Failure of platelet-derived growth factor plus insulin to stimulate sustained proliferation of Swiss 3T3 cells

Requirement for hydrocortisone, prostaglandin E_1, lipoproteins, fibronectin and an unidentified component derived from serum

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Summary

Platelet-derived growth factor (PDGF) has been reported to be a potent mitogen for Swiss 3T3 cells made quiescent by growth to saturation density in high serum, and its activity is further potentiated by high levels of insulin, which alone have little effect. We show here that this is not the case for sparse 3T3 cells made quiescent by plating in low serum. Under these conditions, insulin alone is at least as effective as PDGF and frequently more so. Together, the response is no more than additive at best, and in many cases less than additive, the combined effect being no greater than for insulin alone. Instead, we find that optimal mitogenic stimulation requires the additional presence, besides PDGF and insulin, of hydrocortisone, prostaglandin E_1 and an unidentified, non-dialysable component contained in serum treated with dithiothreitol (DTT) to inactivate endogenous growth factors. Interestingly, overnight pretreatment of the cells with hydrocortisone alone potentiates the subsequent response to PDGF+insulin, i.e. pretreatment induces a long-term memory that persists after the removal of the hydrocortisone from the medium. In short-term (24 h) thymidine incorporation assays, the combination of PDGF, insulin, hydrocortisone, prostaglandin E_1 and DTT-serum, is as effective as optimal levels of whole serum, but is unable to sustain longer-term proliferation (measured over 6 days). For this, high- and low-density lipoproteins, fibronectin and, to some extent epidermal growth factor (EGF), are also necessary.

Key words: PDGF, insulin, cell proliferation.

Introduction

Mouse 3T3 cells have an absolute requirement for growth factors, which is normally met by supplementing the culture medium with serum. On lowering the serum concentration, the 3T3 cell cycle becomes substantially more variable in duration and the minimum cycle time increases (Brooks and Riddle, 1988*a*). More surprisingly, the population (despite being clonal) becomes markedly heterogeneous in its proliferative capacity, some cells ceasing to proliferate while others continue through numerous consecutive cell cycles (Brooks and Riddle, 1988*a*,*b*; Brooks, 1985).

Recently, we reported that platelet-derived growth factor (PDGF) added at the time of step-down to 1% serum (from 10% serum) was able to maintain maximal proliferation rate and prevent the appearance of non-dividing cells (Brooks and Riddle, 1988*a*). This suggested that the heterogeneity might reflect responsiveness to a single growth factor (PDGF). Subsequently, we have found the response to PDGF to be rather variable. In some experiments, as previously reported, 1% serum plus PDGF gave proliferation rates comparable to 10% serum (Brooks and Riddle, 1988*a*). In other experiments (with the same batches of serum and PDGF), the response was extremely poor (unpublished observations), with very little cell division occurring, despite the presence of optimal levels of both PDGF and insulin. (High levels of insulin potentiate the response to PDGF, e.g. see Dicker *et al.* (1981).) The reasons for the variability remain obscure. However, responsiveness could be restored by an overnight pretreatment with 10% serum (unpublished observations). This suggested that the ability to respond to PDGF might depend upon the presence of other growth factors, the effects of which may have been carried over to varying degrees from the previous culture conditions. For this reason, we set out to explore more systematically the growth factor requirements of Swiss 3T3 cells. We report

here that in addition to PDGF and high levels of insulin, optimal mitogenic stimulation of sparse 3T3 cells requires the presence of hydrocortisone, a cyclic AMP elevating agent, and an unidentified, non-dialysable activity present in serum distinct from known peptide growth factors. Furthermore, this combination, though comparable to 10% serum in stimulating thymidine incorporation, fails to stimulate sustained proliferation (i.e. repeated cell cycles). To achieve this requires, in addition, not only the expected transferrin (Barnes and Sato, 1980), but also low- and high-density lipoproteins (LDL and HDL) and fibronectin.

Materials and methods

Cells

The Swiss 3T3 cells used (clone 4A/C5) and the methods for their routine subculture have been described previously (Brooks *et al.* 1984). The medium used throughout was Dulbecco's modification of Eagle's medium (DMEM) equilibrated with 12% CO_2, and normally containing 10% (v/v) newborn calf serum (Tissue Culture Services, Slough, England; or Gibco, Paisley, Scotland) except where stated otherwise. Tissue culture dishes were obtained from Nunc.

Growth factor-inactivated serum

Treatment of newborn calf serum with dithiothreitol followed by iodoacetimide was exactly as described by Van Zoelen *et al.* (1985). The resulting preparation is hereafter referred to as DTT-serum.

Quiescent cultures

Subconfluent quiescent cultures. These were prepared by seeding 2.5×10^4 or 3×10^4 cells, from an exponentially growing culture, onto 13 mm diameter glass coverslips, within the (16 mm diameter) wells of a 24-well plate (Nunc), in 1 ml of DMEM containing 0.5% (v/v) newborn calf serum. The cells were used 3–4 days later.

Density-arrested cultures. These were prepared by seeding 2.5×10^4 or 3×10^4 exponentially growing cells onto coverslips, as above, in 1 ml of DMEM containing 5% (v/v) newborn calf serum. The cells were used 7 days later, i.e. about 3 days after reaching saturation density. Visual inspection confirmed the absence of mitotic cells.

