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SHORT COMMUNICATION

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Glucocorticoid pretreatment reduces the cytotoxic effects of a variety of DNA-damaging agents on rat tibial growth-plate chondrocytes in vitro

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Abstract It is apparent that cytotoxic chemotherapy used to treat childhood malignancies has a major impact on subsequent growth. Our initial studies have demonstrated a direct adverse effect of individual glucocorticoids and cytotoxic agents on the proliferative capacity of rat tibial growth-plate chondrocytes in vitro. In the present study we investigated the interaction between these classes of agents using in vitro cultures of chondrocytes and examined the potential of these cells to recover from the adverse effects of the drugs as applied either alone or in combination. The glucocorticoids prednisolone and dexamethasone significantly reduced the growth rate of chondrocytes when present in cultures for 3 days. The growth rate increased following the removal of prednisolone and dexamethasone from cultures and reached 83.9 ± 0.8% and 62.4 ± 4.0%, respectively, of the control values after 11 days of culture. In contrast, cell numbers were significantly reduced when the DNA-damaging agents cisplatin, carboplatin, etoposide or actinomycin-D were present in cultures for 3 days. Very little recovery of cell growth was observed after removal of the drugs from cultures, with cell loss occurring in the cisplatin- and actinomycin-D-treated cultures. However, pretreatment of chondrocytes with either of the glucocorticoids completely ameliorated the cytotoxic effects of etoposide and carboplatin and significantly reduced those of cisplatin and actinomycin-D. Recovery of the cells treated with a combination of glucocorticoid and DNA-damaging agent was demonstrated by a significant increase in their ability to form colonies in suspension culture. Colony numbers were increased by a factor of between 5 and 80 as compared with the cells receiving medium alone followed by DNA-damaging agent. The glucocorticoids offer a protective effect in terms of the reduced cytotoxicity of DNA-damaging agents and improve the subsequent clonogenicity and recovery of growth-plate chondrocytes. This has important implications for treatment schedules involving both cytotoxic agents and glucocorticoids in childhood malignancies.

Key words Chondrocytes · Chemotherapy · Recovery · Glucocorticoids

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Introduction

With improved survival rates being reported for most childhood malignancies, it has become increasingly apparent that some children grow poorly long after the cessation of chemotherapy (CT). Certainly, CT in association with craniospinal irradiation (XRT) is associated with a greater degree of growth failure than is irradiation alone [12, 18]. Furthermore, the adverse impact of CT on growth may be influenced by the intensity of CT schedules [6, 10]. As far as the mechanism of growth failure is concerned, children treated with CT alone show no disturbance of growth hormone (GH) secretion [15], which would suggest an effect of these agents on the bone itself. This has been demonstrated in growth plate specimens from children with osteosarcoma [1] and from radiological evidence in children with non-Hodgkin's lymphoma who have received sustained and intensive CT [20]. This

is further supported by the final height data reported by Davies et al. [7] in which children treated for acute lymphocytic leukaemia (ALL) with combination CT and cranial XRT showed significant disproportion with a relatively short spine. The effects of CT on the large numbers of epiphyses that are found in the spine provide a potential explanation for the disproportion.

Multi-drug regimens combining cytotoxic drugs with a variety of other agents, including steroids, are increasingly being used in the treatment of paediatric malignancies. For example, in the treatment of acute lymphocytic leukaemia (ALL), steroids are commonly used for a prolonged course of 3–4 weeks during induction of remission and in 5- to 7-day pulses during continuing therapy. They are also frequently given with other anti-emetics for patients receiving intensive CT. Information concerning the cytokinetic responses of chondrocytes to these agents is required if we are to understand the changes in growth-plate physiology associated with CT-induced growth retardation.

