Adenosine 3': 5'-Cyclic Monophosphate in Young and Senescent Human Fibroblasts during Growth and Stationary Phase *in vitro*

EFFECTS OF PROSTAGLANDIN E₁ AND OF ADRENALINE

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Cyclic AMP levels per mg of cell protein were higher in late-passage (senescent) fibroblasts than in early-passage (young) fibroblasts both during growth and stationary phase, but, because the protein concentration per unit volume in senescent cells was lower than in young cells, the molar concentrations of intracellular cyclic AMP were very similar in the two cell types. In both young and senescent fibroblasts cyclic AMP levels declined during growth and no increase in intracellular cyclic AMP occurred in association with density-dependent inhibition of growth. These results indicate that changes in cyclic AMP concentration do not play a role in controlling the growth or in the senescent decline of human fibroblasts. Prostaglandin E₁ (1 μ M) caused maximal increases in fibroblast cyclic AMP concentration of 60–500-fold after 10–30 min, and adrenaline (epinephrine) (10 μ M) caused maximum increases of 5–25-fold after 2–10 min, depending on both the number of passages and the period after subculture. The cyclic AMP level in confluent young cells increased more with prostaglandin E₁ and far less with adrenaline than the cyclic AMP level in confluent senescent cells. During growth to confluence the cyclic AMP response to adrenaline declined in young cells and increased in senescent cells. As these responses to prostaglandin E₁ and to adrenaline changed independently of each other and of the basal cyclic AMP concentration, it is suggested that the expression of hormone receptors is altered both during growth to confluence and during senescence of human fibroblasts.

The cultured human fibroblast provides an excellent model for the study of aging (Hayflick, 1965; Goldstein, 1971; Cristofalo, 1972). After a finite number of cell divisions these cells become senescent in that they cease to grow, become swollen and granular and finally undergo lysis. Although several theories have been proposed (e.g. Cristofalo, 1972; Holliday & Tarrant, 1972), the biochemical basis for this loss of proliferative capacity remains uncertain.

Recent evidence suggests that cyclic AMP may regulate the growth of cultured cells. Thus growth is inhibited by agents that can increase intracellular cyclic AMP concentrations, including methylxanthines (Bürk, 1968), and PGE₁* (Johnson & Pastan, 1971; Naseem & Hollander, 1973), and by N⁶,2'-O-dibutyryl cyclic AMP (Sheppard, 1971; Johnson & Pastan, 1972; Burger *et al.*, 1972; Froehlich & Rachmeler, 1972). Rapidly growing cells have been reported to have lower intracellular cyclic AMP concentrations than more slowly growing cells (Otten *et al.*, 1971; Heidrick & Ryan, 1971; Sheppard, 1972). In addition, some workers have

observed an increase in cyclic AMP concentration associated with density-dependent inhibition of growth (Otten *et al.*, 1971; Heidrick & Ryan, 1971; Otten *et al.*, 1972b; D'Armiento *et al.*, 1973; Anderson *et al.*, 1973b). Considerable evidence has accumulated indicating that oncogenic viruses induce changes in the cell adenylate cyclase–cyclic AMP phosphodiesterase system and in its responses to serum factors, that favour uncontrolled growth (Bürk, 1968; Otten *et al.*, 1971, 1972a; Sheppard, 1972; Seifert & Paul, 1972; Kram *et al.*, 1973; Anderson *et al.*, 1973a). Viral transformation has also been reported to alter the sensitivity to PGE₁ of adenylate cyclase from some cell lines (Peery *et al.*, 1971). The hormonal sensitivity of cultured cells is not restricted to PGE₁. Thus adenylate cyclase from many permanent cell lines and from human skin fibroblasts is also activated by catecholamines (Makman, 1970, 1971; Rao *et al.*, 1971) and both PGE₁ and catecholamines have been reported to increase cyclic AMP amounts in intact human skin fibroblasts (Manganiello *et al.*, 1972; Franklin & Foster, 1973a,b).

In view of the above findings we have explored

* Abbreviation: PGE₁, prostaglandin E₁.

the possible role of cyclic AMP in the senescent loss of proliferative capacity of cultured human skin fibroblasts. Studies have been carried out on the basal cyclic AMP levels of both growing and confluent early-passage (young) and late-passage (senescent) cultures. In addition, the effects of PGE₁ and of adrenaline (epinephrine) on cyclic AMP levels in these cells have been studied to determine whether their hormonal sensitivity changes during the growth cycle or during their lifespan *in vitro*. Some of the results have been reported in a preliminary form (Goldstein & Haslam, 1973).

