

# Coupling of Preadipocyte Growth Arrest and Differentiation.

## I. Induction by Heparinized Medium Containing Human Plasma

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**ABSTRACT** The differentiation of preadipocytes *in vitro* typically required prolonged culture of cells at a high density in high concentrations of serum and added hormones. With such culture conditions it is difficult to design experiments to determine the mechanisms that control the differentiation process. We now describe the rapid and parasynchronous growth arrest and differentiation of low density murine preadipocytes in heparinized medium containing only human plasma. When low density cells are cultured under these conditions, growth arrest at a distinct state in the  $G_1$  phase of the cell cycle occurs within 2 d and the differentiation of 80–100% of the cell population occurs within 4 d thereafter. The factors in human plasma which promote growth arrest and differentiation are heat labile and can be separated by barium adsorption. In the following paper we have used these methods to show that there are five separate phases which regulate the coupling of preadipocyte growth arrest and differentiation. The data reported in this paper establish that: (a) high cell density and extensive cell-to-cell contact are not required for adipocyte differentiation, (b) prolonged culture is not required for adipocyte differentiation, and (c) high concentrations of serum and/or added hormones are not required for adipocyte differentiation.

Cell differentiation is a biological process that results in the expression of stable phenotypic changes in cells. Cell differentiation is typically preceded by growth arrest in the  $G_1$  phase of the cell cycle. For example, *in vitro* preadipocytes (8, 9, 25, 26) and myoblasts (22) growth arrest in  $G_1$  several days before the expression of the differentiated phenotype. *In vivo*, the differentiation of erythrocyte precursors (18) and epithelial precursor cells of the skin (16) also occurs in association with a loss in proliferative capacity and growth arrest in the  $G_1$  phase of the cell cycle.

The *in vitro* growth arrest of undifferentiated cells at  $G_1$  states induced by the deprivation of growth factors or nutrients has previously been suggested to be critically important in the physiological regulation of cell proliferation (2, 23, 29). There is, however, a paucity of data to support the conclusion that deprivation of growth factors or specific nutrients actually influences the control of proliferation of most stem cells *in vivo*. The control of cell proliferation mediated by the coupling of  $G_1$  growth arrest and differentiation appears to be physiologically more relevant (16, 18). We have, therefore, studied

the mechanisms that control the coupling of preadipocyte growth arrest and differentiation. We reported that the coupling process in preadipocytes is mediated at a distinct state in the  $G_1$  phase of the cell cycle, designated  $G_D$  (26). Attempts to delineate in more detail the events that control the coupling process have, however, been limited by technical difficulties. This is so because the induction of adipocyte differentiation by standard methods requires the culture of cells to be at a high density for prolonged intervals, in medium containing high concentrations of the pathological fluid serum and/or supra-pharmacological concentrations of insulin and other hormones (5, 10–12). With such culture conditions, the differentiation process also occurs slowly, is nonsynchronous, and is extremely difficult to modulate experimentally.

We now describe the induction of  $G_D$  arrest and differentiation of low density preadipocytes in the absence of serum and added hormones. The data show that the culture of preadipocytes in heparinized medium containing only human plasma induces rapid and parasynchronous  $G_D$  arrest and adipocyte differentiation. We also describe specific human plasma frac-

tions which have distinct effects on the coupling process. In the accompanying paper (27) we have used this culture system to obtain experimental evidence to support a model that establishes that there are five distinct phases involved in the coupling of proadipocyte growth arrest and differentiation.

## MATERIALS AND METHODS

### Cell Culture and Cell Differentiation

3T3 T proadipocytes (gift of D. L. Diamond, University of Pennsylvania, Philadelphia, PA) were the principal cell line used in this study. They were derived from the clone A31 BALB/3T3 cell line and have been previously characterized (5). Stock cultures of 3T3 T proadipocytes were grown at 37°C in 5% CO<sub>2</sub> atmosphere in Dulbecco's modified Eagle's medium containing 4.5 g/L glucose (DME) (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (KC Biological, Inc., Lenexa, KS). Stock cultures were cultured at low confluence to prevent selection for cells capable of proliferation at high cell densities and in antibiotic free medium to prevent unsuspected microbial contamination. 3T3 T and other cell lines used in this study were indeed found to be free of mycoplasma contamination by cultural analysis and by the staining method of Chen (4). New stock cultures of 3T3 T proadipocytes were recovered from liquid nitrogen storage every 8–10 wk. Swiss 3T3-L1 cells (gift of H. Green, MIT, Boston, MA) were also used; they have characteristics comparable to BALB/3T3 T cells in that they can differentiate into adipocytes *in vitro* (10–12).

A variety of other cell lines that cannot differentiate under physiological conditions were also used. These included BALB/3T3 T (clone A31) cells and simian virus 40 transformed and methylcholanthrene transformed BALB/3T3 cells. These cell lines were kindly provided by Dr. George Todaro, National Institutes of Health, Frederick, MD. Nontransformed C3H/10T½ cells were provided by Dr. Harold Moses, Mayo Clinic, Rochester, MN. Limited studies were also performed on primary cultures of adult human and mouse skin fibroblasts and three human embryo lung-derived cell strains, i.e., WI 38, MRC-5, and HEL-299 (American Type Culture Collection, Rockville, MD). The above cells were grown in either DME containing 10% calf serum, or in 10% fetal calf serum as described above. Passage numbers of the cell strains were as follows: WI 38, passage 13–17; MRC-5, passage 17–20; and HEL-299, passage 6–10.