During chondrogenesis, cells within the resting zone of the growth plate differentiate, proliferate and later become hypertrophic in that they dramatically increase both their volume and their metabolic activity [8]. Using chondrocytes grown in both in vitro monolayer cultures of defined media and suspension cultures stabilised with agarose, this phenotypic progression can be maintained [11]. Using these systems as a model of chondrogenesis, we have previously demonstrated that glucocorticoids (prednisolone and dexamethasone) and a variety of cytotoxics (actinomycin-D, cisplatin, carboplatin and etoposide) reduce the proliferative capacity of the chondrocytes [14]. The present study further examines the relationship between the actions of these agents using in vitro measurements of growth-plate chondrocyte cell kinetics and recovery.

Materials and methods

Isolation and culture of chondrocytes

Chondrocytes were isolated from the tibial epiphyseal growth plates of 21-day-old male Sprague-Dawley rats as previously described [14]. The cells were then diluted in F12 medium supplemented with 10% newborn calf serum (NCS; Gibco Life Tech, Paisley, UK) and ascorbic acid at 50 μg/ml (Sigma, Dorset, UK; F12/NCS/AA) before seeding of 5×10^5 cells per 75-cm^2 tissue-culture flask (Falcon, Oxford, UK). Cells were incubated at 37 °C in air containing 5% CO_2 and the culture medium was changed after 4 days. Cells were cultured for at least 3 days post-confluence, after which they were trypsinised, washed in culture medium and counted. The second culture period was performed in either 12- or 96-well tissue-culture plates (Falcon) to which the chondrocytes were seeded at densities of 10,000 and 2,000 cells/well, respectively.

Chemotherapeutic agents

The following chemotherapeutic agents were employed in all experiments at doses previously shown to inhibit the growth of chondrocytes following a 3-day period of incubation [14]: prednisolone at 5 μg/ml, dexamethasone at 5 μg/ml, cisplatin at 1 μg/ml, carboplatin at 10 μg/ml, etoposide at 1 μg/ml and actinomycin-D at 0.1 μg/ml.

Proliferation

Chondrocyte growth was measured in monolayer culture using a 96-well-plate sulforhodamine B (SRB; Sigma) assay [16]. By means of a direct assessment of protein content, this agent provides a colorimetric endpoint as a highly sensitive measure of drug-induced cytotoxicity.

Cells were incubated for 24 h at 37 °C in air containing 5% CO_2 before the addition of fresh medium with or without chemotherapeutic agents as described below:

1. All agents were present in culture for 3 days, after which they were removed and the cells, washed in F12. F12/NCS/AA alone was then added and the cells were incubated for a further 8 days. Cultures were terminated at varying times during this period.
2. Prednisolone or dexamethasone was present in culture for 3 days, after which these agents were removed and the cells, washed in F12. Medium (F12/NCS/AA) with or without either cisplatin, carboplatin, etoposide or actinomycin-D was then added to the cultures, which were terminated after a further 3-days of incubation.

Following incubation, cultures were fixed in 10% trichloroacetic acid (Sigma) for 1 h at 4 °C. Plates were then washed five times in tap water and air-dried before the addition of 0.4% SRB in 0.1% acetic acid (BDH) for 1 h at room temperature (RT). Following washing of the wells with 0.1% acetic acid to remove unbound dye, 100 μl 10 m*M* TRIS-base (Sigma) was added to each well. Readings were made at 540 nm on a microplate reader (Molecular Devices) within 30 min of the addition of the TRIS-base.