Experimental

Materials

[³H]Inulin (300mCi/mmol) was obtained from Amersham/Searle Corp., Don Mills, Ont., Canada, and cyclic [8-³H]AMP (28 Ci/mmol) from Schwarz/Mann, Orangeburg, N.Y., U.S.A. (–)-Adrenaline (epinephrine) bitartrate was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. PGE₁ was a gift from Dr. J. Pike of the Upjohn Co., Kalamazoo, Mich., U.S.A.; a stock neutral solution of 10mM-PGE₁ in 95% (v/v) ethanol was prepared.

Cell culture

All experiments were carried out with skin fibroblasts derived from the anterior forearm of two normal adult males, J004 and TM. Methods for establishment and propagation of cultures have been described (Goldstein & Littlefield, 1969). Essentially, cells were grown in Eagle's medium supplemented with non-essential amino acids and 15% foetal calf serum (Grand Island Biological Co., Grand Island, N.Y., U.S.A.). All subcultures were carried out from confluent dishes by using a split ratio of 1:8 (Hayflick, 1965). Therefore on average three successive cell divisions, or more correctly three mean population doublings, occurred each time a culture grew to confluence. By this criterion, J004 and TM underwent 85 and 65 mean population doublings respectively before cessation of growth through senescence. In the experiments reported, young and senescent cultures were used within 42–60 and 3–12 mean population doublings of growth cessation respectively. Cells were banked in liquid N₂ at early and late passage. They were reconstituted 1–2 weeks before use and were subcultured at least twice before study. Cultures were harvested with 0.125% (w/v) trypsin (1:250; Difco Laboratories Inc., Detroit, Mich., U.S.A.) dissolved in phosphate-buffered saline (Kelley & Meade, 1971), followed by the addition of growth medium and vigorous pipetting to obtain an even suspension of single cells. Cells were counted with a

Cytograph electronic counter (Biophysics Inc., Mahopac, N.Y., U.S.A.). Although the growth medium was not changed after subculture, the medium pH remained within the range 7.4–7.7 in all experiments.

Determination of cell volume

Measurement with the Cytograph. In this method suspended cells were illuminated with a laser beam and the shadows cast were compared with those produced by spherical latex particles of known size. Thus mean cell radius was determined and the cell volume was calculated assuming that the cells were spherical. This involves some error, particularly in older cultures. The ratios of mean cell volumes for senescent/young cells by this method were 3–6 at various times during cell growth.

Measurement of packed cell volume. Cells were harvested at various times after subculture. A sample was counted and the remaining suspension was centrifuged at 200g for 5 min in a specially designed tube with a constricted lower end marked with a 100μl calibration. After removal of the supernatant, [³H]inulin (0.1μCi) in regular growth medium was added to give a total volume of 100μl (cell pellet plus fluid). The cells were resuspended in this volume, and 25μl micro capillary tubes (Corning Glassware, Corning, N.Y., U.S.A.) were filled with suspension, sealed with cement (Critoseal; Sherwood Medical Industries, St. Louis, Mo., U.S.A.) and centrifuged for 1 min in a micro capillary centrifuge (International Equipment Co., Needham, Mass., U.S.A.; model MB). The lengths of the columns of packed cells were measured so that the pellet volume could be calculated and the tube contents were rapidly frozen to immobilize the fluid. Each tube was filed and broken so that the column of cells as well as a 10mm column of supernatant could be individually transferred to scintillation-counting fluid, which was sonicated to disperse the tube contents and counted for radioactivity. The [³H]inulin space was taken to be equivalent to the trapped extracellular fluid within the cell pellet and ranged from 25 to 35% (mean 31%) of the pellet volume with both young and senescent cells. The total cell volume/culture dish was calculated and used to determine the average molar concentration of intracellular cyclic AMP. With this method the ratios of mean cell volumes for senescent/young cells were 1.5–3.0 at various times during cell growth.

Assay of cyclic AMP

Before determination of cyclic AMP or addition of hormones, the fibroblasts were washed once with serum-free Eagle's medium and then allowed to incubate in a fresh volume of this same medium