[^{3}H]thymidine incorporation

To measure the stimulation of DNA synthesis, coverslips of quiescent cells were transferred to fresh 24-well plates, each well containing 1 ml of the test medium together with [^{3}H]thymidine (TRK120, Amersham International) at 1 μCi ml^{-1} and unlabelled thymidine at 2.5 μM (1 μCi=37 kBq). Incorporation was terminated after 24 h by fixing the cells in formol saline (3.7% (w/v) formaldehyde, 0.5% (w/v) NaCl and 1.5% (w/v) Na_2SO_4). Coverslips were then extracted with 5% (w/v) trichloracetic acid at 4°C (2×5 min), rinsed with 95% (v/v) ethanol (×3), air dried, and transferred to scintillation vials containing 5 ml of toluene-based scintillation fluid (PPO, 5 g l^{-1}; POPOP, 0.2 g l^{-1}) for scintillation counting.

For autoradiography, the coverslips were removed from the scintillation vials, rinsed in toluene, and processed exactly as previously described (Brooks, 1975), except that the emulsion was Ilford K5, the developer, Ilford ID19 diluted 1:1 with water, and the fixer, Kodak Unifix diluted 1:5.

Cell multiplication assays

To avoid any differences in plating efficiency that might arise from plating cells in media of different compositions, cultures were set up initially in DMEM containing 0.5% (v/v) newborn serum, generally at 10^4 cells/16 mm well (24-well plates) in a volume of 1 ml. Approximately 24 h later, 0.9 ml of medium was removed from each well and replaced with 0.9 ml of the test medium. This procedure, which results in a carry-over of 0.05% serum into the assay, was preferred to complete medium replacement, since it minimized cell loss and disturbance, which otherwise invariably occurred with complete medium change. Three days after the shift to test conditions, 0.9 ml of the medium was again removed and replaced with 0.9 ml of fresh medium of the same composition. On the sixth day, the cells were trypsinized, and counted using a Coulter Counter.

Lipoproteins

Low-density lipoprotein (LDL) was prepared exactly as described by Rankin *et al.* (1989). In addition, some samples of LDL were obtained from Sigma, as was the high-density lipoprotein (HDL) used.

Growth factors

Highly purified porcine PDGF was very kindly donated by Dr P. Stroobant (Ludwig Institute for Cancer Research, London W1P 8RT). Partially purified porcine PDGF was obtained from Bioprocessing, Consett, Co. Durham, UK). One unit is equivalent to 14 ng of highly purified PDGF. Epidermal growth factor (EGF) was obtained from Collaborative Research, or generously provided by Dr M. R. Green (Unilever Research, Bedford, UK). Transforming growth factor β (TGFβ) from human platelets was obtained from Bioprocessing. Hydrocortisone, prostaglandins E_1 and $F_{2\alpha}$ and fibronectin, were obtained from Sigma. Human transferrin was obtained from Miles.

Results

Response of quiescent cells to PDGF and insulin

In order to clarify the reasons for the variable response to PDGF, we set out to determine the full set of growth factors required for the multiplication of low-density Swiss 3T3 cells, with the ultimate aim of eliminating the need for serum altogether. In the first instance, we chose to use a standard thymidine incorporation assay, involving the stimulation of quiescent cells in a background of 0.1% serum. This level of serum permits better cell survival than serum-free conditions but has little mitogenic activity of its own.

Most assays of the growth factor requirements of 3T3 cells have been conducted using cultures made quiescent by growth to saturation density in relatively high levels of serum (e.g. see Pledger *et al.* 1977; Dicker *et al.* 1981; Leof *et al.* 1982; Lopez-Rivas *et al.* 1984; Bravo *et al.* 1987). Using such density-arrested cultures, we were able to confirm that PDGF alone was able to stimulate some entry into S phase, as measured by both thymidine incorporation (Fig. 1A) and autoradiography (Fig. 1B). Insulin alone (at 5 μg ml^{-1}) had relatively little activity, but markedly potentiated the response to lower levels of PDGF. Together, the maximum response was substantially greater than that attained with fresh 5% serum. Similar results have been presented by others (Dicker *et al.* 1981; Lopez-Rivas *et al.* 1984; Rozengurt, 1986; Bravo *et al.* 1987).

When cells were made quiescent at low density by plating in low levels of serum (0.5%), the results were somewhat different. In absolute terms, the response of sparse cells to PDGF alone, as measured by autoradiography, was similar to that of density-arrested cells (Fig. 1D) except that the maximal response was achieved at a lower growth factor concentration, as might be expected (Holley, 1975). Relative to 5% serum, however, PDGF was much less effective (Fig. 1C, D). Furthermore, we have found repeatedly that insulin was at least as mitogenic as optimal levels of PDGF (Fig. 1C, D), and in many exper-