Clonogenicity

Chondrocytes seeded in 12-well tissue-culture plates were incubated for 24 h at 37 °C in air containing 5% CO_2 before the addition of fresh medium with or without the chemotherapeutic agents (triplicate wells) as outlined above. Cells were then trypsinised and sub-cultured in agarose according to Benya and Shaffer [3]. In brief, 60-mm non-tissue-culture-treated petri dishes (Falcon) were precoated with 1% "standard low" agarose (autoclaved at 112 °C for 45 min; Bio-Rad, Richmond, Calif. USA). Low-melting-point agarose (Bio-Rad) was autoclaved as described above and mixed with an equal volume of F12 to give an agarose concentration of 1%. Treated cells from the 12-well plate were then trypsinised, counted and mixed with F12 to give a final agarose concentration of 0.5% containing 10,000 cells/ml. A 3-ml vol. of this solution was added to each petri dish (30,000 cells/dish) and the gel was allowed to solidify at 4 °C for 15 min before the addition on top of the gel of Dulbecco's modified Eagle's medium (DMEM; Gibco Life Tech.)/F12 mix + 20% foetal bovine serum (FBS; Biowhittaker, Wokingham, UK). Thereafter, the cultures were screened for adherent cell clusters of more than three cells. No such cluster was seen at the start of culture in any experiment presented in this report. The suspension cultures were maintained for 14 days at 37 °C in air containing 5% CO_2 with a medium change after 7 days of DMEM/F12 mix + 10% FBS alone.

Suspension cultures were terminated by fixation in 4% paraformaldehyde (Sigma) in phosphate-buffered saline (PBS) and were stained with alcian blue (0.5% in 0.04 *M* hydrochloric acid; Sigma) to identify colonies producing glycosaminoglycans. Colonies from triplicate dishes were counted at 100× magnification, and a colony was defined as a cluster of cells with an alcian blue-stained matrix and a diameter of > 50 μm. Colony numbers and any marked variation in colony size were recorded.

Statistical analysis

Mean values were compared by analysis of variance. Comparisons were made between two mean values using Student's

unpaired t-test. Differences were considered to be significant when $P \leq 0.05$.

Results

Recovery of chondrocytes following single-agent treatment

As compared with the untreated control, both prednisolone and dexamethasone reduced chondrocyte proliferation when present in cultures for 3 days (Fig. 1a). At this time, chondrocyte numbers were not significantly different from the day 0 values. This is consistent with our earlier report of an increase in the doubling time of chondrocytes in the presence of the glucocorticoids [14]. Following the removal of either prednisolone or dexamethasone from the cultures, no significant change in proliferation was observed in the first 3 days. However, recovery of the proliferative capacity of chondrocytes occurred between days 6 and 11 of culture for the prednisolone-treated cells and between days 8 to 11 for the dexamethasone-treated cells.