for 1 h. The purpose of this procedure was to standardize incubation conditions and thus to avoid possible short-term effects of altered growth medium on the adenylate cyclase-cyclic AMP phosphodiesterase enzyme system during the variable times of growth in young and senescent cultures. This procedure, which did not affect the amount of cyclic AMP in the cells (see the Results section), also permitted accurate protein determinations on the same cells in which cyclic AMP was measured even when relatively few cells were present. After the fibroblasts had been incubated in the serum-free medium for 1 h, 0.01 vol. of 0.154 M-NaCl or of PGE₁ or adrenaline diluted in 0.154 M-NaCl was added and the incubations were continued for the required times. The final concentrations of PGE₁ and adrenaline used were 1 and 10 μ M respectively. Ethanol was present at a concentration of 0.01% (v/v) in incubations containing PGE₁, but did not affect cell cyclic AMP levels in preliminary experiments. Incubations were stopped by rapid removal of the incubation medium and addition of 2 ml of ice-cold 0.5 M-HClO₄ containing 10000 d.p.m. of cyclic [8-³H]AMP. The cellular material was scraped from the plastic dish with a rubber 'policeman', and after a rinse with 1 ml of ice-cold 0.5 M-HClO₄, the pooled 3 ml of denatured protein and acid extract was centrifuged. The protein in the pellets was determined by the method of Lowry *et al.* (1951). The supernatants were passed through columns containing 2 ml of packed resin (Bio-Rad AG 50W X8; 200–400 mesh; H⁺ form) and cyclic AMP was then eluted with water in the 4.5–10.5 ml fractions, which were freeze-dried in siliconized vials. This procedure simultaneously removed HClO₄ and separated cyclic AMP from other adenine nucleotides. The freeze-dried material was taken up in 0.3 ml of solution containing 50 mM-Tris-HCl buffer, pH 7.4, 8 mM-theophylline and 6 mM-mercaptoethanol. Recovery of cyclic AMP (60–80%) was monitored by counting for radioactivity 50 μ l of this solution. Fractions of the remainder were used for the assay of cyclic AMP by the protein-binding method of Brown *et al.* (1971). Sufficient cyclic [8-³H]AMP was added to each assay tube to give a total of 10000 d.p.m. including that recovered from the column. Better results were obtained by using 6 mg of charcoal to remove unbound cyclic AMP rather than the 10 mg recommended by Brown *et al.* (1971). Standard curves were derived from assay tubes in triplicate containing 0.2–10 pmol of unlabelled cyclic AMP. Optimum sensitivity was encountered with 0.5–5.0 pmol. Assays which did not fall within this range were repeated with more appropriate fractions of the freeze-dried material. The results were adjusted for the recovery of cyclic AMP up to the point at which the assay was done.

Liquid-scintillation counting

³H was counted in a dioxan-based phosphor (Scales, 1967) in a Beckman LS 230 liquid-scintillation spectrometer. The counting efficiency was approx. 33%; 10000 c.p.m. were counted. Quench corrections were applied by the external-standard-ratio method after subtraction of the background.

Results

Basal cyclic AMP levels in J004 fibroblasts

Preliminary results indicated that cyclic AMP levels measured per mg of protein were higher in senescent than in young cells that were grown to confluence (stationary phase). However, measurements of cell volumes with the Cytograph suggested that in confluent cultures senescent cells were 4–6 times larger than young cells despite the fact that the former contained only twice as much protein per cell. This indicated that the molar concentrations of cyclic AMP in the two cell types might not reflect the difference in levels of cyclic AMP expressed per mg of protein and might in fact be lower in the senescent cells. For this reason a more accurate method of measuring cell volume was developed (see the Experimental section) and intracellular cyclic AMP, measured in young and senescent fibroblasts at different times after subculture, was studied in relation to cell growth monitored in terms of protein, cell number and total cell volume.

The growth curves for young and senescent J004 fibroblasts were markedly different regardless of the unit used to measure growth (Figs. 1a, 1b and 1c). Young cells showed a rapidly accelerating growth after subculture for 1 day and achieved a mean doubling time of 24–30 h. They abruptly ceased growing on reaching visual confluence after about 6 days. Senescent cells not only grew more slowly (mean doubling time, 96–120 h), but showed a gradually declining rate of growth after the third day so that visual confluence was approached gradually after about 13 days. Senescent cells never became as tightly packed as young cells. For both young and senescent cells the expansion in total cell volume during growth, relative to the value 1 day after subculture, was proportionately much greater than the increase in protein, so that there was a considerable fall in the mean cell protein concentration (from 156 to 80 g/litre in young cells and from 117 to 54 g/litre in senescent cells in the experiment recorded in Fig. 1). Changes in mean cell volume during growth were relatively small, the younger cells tending to decrease and the older cells to increase in volume, but the difference between young and senescent cells was marked, with final mean cell volumes of 4.7 pl and 12.4 pl respectively in this experiment. These differences

Fig. 1. Basal cyclic AMP levels in young and senescent fibroblasts at various times after subculture in relation to different parameters of growth

Confluent cultures of young and senescent J004 fibroblasts were subcultured at a split ratio of 1:8 in 10cm diameter dishes. At subculture the senescent fibroblasts were six mean population doublings from growth cessation. Cyclic AMP levels were measured on the indicated days 1h after rinsing and replacing the growth medium with serum-free medium. Values are means $\pm$ S.E.M. from three dishes. Cell number and total cell volume were measured on separate replicate dishes grown in parallel. (a), (b) and (c) show growth in terms of protein, cell number and cell volume respectively per dish; (d), (e) and (f) show the intracellular cyclic AMP levels per mg of protein, per 10^6 cells and per litre of cells respectively. Young cells, closed symbols; senescent cells, open symbols.