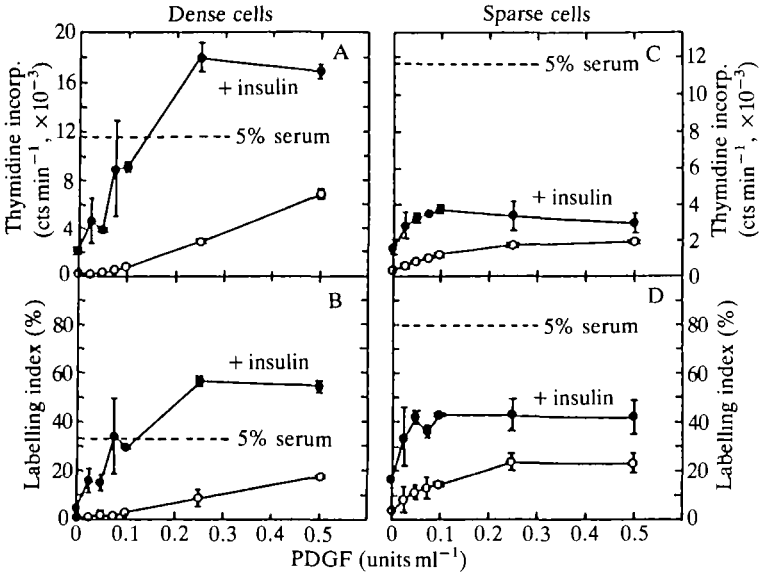

Fig. 1. Response of density-arrested (A, B) or sparse quiescent (C, D) 3T3 cells to PDGF±insulin (5 μg ml^{-1}), in a background of 0.1 % calf serum. Thymidine incorporation (A, C) was measured after 24 h. The same coverslips were then prepared for autoradiography and the proportion of labelled cells (labelling index) determined (B, D). Each point represents the mean±S.D. of triplicate determinations. Mean values for the 5 % serum control are indicated by the horizontal broken lines. Filled symbols, +insulin; open symbols, no insulin.

Table 1. *Interaction between insulin, PDGF, hydrocortisone and PGE_1*

	Thymidine incorporation per coverslip (cts min^{-1}±S.D.)
5 % Serum control	7099±405
No addition	550±27
Insulin	3037±755
PDGF	1952±308
Hydrocortisone	410±33
PGE_1	477±105
Insulin+PDGF	2495±388
Insulin+hydrocortisone	2224±161
Insulin+PGE_1	3297±438
PDGF+hydrocortisone	2223±113
PDGF+PGE_1	3178±324
Hydrocortisone+PGE_1	906±111
Insulin+PDGF+hydrocortisone	5108±114
Insulin+PDGF+PGE_1	5136±526
Insulin+hydrocortisone+PGE_1	4314±404
PDGF+hydrocortisone+PGE_1	3621±312
Insulin+PDGF+hydrocortisone+PGE_1	6097±842

Except for the 5 % serum control, all cultures contained 2.5 % DTT-serum. Growth factors were added at the following concentrations: PDGF, 0.25 unit ml^{-1}; insulin, 5 μg ml^{-1}; hydrocortisone, 1 μg ml^{-1}; PGE_1, 1 μg ml^{-1}. The stimulation of thymidine incorporation in sparse, quiescent 3T3 cells was determined after 24 h.

iments more so (Table 1) despite its relative lack of activity in density-arrested cultures (Fig. 1A, B). In the experiment shown in Fig. 1C, D, insulin augmented the response to PDGF when assayed in combination. There was some indication of synergy at the lower levels of PDGF, but at saturating concentrations of PDGF, the effect of insulin was little more than additive. Even so, the maximum stimulation achieved remained much less than obtained with 5 % serum. In other experiments, insulin and PDGF, though both active on their own, gave no additional effect in combination (Table 1), in marked contrast to the strongly synergistic effects on density-arrested cells (Fig. 1A, B).

It should be pointed out that the experiments shown in Fig. 1 reveal some slight discrepancies between thymidine incorporation and labelling index (LI). In sparse cultures, the maximum stimulation of thymidine incorporation by insulin plus PDGF was roughly 30 % of that obtained with 5 % serum, whereas the labelling indices (of the same coverslips) differed by only 50 %. This indicates that, compared to serum, PDGF plus insulin is more effective at stimulating entry into S phase in sparse cells than it is at stimulating thymidine incorporation. This could be due to less-efficient uptake of thymidine or to a slower rate of DNA replication (i.e. longer S phase) than in the presence of serum. Such differences were less evident with density-arrested cells. Despite the discrepancies, we have continued to use thymidine incorporation as the assay of choice. This was not only because of convenience but because we were interested in reproducing with defined factors all of the effects of serum related to proliferation, and not merely the stimulation of entry into S phase.

Potentiation of the response to PDGF and insulin by serum treated to eliminate peptide growth factors

The inability of PDGF plus insulin to stimulate more than 30 % of the thymidine incorporation achieved with 5 % serum in subconfluent 3T3 cells (Fig. 1C; Table 1) clearly points to a need for other factors. Serum, of course, provides many substances besides peptide growth factors, such as lipids, vitamins, trace elements, antioxidants etc. (Barnes and Sato, 1980; Ham, 1981), and it seemed possible that some such component might have been limiting here. Van Zoelen *et al.* (1985) have described a procedure for destroying the peptide growth factors in serum whilst retaining other growth promoting properties. This exploits the fact that most peptide growth factors contain essential disulphide bridges sensitive to reducing agents such as dithiothreitol (DTT). By adding back purified growth factors to such DTT-treated serum, Van Zoelen *et al.* were able to obtain sustained proliferation of NRK cells, whereas the growth factors alone were inadequate.