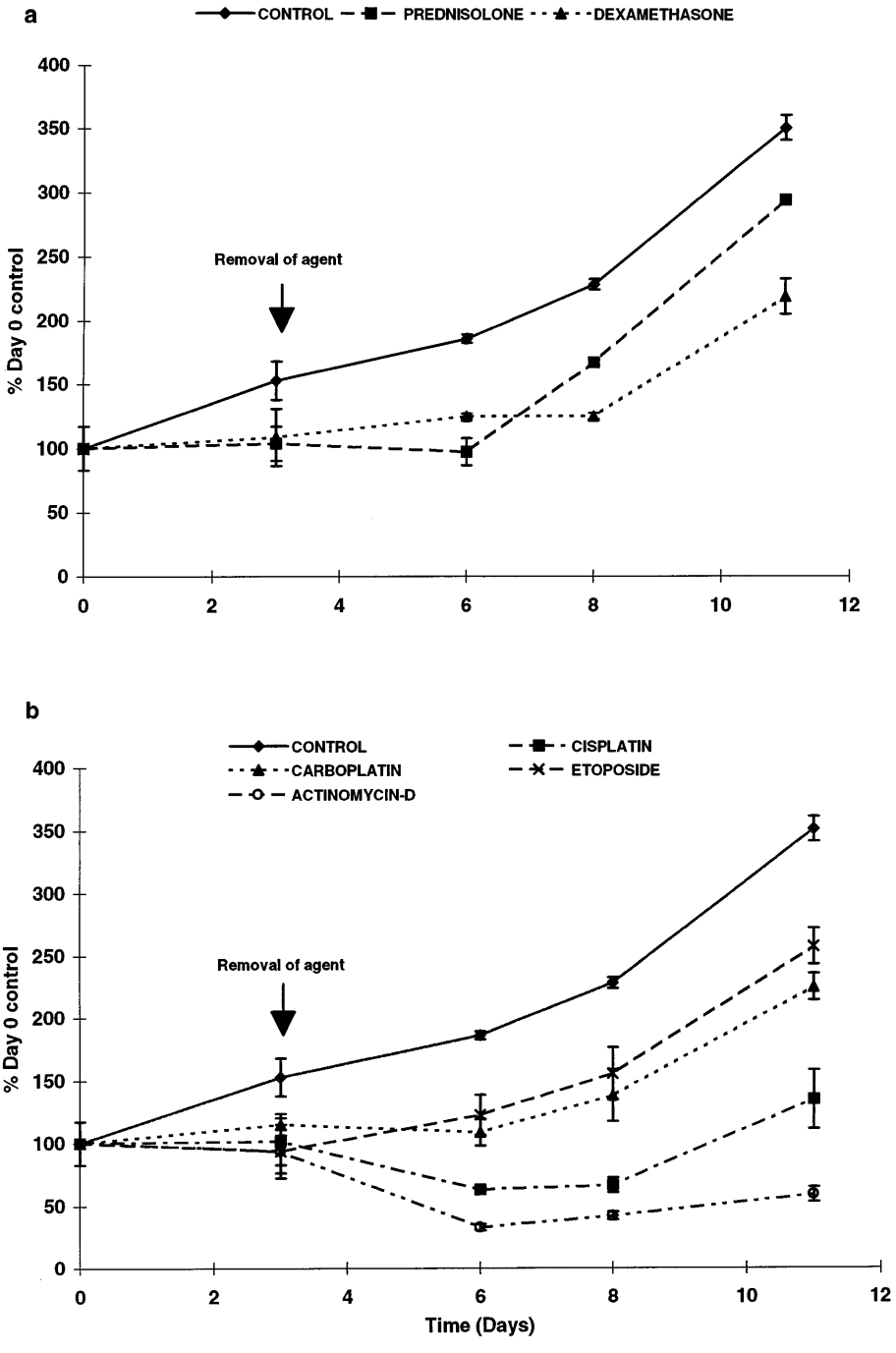

Fig. 1a,b The effects of **a** prednisolone (5 μg/ml) or dexamethasone (5 μg/ml) and **b** etoposide (1 μg/ml), carboplatin (10 μg/ml), cisplatin (1 μg/ml) or actinomycin-D (0.1 μg/ml) on the proliferation of rat proximal tibial growth-plate chondrocytes with time. All agents were continuously present for 3 days in monolayer cultures, after which they were removed, the cells were washed in Hams' F12 and their subsequent growth in F12/NCS/AA was determined at 6, 8 and 11 days in culture. Data are expressed as a percentage of day 0 values and represent mean values ±SD for 6 determinations in 2 separate experiments

That this lag phase (prior to an increase in proliferation) was greater in the dexamethasone-treated as compared with the prednisolone-treated cells was reflected in the final numbers of cells, which corresponded to 62.4 ± 4.0% and 83.9 ± 0.8%, respectively, of the untreated control value ($P < 0.005$, $n = 6$). Growth rates at this time, however did not significantly differ between the prednisolone-, dexamethasone- and control-treated cells.