Fig. 2. Effects of PGE_1 and of adrenaline on cyclic AMP levels in young fibroblasts at different times after subculture

Confluent young J004 fibroblasts were subcultured at a 1:8 split ratio in 6cm diameter dishes and grown for 1 day (a, g), 2 days (b, h), 4 days (c, i), 6 days (d, j), 8 days (e, k) and 10 days (f, l) before the effects of $1 \mu M$ - PGE_1 (●) and of $10 \mu M$ -adrenaline (■) on cyclic AMP levels were determined in incubations lasting 0.5, 2, 10 and 30 min. Control incubations without these additions were also performed (▲). Mean cyclic AMP values from three identically treated dishes are given; the S.E.M. is shown where it extends beyond the symbol.

Fig. 3. Effects of PGE₁ and of adrenaline on cyclic AMP levels in senescent fibroblasts at different times after subculture

Confluent senescent J004 fibroblasts (six mean population doublings from growth cessation) were subcultured at a 1:8 split ratio in 6 cm-diameter dishes and grown for 1 day (a, g), 3 days (b, h), 6 days (c, i), 9 days (d, j), 12 days (e, k) and 16 days (f, l) before the effects of 1 μM-PGE₁ (O) and of 10 μM-adrenaline (□) on cyclic AMP levels were determined in incubations lasting 0.5, 2, 10 and 30 min. Control incubations without these additions were also performed (Δ), but cyclic AMP levels were too low for accurate measurement on cells subcultured for only 1 and 3 days. Mean cyclic AMP values from three identically treated dishes are given; the S.E.M. is shown where it extends beyond the symbol.

between young and senescent cells and the changes that occurred during growth make comparison of cyclic AMP levels in different cells at different times difficult and critically dependent on the denominator of the units in which they are expressed (Figs. 1*d*, 1*e* and 1*f*).

On a per mg of protein basis, cyclic AMP levels declined during the first 2–3 days of growth in both young and senescent cells and then remained relatively constant both before and after confluence was reached. In the experiment shown in Fig. 1(*d*), the levels in senescent cells were approximately twice those in young cells (15 and 7.5 pmol/mg of protein respectively after confluence). Expression of cyclic AMP levels per 10⁶ cells (Fig. 1*e*) or per unit volume (Fig. 1*f*) increased the rate, extent and duration of decline during growth towards confluence, but whereas the amount of cyclic AMP in the large senescent cells was consistently greater than in the smaller younger fibroblasts, the molar concentrations of cyclic AMP in senescent cells were similar to those in the younger cells only 1–2 days earlier in the growth cycle. The intracellular cyclic AMP concentrations at confluence in these young and senescent cells were determined to be $0.66 \pm 0.06 \mu\text{M}$ and $0.73 \pm 0.05 \mu\text{M}$ respectively (means of three values $\pm$ S.E.M.). These values are not significantly different (Student's *t* test).

None of the methods of calculating cyclic AMP levels showed an increase related to density-dependent inhibition of growth (Fig. 1). However, as noted in the Experimental section, the regular growth medium was replaced by fresh serum-free medium 1 h before cyclic AMP was extracted from the cells. It follows that an elevation of cyclic AMP levels resulting from an accumulation of metabolites or depletion of medium components during growth might not have been detected. In particular, removal of growth factors present in serum could have increased cyclic AMP levels in the growing cultures so that no differences between these and confluent serum-exhausted cultures could be observed. To investigate these possibilities the cyclic AMP present in cultures processed by the standard methods was compared with that in identical unwashed cultures extracted immediately after removal of the regular growth medium. In neither rapidly growing nor confluent growth-inhibited cultures of young or senescent fibroblasts did removal of the growth medium followed by washing and incubation for 1 h in serum-free medium significantly affect the amounts of cyclic AMP in the cells.

Effects of PGE₁ and of adrenaline on cyclic AMP levels in J004 fibroblasts

With young confluent cells, PGE₁ (Figs. 2*d*, 2*e*

and 2*f*) induced a 500-fold increase in intracellular cyclic AMP to around 4000 pmol/mg of protein (about 320 μM) after 10–30 min. In contrast, with adrenaline (Figs. 2*j*, 2*k* and 2*l*), maximum levels of only about 60 pmol/mg of protein were encountered at 2 min, after which a slow decline towards basal values occurred. Different results were obtained before young cells became confluent. Although 1 day after subculture the response to PGE₁ was similar to that observed in confluent cells (Fig. 2*a*), the response to adrenaline was much greater than after confluence, with peak cyclic AMP levels of 400 pmol/mg of protein after 2–10 min (Fig. 2*g*). During the most rapid growth of young fibroblasts around 2–4 days after subculture the responses to both PGE₁ and adrenaline were depressed, but although the former recovered the latter declined further.