The response of 3T3 cells to two independent preparations of DTT-treated newborn calf serum is shown in Fig. 2. Concentrations in the range of 2–3 % (v/v) consistently potentiated the response to PDGF plus insulin whilst having no mitogenic effect on their own (see Fig. 2, inset). At levels above 3 %, thymidine incorporation declined from the maximum, falling below the control (PDGF plus insulin only) above 6 % DTT-serum. This suggests either that the stimulatory component has a sharp optimum, with higher levels being inhibitory, or that the DTT-serum contains both stimulatory and inhibitory components. We have so far made no attempt to distinguish between these possibilities, but have used the optimal level of 2.5 % in subsequent experiments.

Lack of response to epidermal growth factor (EGF) and transforming growth factor β (TGFβ)

Even in the presence of optimal levels of DTT-serum (2.5 %), the response to insulin plus PDGF remained less than achieved with serum. No further enhancement was obtained with up to 20 ng ml^{-1} of EGF (not shown), even though this factor has been widely reported to be mitogenic for other strains of 3T3 cells (e.g. see Dicker *et al.*

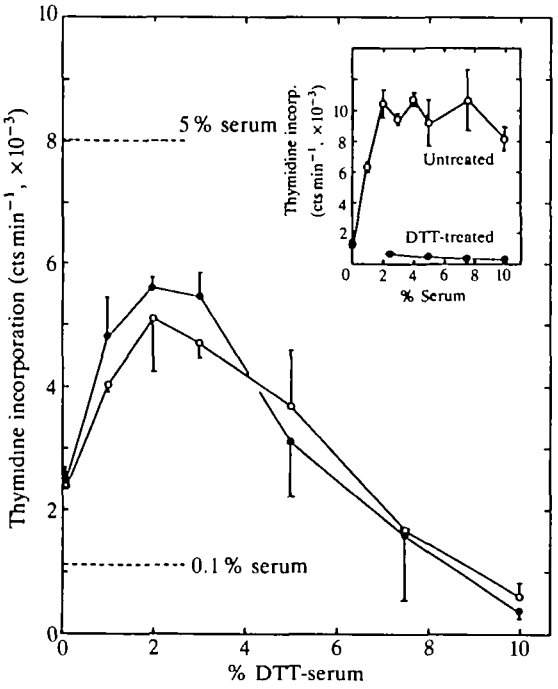

Fig. 2. Response of sparse quiescent 3T3 cells to PDGF (0.5 unit ml^{-1}) plus insulin (5 μg ml^{-1}) in the presence of various amounts of DTT-treated serum (two independent batches, different symbols). Thymidine incorporation was determined after 24 h. Each point is the mean of triplicate determinations, with the S.D. indicated by the vertical bars. The horizontal broken lines indicate the mean level of thymidine incorporation stimulated by 0.1 % or 5 % untreated serum (S.D. 66 and 1521 cts min^{-1}, respectively). Inset: stimulation of thymidine incorporation by either DTT-treated or untreated serum in sparse, quiescent 3T3 cells, determined after 24 h in triplicate. Points for both batches of DTT serum were superimposable.

1981; Leof *et al.* 1982; Richmond *et al.* 1984; Bravo *et al.* 1987). Similarly, no response has been noted with up to 3 ng ml^{-1} of TGFβ (not shown), a factor known to be present in serum, derived from platelets, and mitogenic for some fibroblast cell types (Sorrentino and Bandyopadhyay, 1989).

Requirement for hydrocortisone

Rather greater success was obtained with hydrocortisone. Glucocorticoids such as hydrocortisone have been previously shown to potentiate the response to fibroblast growth factor (FGF) (Holley and Kiernan, 1974; Shipley and Ham, 1983). As shown in Table 1, this is also the case for PDGF plus insulin. No effect was obtained with hydrocortisone alone, or in combination with either insulin or PDGF (Table 1). Only when both insulin and PDGF were present together did hydrocortisone further increase thymidine incorporation (Table 1). These experiments utilized a saturating level of hydrocortisone (1 μg ml^{-1}). In other experiments, the maximal response was shown to be obtained with 0.33 μg ml^{-1}. Interestingly, pretreatment with hydrocortisone for 22 h, followed by withdrawal at the time of transfer to PDGF plus insulin, was more effective than when the hydrocortisone was added with the PDGF plus insulin (Table 2). Evidently, the hydrocortisone need not be present simultaneously but is able to prime the cells in some way so as to promote their subsequent response to PDGF plus insulin.

Table 2. *Effect of pretreatment with hydrocortisone on the response of sparse, quiescent 3T3 cells prior to PDGF plus insulin*

	Thymidine incorporation per coverslip (cts min^{-1} ± S.D.)
5 % Serum control	7949±291
No additions	191±56
Insulin+PDGF	3790±154
Insulin+PDGF+hydrocortisone	4727±317
Insulin+PDGF only, after pretreatment for 22 h with hydrocortisone	5343±627

Except for the 5 % serum control, all cultures contained 2.5 % DTT-serum. Growth factors were added at the following concentrations: PDGF, 0.5 unit ml^{-1}; insulin, 5 μg ml^{-1}; hydrocortisone, 1 μg ml^{-1}. Thymidine incorporation was determined in triplicate after 24 h.