The DNA-damaging agents cisplatin, carboplatin, etoposide and actinomycin-D significantly reduced the numbers of rat proximal tibial chondrocytes ($P < 0.05$, $n = 6$) when present in cultures for 3 days (Fig. 1b). In the presence of cisplatin and actinomycin-D only, cell numbers fell significantly below the day 0 values ($P < 0.05$, $n = 6$), indicating that cell loss had occurred. Following the removal from cultures at day 3 of cisplatin and actinomycin-D, cell numbers continued to decline, and at day 6 we observed a maximal cell loss of 36.9 ± 1.6% and 67.4 ± 2.7%, respectively, as compared with the day 0 values ($P < 0.05$, $n = 6$). In contrast, chondrocytes treated with etoposide increased their rate of growth immediately following the removal of this agent, and at day 11 of culture, cell numbers had reached 73.4 ± 4.1% of the untreated control-cell population at this time. Carboplatin-treated cultures demonstrated a similar, although delayed, increase in cell number following the removal of this agent, such that by day 11 of culture, cell growth was 64.1 ± 3.1% of the untreated control-cell population at this time.

Chondrocyte proliferation following pretreatment with glucocorticoids

For chondrocytes pretreated with medium alone, the addition for a further 3 days of either cisplatin, carboplatin, etoposide or actinomycin-D significantly reduced proliferation ($P < 0.05$, $n = 6$). For example, the numbers of chondrocytes were reduced to 12.8 ± 1.95%, 84.6 ± 3.38%, 66.8 ± 5.11% and 32.9 ± 3.16%, respectively, of the control treated with medium alone (Fig. 2a). By comparison, the cytotoxic effects of these agents (in terms of relative cell loss) were reduced when chondrocytes were pretreated for 3 days with either prednisolone or dexamethasone (Fig. 2a). Specifically, neither carboplatin nor etoposide had any significant effect on the final number of chondrocytes in the dexamethasone-pretreated cultures and, whereas cisplatin and actinomycin-D remained cytotoxic to the prednisolone- and dexamethasone-pretreated cells, this cytotoxicity was significantly lower than that observed for the cells pretreated with medium alone. Cisplatin cytoxicity was reduced by 37.8 ± 2.58% and 49.4 ± 4.86% and actinomycin-D cytotoxicity, by 5.9 ± 4.86% and 30.3 ± 6.99%, respectively, in the prednisolone- and dexamethasone-pretreated cells as compared with the cells pretreated with medium alone.

Recovery of chondrocytes following combination treatment

Control cells treated with either cisplatin, carboplatin or actinomycin-D for a further 3 days demonstrated an absence of colony formation when subsequently grown in suspension culture (Fig. 2b). However, a number of small colonies of cells (< 50 μm) were noted. In the actinomycin-D treatment arm, the loss of colony formation was also accompanied by a loss of single cells in the suspension cultures. In contrast, cells that had been treated with either prednisolone or dexamethasone before the addition of the DNA-damaging agents and had then been grown in suspension culture formed colonies that exceeded 50 μm in size. In general, colony formation was between 5 and 20 orders of magnitude higher ($P < 0.05$, $n = 3$) for the cells derived from monolayer cultures pretreated with either of the glucocorticoids (Fig. 2b). A 72.7 ± 10.2-fold increase in colony formation, however, was observed for the cells derived from monolayer cultures treated with dexamethasone followed by actinomycin-D. Overall, chondrocytes derived from the glucocorticoid pretreatment arms formed colonies that were extremely large (> 100 μm) as compared with the small (< 50 μm), irregular colonies noted for chondrocytes derived from medium alone pretreatment arms.

Discussion

Glucocorticoids are important regulatory molecules that govern metabolism and development. They have potent anti-proliferative effects in many cell types. Glucocorticoids can induce a G1 cell-cycle arrest and programmed cell death in several leukaemic cell lines [17]. They also inhibit the proliferation of mammary epithelial cells [5] and induce differentiation of osteoblasts [13]. In our studies, both prednisolone and dexamethasone significantly reduced the proliferative ability of rat tibial chondrocytes in primary cultures, which was not the result of significant cell loss. Following the removal of glucocorticoids from the cultures a lag period occurred (before recovery of cell growth) that was greater for dexamethasone-treated as compared with prednisolone-treated cells, and this may have been related to the increased potency of dexamethasone as compared with prednisolone previously described in vivo [9]. Although an increase in potency would clearly be important for the treatment of childhood leukaemia/lymphoma, our results suggest that this also impacts upon the normal chondrocytes of the growth plate. Thus, although subsequent growth rates of glucocorticoid-treated chondrocytes were not significantly different from those of the untreated cells, chondrocyte numbers were significantly reduced, and it should be emphasised that this presents a potential loss of linear growth. To investigate this possibility we examined the clonogenicity of glucocorticoid-treated chondrocytes in suspension culture. These cells retained their ability to form colonies and to