In senescent fibroblasts, as in young cells, the effect of PGE₁ was maximal in 30 min incubations, whereas the effect of adrenaline tended to peak after 2 min (Fig. 3). However, with senescent cells PGE₁ induced smaller increases in cyclic AMP than with young cells. The maximum cyclic AMP levels encountered after addition of PGE₁ to senescent fibroblasts were about 2000 pmol/mg of protein (about 110 μM). Although there were no significant changes in the cyclic AMP observed after 10 min exposure to PGE₁ at different times after subculture (Figs. 3*a*–3*f*), larger amounts were observed in the 9–16-day cultures than in the 3-day culture after 30 min with PGE₁. Addition of adrenaline to confluent senescent cells (Figs. 3*k* and 3*l*) caused a much larger and more persistent increase in cyclic AMP level (250 pmol/mg of protein after 2–10 min) than observed with confluent young cells (see Figs. 2*j*, 2*k* and 2*l*). During shorter periods after subculture the response to adrenaline was variable, but in general the opposite trend to that seen with young cells was observed, i.e. the effect of adrenaline increased during growth (Figs. 3*g*–3*j*).

The most marked differences in the responses of young and senescent fibroblasts to PGE₁ and to adrenaline shown in Figs. 2 and 3 are summarized in Fig. 4, in which the increases in cell density and the ratios of the maximum increases in cyclic AMP per mg of protein seen with the two hormones are plotted against time after subculture. The ratio of the hormonal responses changed much more during growth of young than of senescent cells. Although initially similar in the two cell types the ratios diverged sharply as growth of the young cells accelerated. With confluent young cells PGE₁ increased cyclic AMP to about 60-fold the maximum value obtained with adrenaline, whereas in the senescent cells this ratio was only about 8. Comparison of Figs. 4(*a*) and 4(*b*) shows that the

Fig. 4. Ratios of the peak cyclic AMP levels obtained with PGE₁ and with adrenaline in relation to growth of young and senescent fibroblasts

(a) Increases in cell density associated with the growth of the young (■) and senescent (□) J004 fibroblasts used in the experiments described in Figs. 2 and 3; (b) ratios from the data in Figs. 2 and 3 of the cyclic AMP levels observed 30 min after addition of 1 μM-PGE₁ to those observed 2 min after addition of 10 μM-adrenaline during the growth of young (●) and senescent (○) fibroblasts.

relative effects of PGE₁ and adrenaline were very roughly correlated with the cell density.

Effects of senescence on cyclic AMP levels per mg of protein in TM fibroblasts

The basal cyclic AMP levels and the effects on cellular cyclic AMP of incubation for 10 min with PGE₁ or with adrenaline were also measured in confluent fibroblasts from another donor (TM) as the cells approached senescence (Table 1). Both the mass culture and a clone derived from it were studied. As with J004 fibroblasts, senescence was associated with an increase in the basal level of cyclic AMP measured in terms of pmol/mg of protein, a decrease in the effect of PGE₁ on the intracellular cyclic AMP level and an enhancement of the effect of adrenaline. These changes, particularly the first, were clearly relatively terminal events in the lifespan of the cells *in vitro*.

Table 1. Effects of senescence on cyclic AMP levels in TM fibroblasts

A mass culture of TM cells and a subclone derived therefrom (TM-20) were studied as they approached senescence during serial subculture at a 1:8 split ratio. Cyclic AMP determinations were performed on confluent single 6 cm-diameter dishes preincubated with serum-free medium for 1 h and then incubated with 0.154 M-NaCl, 1 μM-PGE₁ or 10 μM-adrenaline for 10 min.

Cell type	Mean population doublings to cessation of growth	Cyclic AMP (pmol/mg of protein)		
		Control	PGE ₁	Adrenaline
TM	15	11.7	2261	37.1
	12	9.7	—	49.9
	9	11.7	—	92.3
	6	27.3	1688	651
TM-20	9	11.8	2286	220
	3	34.6	1008	1416

Discussion

The results show that when the cyclic AMP levels in different cells are compared the denominator of the units in which cyclic AMP is expressed may radically affect the results. Although when expressed per mg of cell protein cyclic AMP levels in senescent fibroblasts were considerably higher than those in young cells both during growth and after the cells had become confluent, this difference largely disappeared when molar concentrations of cyclic AMP derived from measurements of total cell volume were compared. This anomaly was caused by the higher protein concentration per unit volume in young compared with senescent fibroblasts. The packed-cell-volume measurements used in these studies may lead to a slight underestimate of the total volume of the larger and more fragile senescent cells, some of which could be lost during the necessary manipulations. In this case the molar cyclic AMP concentration in senescent cells could be equal to or even slightly lower than that in young cells. In fact, a much less accurate optical method of measuring cell volume used in preliminary studies did suggest that the molar cyclic AMP concentration was lower in the senescent cells (Goldstein & Haslam, 1973). As a whole the results suggest that there is probably no biologically significant difference between the mean concentrations of cyclic AMP in young and senescent fibroblasts, although we cannot eliminate the possibility that different degrees of protein binding or some other form of compartmentation results in different effective concentrations of the compound. The basal cyclic AMP levels of young and senescent human fibroblasts have not previously

been compared, but Barnes *et al.* (1972) briefly reported that the adenylate cyclase activity of WI 38 cells did not change during aging, although viral transformation did lower the activity of the enzyme markedly. We conclude that changes in components of the adenylate cyclase-cyclic AMP phosphodiesterase enzyme system are unlikely to be responsible for the senescent loss of proliferative capacity of human diploid fibroblasts.