Requirement for cyclic AMP elevating agents

Rozengurt and colleagues have shown that elevation of the cytoplasmic concentration of cyclic AMP is an important element in the mitogenic response of another strain of Swiss 3T3 cells (Rozengurt *et al.* 1981). The effectiveness of PDGF as a mitogen on its own was reported to be due in part to its ability to raise cyclic AMP indirectly through the release of E type prostaglandins, most probably as a consequence of increased arachidonic acid mobilization (Rozengurt *et al.* 1983). Since these observations were made with density-arrested 3T3 cells, it seemed likely that prostaglandin accumulation might be inadequate in subconfluent cultures maintained at the high medium/cell ratios used here. We therefore examined the effect of supplementing the medium with prostaglandin E_1 (PGE_1). In the presence of insulin, PDGF and hydrocortisone, PGE_1 further stimulated thymidine incorporation, which reached levels comparable to 5 % serum at concentrations around 100 and 1000 ng ml^{-1} (Fig. 3A). In contrast, $PGF_{2\alpha}$ (which stimulates phosphatidyl inositol turnover rather than adenylate cyclase; Macphee *et al.* 1984) gave little consistent stimulation, and if anything was slightly inhibitory at the higher levels. Interestingly, the stimulation of thymidine incorporation by PGE_1 was not confined to sparse cells. Essentially identical results were obtained with density-arrested cells (Fig. 3B). This suggests that this particular clone of 3T3 may be less able to accumulate adequate levels of E-type prostaglandins, in response to PDGF, than the strain used by Rozengurt and colleagues.

The stimulation of thymidine incorporation by PGE_1 occurred in the presence of PDGF, or in combination with both hydrocortisone and insulin. On its own, PGE_1 was inactive (Table 1). That the effects of PGE_1 were due to an increase in cyclic AMP was suggested by the fact that similar results (not shown) have been obtained with cholera toxin (10^{-9} M optimum).

Requirement for EGF, lipoproteins and fibronectin for maximal cell growth

The combination of PDGF, insulin, hydrocortisone and PGE_1 in a background of 2.5 % DTT-serum, stimulated sparse, quiescent cells to a level comparable to 5 % serum, in a short-term (24 h) thymidine incorporation assay (Fig. 3A, Table 1). In contrast, cell multiplication over a 6-day period was very poor (Fig. 4). This was not due to growth factor depletion over the longer interval, since similar results were obtained by adding higher levels of growth factors, or by using an assay volume of 5 ml instead

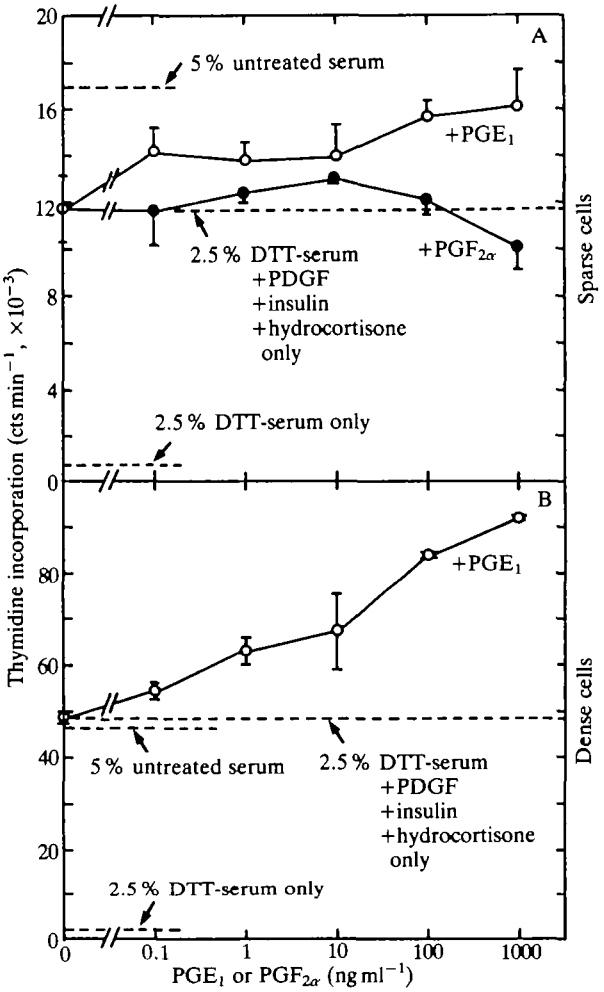

Fig. 3. Response of quiescent 3T3 cells to PGE_1 or $PGF_{2\alpha}$ in the presence of 2.5% DTT-serum+insulin (5 μg ml^{-1})+PDGF (0.5 unit ml^{-1}) and hydrocortisone (1 μg ml^{-1}). Thymidine incorporation was determined after 24 h in triplicate, with the S.D. indicated by the vertical bars. The horizontal broken lines indicate the mean level of thymidine incorporation in control cultures stimulated as indicated. A. Sparse quiescent cells; B, density-arrested cells.

