culture with dexamethasone at a concentration known to induce their differentiation. Moreover, in HOB cells, AP activity was increased following incubation with these agents. This may be a consequence of agents targeting the proliferating AP negative cells. The increase in AP activity seen in this study is in complete contrast to the recent results published by Uehara et al. [44]. They, however, used human SaOS-2 and mouse MC3T3-E1 cell lines.

Osteoblast maturation is initially characterized by proliferation of less differentiated osteoblast phenotypes during which genes are expressed that are associated with the synthesis of proteins for the formation of the extracellular matrix (e.g., type 1 collagen, fibronectin, and transforming growth factor β) [45]. Thus, chemotherapeutic agents may contribute to osteopenia by inhibition of new bone matrix synthesis as a result of reduced precursor cell numbers. Failure to recruit osteoprogenitors to mature osteoblasts may lead further to diminished bone mineralizing ability [46].

In summary, our data indicate that chemotherapeutic agents reduce osteoblast numbers at concentrations achievable *in vivo*. These findings suggest that more chemotherapeutic agents than previously appreciated have deleterious effects on bone metabolism in the clinical setting. The mechanisms involved in the effects of these individual chemotherapeutic agents on osteoblast cell numbers, as well as their effects on osteoblast differentiation, are the subjects of our ongoing research projects.

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