Several workers have suggested that density-dependent inhibition of cell growth is mediated by an increase in intracellular cyclic AMP concentration, though some contrary evidence does exist. Thus addition of *N*⁶,2'-*O*-dibutyryl cyclic AMP, particularly with theophylline, to transformed cells has been reported to restore density-dependent inhibition of cell growth (Hsie & Puck, 1971; Johnson *et al.*, 1971; Sheppard, 1971). However, other workers have found that the inhibition of growth this causes differs from that in dense cultures of normal cells (Paul, 1972; Smets, 1972; Grimes & Schroeder, 1973; Van Wijk *et al.*, 1973). Zacchello *et al.* (1972) reported that the adenylate cyclase activity per mg of cell protein in human diploid fibroblasts increases during growth to confluence. An increase in intracellular cyclic AMP concentration associated with density-dependent inhibition of growth of permanent fibroblast lines has been observed by some (Otten *et al.*, 1971; Heidrick & Ryan, 1971; Otten *et al.*, 1972*b*), but not all (Sheppard, 1972; Oey *et al.*, 1974). Otten *et al.* (1972*b*) observed no increase in cyclic AMP during density-dependent inhibition of the growth of a strain of human diploid fibroblasts (WI 38), when the medium was replaced frequently, although in a later study (D'Armiento *et al.*, 1973) different results were obtained. There is conflicting evidence as to whether contact between cells (e.g. Otten *et al.*, 1972*b*) or depletion of serum factors (e.g. Seifert & Paul, 1972; Kram *et al.*, 1973; Oey *et al.*, 1974) is responsible for the increase in cyclic AMP when it occurs. In the study reported here not only was there no increase in cyclic AMP concentration associated with density-dependent inhibition of fibroblast growth, but cyclic AMP concentrations decreased during growth of both young and senescent cells. The same results were obtained whether or not the cells were incubated with serum-free medium before extraction of cyclic AMP. It follows that neither cell contact nor depletion of serum factors affected cyclic AMP levels in the human diploid fibroblasts used in this study. The molar concentration of cyclic AMP in our confluent cells was very low compared with the intracellular concentrations reported by some workers in confluent fibroblasts of several types, including a human diploid strain (MA 308) (Otten *et al.*, 1971, 1972*b*). However, the cyclic AMP level in our confluent young

fibroblasts (as pmol/mg of protein) was comparable with that reported in confluent human skin fibroblasts by Froehlich & Rachmeler (1972), who found evidence that in their cells cyclic AMP limited growth in that re-feeding with fresh medium induced a fall in the cyclic AMP level together with an increase in DNA synthesis that was blocked by exogenous cyclic AMP. These workers did not measure cyclic AMP levels during the development of confluence. Although it is possible that in our cultures cell contact or serum depletion mediated density-dependent inhibition of growth without any involvement of cyclic AMP, it is also possible, as initially suggested by Froehlich & Rachmeler (1972), that the growth-inhibitory activity of cyclic AMP is enhanced as a result of biochemical changes initiated by cell contact or serum depletion. The latter hypothesis would reconcile considerable evidence that depression of cyclic AMP levels by addition of serum, trypsin or insulin to confluent fibroblasts is associated with a stimulation of growth (Sheppard, 1972; Froehlich & Rachmeler, 1972; Burger *et al.*, 1972; Otten *et al.*, 1972*b*) with the failure to observe an increase in cyclic AMP associated with density-dependent inhibition of growth in this and some other studies. Moreover, a report (Seifert & Rudland, 1974) that addition of fresh serum increases cyclic GMP levels in confluent fibroblasts and that exogenous cyclic GMP can induce DNA synthesis in these cells suggests that the cyclic AMP/cyclic GMP ratio could be more important for the regulation of cell growth than the absolute amount of cyclic AMP. Studies on the cyclic GMP levels in human skin fibroblasts during growth to confluence and during senescence *in vitro* are now needed. The relatively high concentrations of cyclic AMP found in cells in subculture for 1–2 days cannot at present be explained. The manipulation of the cells during subculture, the low initial cell density and the role of serum factors all require investigation.