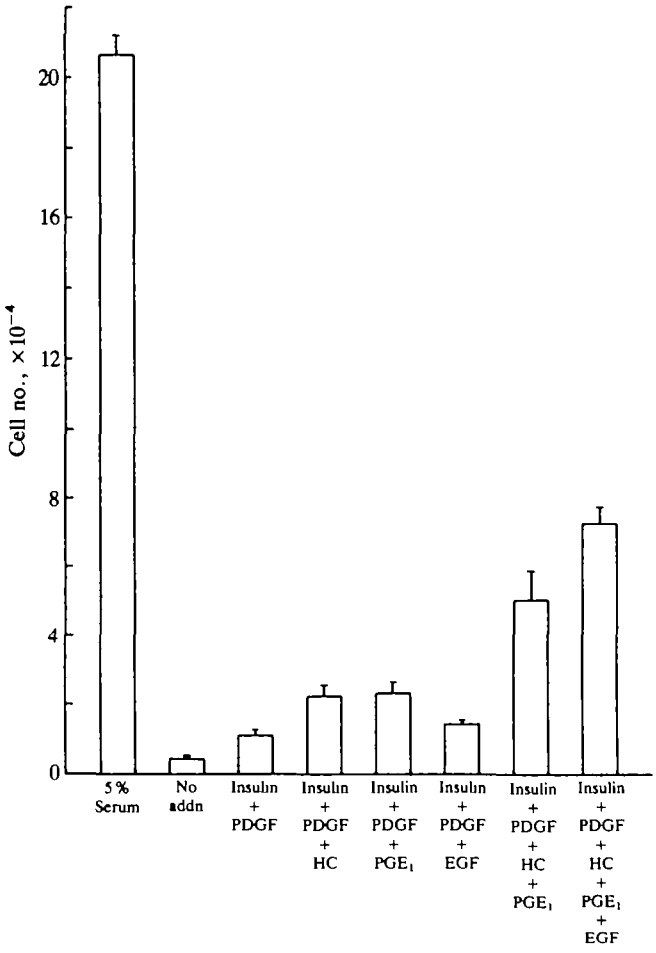

Fig. 4. Cell multiplication in response to insulin (5 μg ml^{-1}), PDGF (0.5 unit ml^{-1}), hydrocortisone (HC; 1 μg ml^{-1}) PGE_1 (1 μg ml^{-1}) and EGF (20 ng ml^{-1}). Except for the control (5% serum), all media contained 2.5% DTT serum, human transferrin (5 μg ml^{-1}), inosine (25 μM) and thymidine (1 μM). Cells were seeded into 16 mm diameter wells of a 24-well plate at 10^4/well, in 1 ml of 0.5% serum, and switched to test conditions after 24 h (=day 0). Cell number was determined in triplicate 6 days later, with medium change on day 3. Vertical bars indicate the S.D.

of the usual 1 ml (not shown). Some slight additional stimulation was obtained by adding EGF (despite the previous lack of response in the short-term thymidine incorporation assay), so EGF is now added routinely. Even so, cell growth remained far less than attained with 5% serum.

Heath and Deller (1983) have developed a serum-free medium for the growth of mouse embryonal carcinoma cells, in which low-density lipoprotein (LDL) and high-density lipoprotein (HDL) from human plasma were essential components, together with the use of fibronectin-treated dishes. Van Zoelen *et al.* (1985) reported that DTT-serum contains substantial quantities of cholesterol and other lipids, as well as cell attachment factors, and we did not therefore anticipate any benefit from further supplementation with LDL, HDL or fibronectin. Nevertheless, addition of LDL (100 μg protein ml^{-1}) and HDL (50 μg protein ml^{-1}) to the growth factor cocktail (PDGF, insulin, EGF, hydrocortisone and PGE_1) significantly improved growth, even in the presence of 2.5% DTT-serum (Fig. 5). Significant stimulation was also obtained with the addition of fibronectin at 5 μg ml^{-1} (Fig. 5) (see also Shipley and Ham, 1983). Moreover, LDL, HDL and fibronectin together gave growth comparable to, or even greater than, that in 5% serum (Fig. 6). As can also be seen in Fig. 6, the optimal level of DTT-serum remained at 2.5% (Fig. 6; cf. Fig. 2), even in the presence of lipoproteins and fibronectin. This suggests that the DTT-serum is supplying something other than lipid and cell attachment factors.

In other experiments (not shown), the optimal level of LDL was found to be in the range of 50–100 μg protein ml^{-1}, and for HDL, 50 μg protein ml^{-1}. Of the two, LDL gave the greater stimulation. Broadly similar results were obtained with commercial samples of LDL (from Sigma) and freshly prepared LDL, though in general the freshly prepared material was more active. In preliminary experiments, acetylation of LDL, which destroys its capacity to bind to the LDL receptor without affecting its lipid content (Basu *et al.* 1976), was found to eliminate the stimulation of growth.