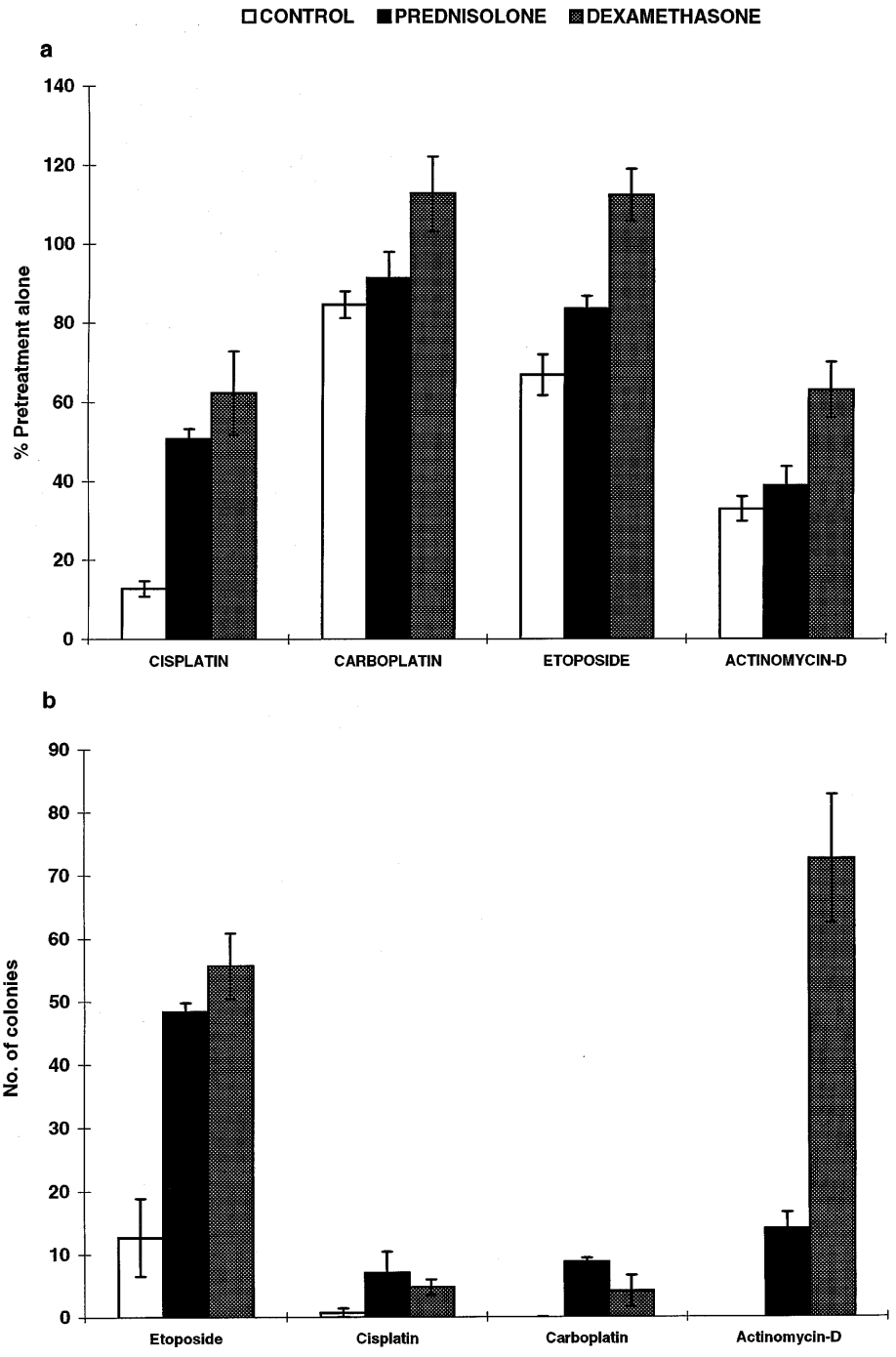

Fig. 2 **a** Effects of DNA-damaging agents on the growth of rat proximal tibial growth-plate chondrocytes in monolayer culture following their pretreatment for 3 days with either prednisolone or dexamethasone. Data are expressed as a percentage of those values obtained in chondrocytes receiving the pretreatment agent alone and represent mean values ±SD for 6 determinations in 2 separate experiments. **b** Clonogenicity of rat proximal tibial growth-plate chondrocytes in suspension culture following their treatment as described above. Data are expressed as the number of colonies exceeding 50 μm in size and represent mean values ±SD for 3 determinations within a single experiment

progress through the various stages of chondrogenic maturation.

Despite their differing toxicities, all DNA-damaging agents affected rapidly proliferating cells, causing substantial cell loss. In terms of chondrogenic maturation within the epiphyseal growth plate, it therefore seems likely that cells would be lost from the proliferative layer and also, perhaps more importantly, from the stem-cell population. This is further supported by both the absence of colony formation and the formation of small, irregular colonies observed when cytotoxic-pretreated chondrocytes were subcultured in agarose.

These cytokinetics studies yielded important data suggesting that the pretreatment of chondrocytes with glucocorticoids, due to their ability to suppress proliferation, would significantly reduce the cytotoxic effects of the DNA-damaging agents. Certainly, in dexamethasone-pretreated cultures the cytotoxic effects of the DNA-damaging agents were significantly reduced. Although only the cytotoxic effects of cisplatin and etoposide were reduced in prednisolone-pretreated cultures, it is possible that this is attributable merely to differences in potency between the two glucocorticoids. However, other interactions between these agents cannot