Although the changes in the cyclic AMP levels in response to PGE₁ or adrenaline depended in a complex manner on whether early- or late-passage cultures were used and on the period of growth in subculture, the effect of each hormone was clearly identifiable. PGE₁ at 1 μ M caused a 60–500-fold increase in cellular cyclic AMP after 10–30 min, whereas adrenaline at 10 μ M increased cyclic AMP levels by 5–25-fold with peak values after 2–10 min. The increase in cyclic AMP with PGE₁ was massive compared with the effects of PGE₁ on permanent cell lines (Manganiello & Vaughan, 1972; Otten *et al.*, 1972*b*), but was similar to that briefly reported to occur in human skin fibroblasts by Manganiello *et al.* (1972). Confluent young fibroblasts showed a much greater cyclic AMP response to PGE₁ than confluent senescent fibroblasts, whereas with adrenaline the reverse was the case. During growth of young

cells the peak response to adrenaline decreased from an initial value of 400 pmol/mg of protein to 60 pmol/mg of protein at confluence, whereas the response to PGE₁ was depressed only during the most rapid phase of growth. With our senescent cells the changes during growth to confluence were less marked than in young cells and in contrast with young cells an increase in the cyclic AMP response to adrenaline occurred. In a preliminary report Manganiello *et al.* (1973) have described changes in the cyclic AMP responses of young human fibroblasts to PGE₁ and isoproterenol associated with changes in cell density. These were similar to those reported for PGE₁ and adrenaline here. The question arises of whether the differences in hormonal response between young and senescent cells may be determined by the different cell densities to which they grow. The results show that the ratio of the responses to PGE₁ and adrenaline in young and senescent cells correlates very roughly with the cell density. However, as an increasing cell density is associated with a decreasing response to adrenaline in young cells and an increasing response in senescent cells, other factors must also be involved. Increases in the activation by PGE₁ and by adrenaline of adenylate cyclase from cultures of several cell lines grown to increasing cell densities have been described (Makman, 1971) and may relate to some of our results. However, our findings show that the two hormonal responses studied change independently of each other and of the basal cyclic AMP levels both during growth of the cells after subculture and during senescence. This suggests that changes in the relative amounts or activities of the membrane receptors for PGE₁ and adrenaline occurred, as changes in the amount of adenylate cyclase or in the activity of cyclic AMP phosphodiesterase should alter the basal cyclic AMP and the responses to the two hormones all in the same direction. As the conditions under which the hormones were added were deliberately standardized, the changes in hormonal sensitivity observed cannot be attributed to short-term effects of changes in the pH or composition of the growth medium. The remaining factor that could affect intracellular cyclic AMP concentrations, leakage of the compound into the medium, has been reported to occur after addition of hormones to some cells, but probably has no significant effect on the amount of intracellular cyclic AMP in incubations shorter than 15 min (Franklin & Foster, 1973a; Penit *et al.*, 1974). In any case, as leakage would not be expected to discriminate between increases in cyclic AMP caused by different hormones, it is very unlikely to account for the independent changes in responsiveness to PGE₁ and adrenaline that were observed. The results with J004 fibroblasts are supported by those with cells derived from another donor (TM). The observation that a TM subclone

behaved similarly to the parent mass culture implies that clonal selection is an unlikely explanation for the shifting responses to PGE₁ and adrenaline seen during serial subculture. However, studies on several clones plus parental uncloned cultures would be required to prove this point. As the most probable explanation of our results we suggest that the alterations in cell function associated with density-dependent inhibition of cell growth and with senescence *in vitro* may include changes in the expression of the genes responsible for the synthesis of hormone receptors.

Note Added in Proof (Received 23 September 1974)

After submission of this paper for publication, a report (Kelly & Butcher, 1974) has appeared of effects of PGE₁ and of adrenaline on cyclic AMP in WI-38 fibroblasts that are substantially the same as those we observed in young J004 fibroblasts.

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References

- Anderson, W. B., Johnson, G. S. & Pastan, I. (1973a) *Proc. Nat. Acad. Sci. U.S.* **70**, 1055-1059
- Anderson, W. B., Russell, T. R., Carchman, R. A. & Pastan, I. (1973b) *Proc. Nat. Acad. Sci. U.S.* **70**, 3802-3805
- Barnes, E., Brandes, D., Barnes, P., Ryser, H. & Barnard, S. (1972) *In Vitro* **7**, 274-275
- Brown, B. L., Albano, J. D. M., Ekins, R. P., Sgherzi, A. M. & Tampion, W. (1971) *Biochem. J.* **121**, 561-562
- Burger, M. M., Bombik, B. M., Breckenridge, B. M. & Sheppard, J. R. (1972) *Nature (London) New Biol.* **239**, 161-163
- Bürk, R. R. (1968) *Nature (London)* **219**, 1272-1275
- Cristofalo, V. (1972) *Advan. Gerontol. Res.* **4**, 45-79
- D'Armiento, M., Johnson, G. S. & Pastan, I. (1973) *Nature (London) New Biol.* **242**, 78-80
- Franklin, T. J. & Foster, S. J. (1973a) *Nature (London) New Biol.* **246**, 119-120
- Franklin, T. J. & Foster, S. J. (1973b) *Nature (London) New Biol.* **246**, 146-148
- Froehlich, J. E. & Rachmeler, M. (1972) *J. Cell Biol.* **55**, 19-31
- Goldstein, S. (1971) *New Engl. J. Med.* **285**, 1120-1129
- Goldstein, S. & Haslam, R. J. (1973) *J. Clin. Invest.* **52**, 35a
- Goldstein, S. & Littlefield, J. W. (1969) *Diabetes* **18**, 545-549

