Effects of steroid hormones on human fibroblasts *in vitro*

I. Glucocorticoid action on cell growth and collagen synthesis

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Fibroblasts are the predominant cells of connective tissue and their integrity is therefore dependent upon factors which influence the metabolism of these cells. The sensitivity of fibroblasts to anti-inflammatory steroids has been shown by the suppression of collagen and mucopolysaccharide synthesis *in vivo* (Castor and Baker, 1950; Mancini, Stringa, and Canepa, 1960; Houck, Sharma, Patel, and Gladner, 1968) and *in vitro* (Castor, 1965), and the inhibition of cell growth *in vitro* (Grossfeld, 1959; Wellings and Moon, 1961; Berliner and Ruhmann, 1967).

During prolonged therapy with anti-inflammatory steroids the inhibition described at a cellular level *in vitro* is reflected *in vivo* by an increased susceptibility to skin bruising (West, 1961) and by a decrease in skin thickness (Grahame, 1969). At the site of 'steroid bruising' there is a loss and disorganization of dermal collagen (Scarborough and Shuster, 1960).

In order to study the mode of action of anti-inflammatory steroids on skin, an *in vitro* fibroblast culture system has been used, and in this paper the effects of two anti-inflammatory steroids (prednisolone and cortisol) on fibroblast growth and metabolism will be described.

Materials and methods

I CELLS AND CELL MAINTENANCE

Primary diploid human embryonic skin and muscle fibroblasts (Flow Laboratories, Irvine, Scotland) were grown in tissue culture medium (TCM) consisting of Eagle's Minimal Essential Medium (MEM) containing 10% fetal bovine serum (Flow), 20 mmol/l. Hepes buffer (pH 7.2), 200 µg/ml benzyl penicillin, 100 µg/ml streptomycin sulphate, 2 mmol/l. L-glutamine, and 10 µg/ml ascorbic acid. Cells were replaced from frozen stock after 5 weeks in culture, maintained in 100 ml glass 'medical flat' bottles, and subcultured every 3 days. Cell suspensions were prepared by discarding the culture medium, washing the cell monolayer with 0.25% trypsin (British Drug Houses, Poole) in MEM, incubating at 37°C for 10 minutes,

adding 2 ml TCM, and vigorously pipetting through a fine Pasteur pipette.

II DRUGS

Cortisol (Sigma, London) and prednisolone (Sigma) were dissolved in dimethylacetamide (DMA) (British Drug Houses) before dilution in TCM. The maximum concentration of DMA under experimental conditions was 20 µg/ml. Concentrations of steroids between 0.01 µg/ml and 10 µg/ml were chosen because they extended over the range of physiological and therapeutic serum concentrations.

III EXPERIMENTAL CELL CULTURES

Cells were grown at 37°C in 5 cm sterile plastic tissue-culture petri dishes (Sterilin Ltd., Richmond). To each dish was added 0.5 ml TCM containing 7.5×10^4 cells, 0.5 ml of the dissolved steroid (DMA in the case of the control dishes) appropriately diluted in TCM, and 4 ml TCM. Five replicate dishes were used for each dose level of steroid.

IV ASSESSMENT OF DRUG EFFECTS

(i) Incorporation of tritiated thymidine

Experimental cells were incubated for 48 hours before an 8-hour exposure to 1.0 µCi/ml thymidine-6-³H (specific activity 5,000 mCi/mmol/l.; Radiochemical Centre, Amersham). Cells were harvested and resuspended in 2 ml 0.9% saline solution. 1.0 ml was taken for cell counting and the remainder precipitated in 5% trichloroacetic acid (TCA). The precipitates were washed in fresh 10% TCA, filtered onto glass-fibre discs (2.5 cm GF/C: Whatman Biochemicals Ltd., Maidstone), washed with methanol, and each disc was then placed in a counting vial to which was added 5 ml scintillation fluid containing 1% Soluene-350 (Packard Chemicals Ltd., Reading) and 0.5% Permablend (Packard) in toluene. After overnight incubation at 37°C, radioactivity was measured in a Beckman liquid scintillation counter and counting efficiency was measured by an external standard method and results were expressed as disintegration per minute (dpm) per 10^4 cells.