Finally, it should be noted that the growth experiments reported in Figs 4–6 were conducted in the presence of human transferrin (5 μg ml^{-1}). Transferrin has been

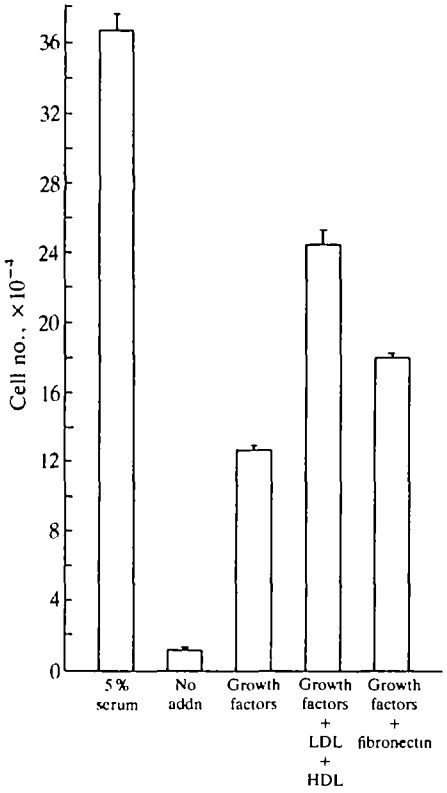

Fig. 5. Effect of LDL (100 μg protein ml^{-1})+HDL (50 μg protein ml^{-1}) and fibronectin (5 μg ml^{-1}) on the multiplication of 3T3 cells. Except for the 5 % serum control, all media contained 2.5 % DTT-serum, human transferrin (5 μg ml^{-1}), inosine (25 μM) and thymidine (1 μM). Growth factors: insulin (5 μg ml^{-1})+PGDF (0.5 unit ml^{-1})+EGF (20 ng ml^{-1})+hydrocortisone (1 μg ml^{-1})+PGE$_1$ (1 μg ml^{-1}). Cells were seeded into 16 mm diameter wells of a 24-well plate at 10^4/well, in 1 ml of 0.5 % serum, and switched to test conditions after 24 h (=day 0). Cell number was determined in triplicate 6 days later, with medium change on day 3. Vertical bars indicate the S.D.

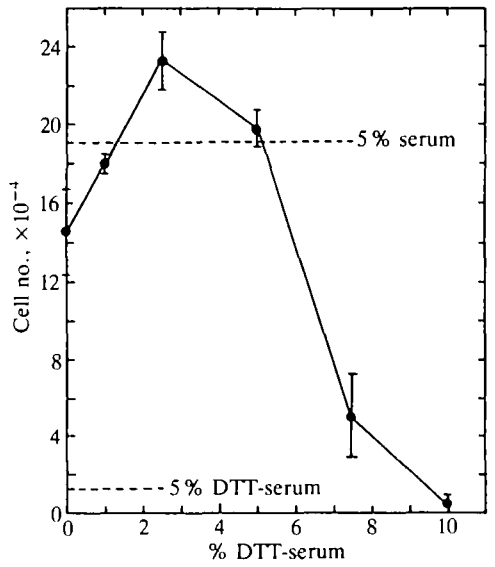

Fig. 6. The effect of DTT-serum on the multiplication of 3T3 cells stimulated by the following: PDGF (0.5 unit ml^{-1}), insulin (5 μg ml^{-1}), EGF (20 ng ml^{-1}), hydrocortisone (1 μg ml^{-1}), fibronectin (5 μg ml^{-1}), LDL (50 μg protein ml^{-1}), HDL (50 μg protein ml^{-1}), and human transferrin (5 μg ml^{-1}), inosine (25 μM) and thymidine (1 μM). Cells were seeded into 16 mm diameter wells of a 24-well plate at 2.5×10^4/well, in 1 ml of 0.5 % serum, and switched to test conditions after 24 h (=day 0). Cell number was determined in triplicate 6 days later, with a medium change on day 3. Vertical bars indicate the S.D. The horizontal broken lines represent the cell number in control cultures stimulated with 5 % untreated or DTT-treated serum.

widely reported to be an essential component of serum-free media (Barnes and Sato, 1980) and we have confirmed that its omission does impair the growth response (not shown).

Discussion

PDGF, on its own, has been reported to be an effective mitogen for quiescent, density-arrested Swiss 3T3 cells. Its activity is potentiated by high levels of insulin (which alone are inactive), resulting in a stimulation of DNA synthesis as great as that obtained with serum (Dicker *et al.* 1981; Lopez-Rivas *et al.* 1984; Rozengurt, 1986; Bravo *et al.* 1987). We show here that this is not the case for *subconfluent* quiescent cells plated in low-serum medium. Under these conditions, PDGF is a relatively poor mitogen. With sparse 3T3 cells, insulin alone is at least as effective as PDGF, and frequently more so. When added together, the maximal response is no more than additive at best, and in many experiments we found no further stimulation of DNA synthesis over that obtained with insulin alone. Furthermore, the maximal response to PDGF plus insulin is usually far less than 50 % of that seen with an optimal level of serum (5 %).

We have made some progress in identifying other factors required for the proliferation of sparse 3T3 cells. In addition to PDGF plus insulin, maximal stimulation of DNA synthesis requires the presence of hydrocortisone, a cyclic AMP elevating agent such as PGE$_1$ or cholera toxin, and the inclusion (at 2.5 %) of serum treated with DTT so as to destroy the majority of its content of peptide growth factors. Together, these supplements stimulated thymidine incorporation (in a 24 h assay) to a level comparable to that achieved with 5 % serum. Despite this, cell multiplication over a 6-day period remained poor. (This was not due to an absence of transferrin, since this was added routinely.) Slight improvement was seen on adding EGF (despite the lack of response in the short-term thymidine incorporation assay), but much greater effects were obtained with fibronectin and the lipoproteins HDL and LDL (especially the latter). A similar requirement for lipoproteins and fibronectin was reported by Heath and Deller (1985) for PC13 mouse embryonal carcinoma cells.