- Grimes, W. J. & Schroeder, J. L. (1973) *J. Cell Biol.* **56**, 487-491
- Hayflick, L. (1965) *Exp. Cell Res.* **37**, 614-636
- Heidrick, M. L. & Ryan, W. L. (1971) *Cancer Res.* **31**, 1313-1315
- Holliday, R. & Tarrant, G. M. (1972) *Nature (London)* **238**, 26-30
- Hsie, A. W. & Puck, T. T. (1971) *Proc. Nat. Acad. Sci. U.S.* **68**, 358-361
- Johnson, G. S. & Pastan, I. (1971) *J. Nat. Cancer Inst.* **47**, 1357-1364
- Johnson, G. S. & Pastan, I. (1972) *J. Nat. Cancer Inst.* **48**, 1377-1387
- Johnson, G. S., Friedman, R. M. & Pastan, I. (1971) *Proc. Nat. Acad. Sci. U.S.* **68**, 425-429
- Kelley, W. N. & Meade, J. C. (1971) *J. Biol. Chem.* **246**, 2953-2958
- Kelly, L. A. & Butcher, R. W. (1974) *J. Biol. Chem.* **249**, 3098-3102
- Kram, R., Mamont, P. & Tomkins, G. M. (1973) *Proc. Nat. Acad. Sci. U.S.* **70**, 1432-1436
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- Makman, M. H. (1970) *Science* **170**, 1421-1423
- Makman, M. H. (1971) *Proc. Nat. Acad. Sci. U.S.* **68**, 2127-2130
- Manganiello, V. & Vaughan, M. (1972) *Proc. Nat. Acad. Sci. U.S.* **69**, 269-273
- Manganiello, V., Breslow, J. & Vaughan, M. (1972) *J. Clin. Invest.* **51**, 60a-61a
- Manganiello, V., Breslow, J. & Vaughan, M. (1973) *Pediat. Res.* **7**, 391
- Naseem, S. M. & Hollander, V. P. (1973) *Cancer Res.* **33**, 2909-2912
- Oey, J., Vogel, A. & Pollack, R. (1974) *Proc. Nat. Acad. Sci. U.S.* **71**, 694-698
- Otten, J., Johnson, G. S. & Pastan, I. (1971) *Biochem. Biophys. Res. Commun.* **44**, 1192-1198
- Otten, J., Bader, J., Johnson, G. S. & Pastan, I. (1972a) *J. Biol. Chem.* **247**, 1632-1633
- Otten, J., Johnson, G. S. & Pastan, I. (1972b) *J. Biol. Chem.* **247**, 7082-7087
- Paul, D. (1972) *Nature (London) New Biol.* **240**, 179-181
- Peery, C. V., Johnson, G. S. & Pastan, I. (1971) *J. Biol. Chem.* **246**, 5785-5790
- Penit, J., Jard, S. & Benda, P. (1974) *FEBS Lett.* **41**, 156-160
- Rao, G. J. S., Del Monte, M. & Nadler, H. L. (1971) *Nature (London) New Biol.* **232**, 253-255
- Scales, B. (1967) *Int. J. Appl. Radiat. Isotop.* **18**, 1-6
- Seifert, W. E. & Paul, D. (1972) *Nature (London) New Biol.* **240**, 281-283
- Seifert, W. & Rudland, P. S. (1974) *Nature (London)* **248**, 138-140
- Sheppard, J. R. (1971) *Proc. Nat. Acad. Sci. U.S.* **68**, 1316-1320
- Sheppard, J. R. (1972) *Nature (London) New Biol.* **236**, 14-16
- Smets, L. S. (1972) *Nature (London) New Biol.* **239**, 123-124
- Van Wijk, R., Wicks, W. D., Bevers, M. M. & Van Rijn, J. (1973) *Cancer Res.* **33**, 1331-1338
- Zacchello, F., Benson, P. F., Giannelli, F. & McGuire, M. (1972) *Biochem. J.* **126**, 27 P