(ii) Incorporation of tritiated proline

Experimental cells were incubated for 72 hours before exposure to 1.0 µCi/ml L-proline-³H(G) (specific activity

500 mCi/mmol/l.; Radiochemical Centre) for 24 hours. After harvesting, the TCA (5%) precipitates of separated aliquots of cells and culture medium were washed, filtered, and the radioactivity measured as previously described.

Collagen was extracted from duplicate aliquots by incubating the precipitates in 5% TCA for 90 minutes at 90°C and washing twice in cold 10% TCA (Manner, 1971, modified from Fitch, Harkness, and Harkness, 1955). The collagen-free precipitates were filtered and radioactivity measured. The difference between the radioactivity of total precipitates and collagen-free precipitates indicated the collagen content of the samples. Collagen extracted from the culture medium was termed soluble collagen and that extracted from the cell monolayer was termed fibrous collagen (Castor, Rowe, Dorstewitz, Wright, and Ritchie, 1970).

(iii) *Cell proliferation*

Counts of single-cell suspensions were performed on a Model D Coulter Counter (Coulter Electronics Ltd., Dunstable) using a 100 μ m aperture.

Results

The Table and Figs 1–4 show representative results of each series of experiments; each experiment was repeated at least 3 times.

EFFECTS OF DMA ON CELL GROWTH

DMA was used to dissolve the steroids before dilution in TCM because of their relative insolubility in aqueous media. There was no effect of DMA upon cell proliferation or on DNA synthesis within the concentration range used in the experimental cultures (0.02–20 μ g/ml), though at 100 μ g/ml DNA synthesis by fibroblasts was inhibited (Fig. 1).

EFFECTS OF CORTISOL AND PREDNISOLONE

(1) *Fibroblast proliferation*

Cortisol and prednisolone inhibited cell proliferation at 10 μ g/ml (Student's 't' test; $P < 0.05$) (Fig. 2), but decreased the numbers of free cells in the culture

FIG. 1 Effect of dimethylacetamide on DNA synthesis (upper graph) and on cell proliferation (lower graph) after 3 days' culture. Vertical lines represent one SEM above and below the mean

FIG. 2 Effect of cortisol and prednisolone on cell proliferation after 3 days' culture. Cell counts were performed on an electronic cell counter

medium at all the steroid concentrations tested (Table). The reduction in the ratio of free to attached cells was statistically significant at 0.1 µg/ml cortisol and 0.01 µg/ml prednisolone ($P < 0.05$).

(2) DNA synthesis

DNA synthesis, measured as the rate of ^3H -thymidine incorporation into the TCA-precipitable cell fraction, was significantly stimulated above control levels by 0.01 µg/ml of both cortisol and prednisolone ($P <$

0.01), but inhibited by 20% at 1.0 µg/ml ($P < 0.05$) and by 50% at 10 µg/ml ($P < 0.01$) (Fig. 3).

(3) Collagen production

Cortisol and prednisolone stimulated the synthesis of both soluble and fibrous collagen, with a maximum stimulation occurring at 0.1 µg/ml of each steroid (Fig. 4a and b). The production of soluble collagen was stimulated to a significantly greater degree than that of fibrous collagen ($P < 0.05$). 73 %

Table Effect of cortisol and prednisolone on cell detachment expressed as a percentage of the cell total $\pm$ standard error of the mean (SEM)

	Concentration (µg/ml)				
	0	0.01	0.1	1.0	10
Cortisol	5.6 $\pm$ 0.63	4.2 $\pm$ 0.8	2.9 $\pm$ 0.5*	3.6 $\pm$ 0.5	4.3 $\pm$ 0.6
Prednisolone	5.6 $\pm$ 0.63	3.4 $\pm$ 0.4*	3.5 $\pm$ 0.8	3.5 $\pm$ 0.9	4.2 $\pm$ 0.3

* Indicates a significant difference from the control value (Student's 't' test; $P < 0.05$).