The nature of the components provided by the DTT-serum is, at present, unknown. The very sharp optimum at 2.5 % most likely indicates the presence of both stimulatory and inhibitory factors. It is conceivable that the latter may have been generated by the treatment with DTT and iodoacetimide, though serum is known to contain growth inhibitors for a variety of cell types (Wille *et al.* 1984; Wier and Scott, 1986; Loo *et al.* 1987). It is possible that these may have been preferentially unmasked by the DTT treatment as a result of elimination of other stimulatory or compensatory activities. If so, then the DTT-serum might be a convenient starting point for their further characterization. As for the stimulatory component(s), the original rationale for including DTT-serum was to provide a source of all those components normally

supplied by serum other than peptide growth factors. Of these, we expected lipid to be one of the most important (Ham, 1981). We were therefore initially surprised to find that the DTT-serum did not eliminate a requirement for LDL and HDL. However, the LDL content of newborn calf serum at 2.5% may be less than the optimal level of 50–100 μg protein ml^{-1} required. In addition, preliminary experiments have indicated that acetylation of LDL, which destroys its capacity to bind to the LDL receptor without affecting lipid content (Basu *et al.* 1976), eliminates the stimulation of growth. This suggests that the uptake of lipid may depend primarily upon a receptor-mediated process, and it is conceivable that the lipid content of DTT-serum is unavailable to the cells. Be that as it may, it is probable that the DTT-serum is contributing something other than lipid. It is clearly not one of the 'well-known' peptide growth factors, since these are sensitive to reduction, though there is at least one platelet-derived factor active on endothelial cells, which is resistant to DTT (Miyazono *et al.* 1987). Further work is required to clarify the matter.

In view of the variability noted in the response of sparse 3T3 cells to PDGF, insulin and 1% serum, the effects of hydrocortisone are interesting. We had previously found that overnight exposure to 10% serum enhanced the effects of PDGF (unpublished observations), suggesting that responsiveness depended on some cellular state induced (to varying degrees) by previous exposure to high levels of serum. Pretreatment with hydrocortisone similarly increased the responsiveness of the cells to subsequent challenge with PDGF plus insulin (Table 2). Whether the effects of pretreatment with serum can be ascribed to its glucocorticoid content remains to be seen.

As for the nature of the state induced by hydrocortisone pretreatment, this might reflect an elevated expression of PDGF receptors or some other element in the mitogenic pathway. Alternatively, hydrocortisone might activate an autocrine loop by switching on the production of endogenous growth factors, which augment the response to added PDGF.

Regardless of the mechanism of action, the long-term memory of exposure to hydrocortisone clearly demonstrates that responsiveness to growth factors is not an absolute property of the cells but depends on their previous culture history. In this context, the difference between sparse quiescent cells and density-arrested cells in responsiveness to PDGF and insulin is not too surprising. The former are produced by plating trypsinized cells directly in low serum (0.5% or less) whereas the latter become quiescent partly through cell crowding (density inhibition) and partly through depletion of (initially) high levels of serum. It is unlikely that this depletion affects all serum growth factors equally. Consequently, at the time they are taken, density-arrested cells will be bathed in a complex residual mixture (and subset) of serum-derived growth factors. The suggestion that PDGF *alone* is sufficient to stimulate DNA synthesis in (density-arrested) Swiss 3T3 cells in the absence of any other factor (Rozengurt, 1986) is therefore a simplification in need of careful qualification.

Finally, the experiments reported here were conducted using Dulbecco's modification of Eagle's medium (DMEM), which is widely used for the culture of this cell type. We have previously found this to be superior to a 1:1 mixture of DMEM and F12 favoured by others (e.g. see Barnes and Sato, 1980) in attempts to replace serum (Brooks and Riddle, 1988*a*). Nevertheless, DMEM is not optimized for 3T3 cells and it is possible that MCDB 402 (developed specifically for 3T3 cells; see Shipley and Ham, 1983) will support better long-term growth under serum-free conditions. Preliminary experiments using MCDB 402 have not so far revealed any major improvements (except at very low cell densities) but this needs to be examined more closely.

In summary, we have established that sustained proliferation of Swiss 3T3 cells depends not only on PDGF and insulin, but also requires the presence of hydrocortisone, a cyclic AMP elevating agent such as PGE_1, EGF, LDL, HDL, fibronectin and an unidentified component present in DTT-treated serum. The individual effect of each of these factors is relatively small. It is therefore unlikely that the proliferative heterogeneity shown by individual cells at low serum concentrations (Brooks *et al.* 1984; Brooks, 1985; Brooks and Riddle, 1988*b*) is the consequence of a deficiency of any single factor. Nevertheless, we are now in a position to address the question of how these factors interact to determine why some cells proliferate whilst others do not in an otherwise homogeneous environment.

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