be ruled out. More importantly, in terms of chondrogenesis, those chondrocytes treated with the DNA-damaging agents following glucocorticoid pretreatment were capable of forming large colonies of cells on their subsequent growth in suspension culture. It is possible that this is due to an ability of the glucocorticoids to suppress the differentiation and, thus, clonal expansion of the stem-cell population and, hence, to protect and maintain a pool of cells committed to chondrogenesis. These cells would then be capable of differentiating, proliferating and maturing once these agents were removed, consistent with the phenomenon of ''catch-up'' growth that is observed following the removal of glucocorticoids in both animals [2] and children [4].

Clearly, further studies will be necessary to examine individual chondrocyte populations in isolation and to determine the precise mechanisms involved during glucocorticoid action. Furthermore, although we examined these effects in the ''normal'' setting, we should be aware of the implication of a similar effect in malignant cells. In a recent report, therapeutic concentrations of dexamethasone attenuated the cytotoxicity and growth inhibition of human malignant glioma cells induced by exposure to several chemotherapeutics [19]. Thus, the clinical implications for the management of paediatric cancer patients will become clearer once the role of these agents in malignant osteosarcoma cells has been explored.

In summary, the ability of growth plate chondrocytes to recover following their treatment with glucocorticoids is of great importance in considerations of the use of these agents in clinical paediatrics, particularly their role in CT schedules combining a variety of cytotoxics. Further studies should examine their role in the complex mechanisms regulating the final height attained in vivo and, hence, the rationale behind the use of growth hormone to stimulate/enhance growth in children following treatment for cancer.

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