FIG. 3 Effect of cortisol and prednisolone on DNA synthesis. 1.0 µCi/ml ^3H -thymidine was added to the culture medium after 3 days and the cells harvested 8 hrs later

FIG. 4 (a) and (b) Effect of cortisol and prednisolone on collagen synthesis. 1.0 µCi/ml ^3H -proline was added to the culture medium after 3 days and collagen was extracted from the culture medium and cells after a further 24 hrs

($\pm 6\%$) of the total ^3H -proline incorporated into protein in the culture medium was associated with extractable collagen, whereas in the cell monolayer 26% ($\pm 8\%$) of the radioactivity was associated with collagen. These ratios were not significantly altered on exposure of the fibroblasts to cortisol or prednisolone.

Discussion

The cellular effects of glucocorticoids *in vitro* vary with both the origin (species and organ) of the cells and with the nature and concentration of the steroid employed. Thus, cortisol at concentrations between $0.1\text{ }\mu\text{g/ml}$ and $40\text{ }\mu\text{g/ml}$ has been reported to increase the proliferation of adult human fibroblast strains (Castor, 1962), yet suppresses the growth of L-strain mouse fibroblasts at $0.1\text{ }\mu\text{g/ml}$ (Berliner and Ruhmann, 1967). Because of this variation in response to cortisol by fibroblasts of different origin, it was first necessary to standardize a culture system in order to quantitate the effects of glucocorticoids on human fibroblasts under controlled conditions.

The difference in steroid effect on cell division when measurement by cell counts is compared with the incorporation of ^3H -thymidine may reflect an alteration in the intracellular availability of labelled precursor; such an alteration could be induced by a reduction in thymidine uptake or a change in the utilization rate of nucleic acid precursors on exposure to cortisol. Experiments in progress suggest that the suppression of ^3H -thymidine incorporation precedes a reduction in the rate of cell proliferation, and inhibition of the latter is accentuated at high doses of cortisol and prednisolone when the culture period is extended to 9 days.

The reduction in cell numbers by $10\text{ }\mu\text{g/ml}$ cortisol and prednisolone (Fig. 2) reflects an inhibition of the rate of cell division rather than a loss of cells from the culture surface through detachment (Table). This finding is at variance with reports that anti-inflammatory steroids cause fibroblast detachment

(Berliner, Bartley, Kenner, and Jee, 1970), but is in agreement with the observation that they cause increased adhesion of hepatoma cells *in vitro* (Ballard and Tomkins, 1969).

In contrast with a reported inhibition of collagen synthesis by cortisol at $1.0\text{ }\mu\text{g/ml}$ (Castor and others, 1970), in the present study both cortisol and prednisolone stimulated the production of soluble and fibrous collagen over the range of steroid concentrations and culture duration employed; this disparity remains unexplained.

The stimulation of DNA synthesis with $0.01\text{ }\mu\text{g/ml}$ of steroid and of collagen synthesis by $0.1\text{ }\mu\text{g/ml}$ of steroid may indicate that physiological concentrations of glucocorticoids are required for normal DNA metabolism and collagen production *in vitro*. These concentrations are comparable to normal plasma corticosteroid levels of $0.04\text{--}0.2\text{ }\mu\text{g/ml}$ (Asfeldt, 1971).

The results of this study have shown that cortisol and prednisolone cause an inhibition of DNA synthesis and a reduction in cell numbers. These *in vitro* effects suggest an explanation for the phenomenon of skin-thinning observed during corticosteroid therapy.

Summary

Human embryonic skin and muscle fibroblasts were grown *in vitro* and the effects of cortisol and prednisolone on cell proliferation, DNA synthesis, and collagen synthesis were studied. Both the steroids stimulated collagen synthesis at concentrations of $0.01\text{--}1.0\text{ }\mu\text{g/ml}$ and DNA synthesis at $0.01\text{ }\mu\text{g/ml}$. Higher concentrations of the steroids caused a marked suppression of DNA synthesis but a smaller reduction in the rate of cell proliferation over a 3-day culture period.

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