Preparation of cells for differentiation assays involved passage of rapidly growing cells at high split ratios so that low density cultures resulted. Cells were then grown for 1–3 d or until they reached a density of  $\sim 1 \times 10^4$  cells/cm<sup>2</sup>. These rapidly growing cells were then fed heparinized DME containing human plasma. Heparin, sterile, was purchased from Abbott Diagnostics, Diagnostic Products, North Chicago, IL. They were assayed for adipocyte differentiation over a two-wk interval, during which time they were repeatedly fed at 4-d intervals. In selected studies high density cells were also used. Various modifications of this protocol were also tested (see Results). The concentration of plasma was varied from 5 to 25% and the concentration of heparin was varied from 1 to 150 U/ml. Some plasma preparations were heat-inactivated at 60° for intervals up to 3 h and some preparations were adsorbed with barium chloride as detailed below. Serum was prepared from blood which was drawn and allowed to clot in the absence of an anticoagulant.

Various terms are used in this paper that describe the densities of cells at different states. The approximate number of cells/cm<sup>2</sup> at these states is indicated in the following summary: low density (0.5 to  $1 \times 10^4$  cells/cm<sup>2</sup>); density at which significant cell-to-cell contact occurs ( $\sim 2 \times 10^4$  cells/cm<sup>2</sup>); confluence 4–5  $\times 10^4$  cells/cm<sup>2</sup>; high density differentiated cultures (6–8  $\times 10^4$  cells/cm<sup>2</sup>).

### Preparation and Fractionation of Plasma

Plasma from humans and from a variety of animal species was prepared as follows. Venous blood was drawn and immediately placed in sodium citrate (final concentration 0.38%). Citrate-anticoagulated blood was sedimented by centrifugation at 1000 g for 30 min at 22°C. The supernatant was again sedimented by centrifugation at 25,000 g for 30 min at 22°C to remove platelets. The citrate anticoagulated platelet poor plasma was frozen at –70°C overnight and subsequently thawed slowly at 4°C. It was sedimented by centrifugation at 1000 g for 10 min at 4°C and then stored at 4°C following Millipore filtration (Millipore Corp., Bedford, MA). To prepare medium containing plasma, DME (minus an appropriate volume) was heparinized at the desired concentration, and then the citrate anticoagulated plasma was added. This medium was used to induce proadipocyte differentiation.

Various plasma preparations were tested for differentiation-promoting activity. In general, freshly drawn platelet-poor plasma was more effective. The activity of such specimens was stable for between 1 and 6 months at 4°C. Outdated plasma and platelet-rich plasma, however, were less potent promoters of differ-

entiation because they induced significant cell proliferation as a result of the release of platelet growth factors.

In selected studies fractionated human plasma was used. Fractionation was achieved using a modification of the method described by Mann (20). BaCl was added to citrate-anticoagulated human plasma (pH 7.8) to a final concentration of 0.1 M. This was mixed for 30 min at 4°C and sedimented by centrifugation at 3600 g for 25 min. The supernatant was saved and further processed as described below. The barium citrate sediment was partially solubilized with one-third vol of 0.9% NaCl-0.02 M sodium citrate. This suspension was separated by centrifugation at 3600 g for 25 min at 4°C. The supernatant fraction was designated CEP i.e., citrate eluate of barium-precipitated plasma. The residual nonsolubilized barium citrate cake was subsequently completely solubilized by addition of 0.2 M EDTA (pH 7.4) at one third the original plasma volume. This fraction was designated EDTAP. The CEP and EDTAP fractions were extensively dialyzed against 6.8 mM sodium citrate (pH 7.4), reconcentrated to the original volume, and stored at 4°C.

The supernatant of the initial barium adsorption described above was further fractionated. First, 20 ml of 3.8% sodium citrate was added to 200 ml of once-adsorbed plasma. Barium chloride was then added to this suspension at a final concentration of 0.2 M. This suspension (pH 7.0) was mixed for 30 min at 4°C then separated by centrifugation as above. The supernatant represents twice barium-adsorbed plasma; the sediment was discarded. Twice-adsorbed plasma was then adjusted to pH 8.7 with 1 N NaOH to induce precipitation. The precipitate was removed by centrifugation and the resulting supernatant was designated barium adsorbed plasma (BaP). It was extensively dialyzed as above, concentrated to its original volume, and stored at 4°C. The mean concentration of protein plus or minus standard deviation of the plasma preparations used in this study was: whole plasma, 57.8  $\pm$  0.4 g/L; BaP, 47.8  $\pm$  18.1 g/L; CEP, 9.9  $\pm$  2.9 g/L; and EDTAP, 1.5  $\pm$  0.6 g/L.

### Differentiation Assays

Adipocyte differentiation was routinely quantitated morphologically. Using phase microscopy, cells were designated to show adipocyte differentiation if they contained numerous large, golden, refractile cytoplasmic lipid droplets, which could be stained red with the oil red "O" dye. An enzyme assay of adipocyte differentiation was also used. Lipoprotein lipase activity in the medium in which proadipocytes or adipocytes were cultured was assayed by a previously described procedure (7). Protein assays were performed by the method of Lowry (19) using crystalline bovine serum albumin as a standard.

### Cell Proliferation Assays

Two methods were used to measure the extent of DNA synthesis in cell cultures. This served to establish the extent of cell proliferation as a function of S-phase cell cycle traverse. Both measurements employed incubation of cells in [<sup>3</sup>H]thymidine. In the rapid method, cells were exposed to [<sup>3</sup>H]thymidine for 1 h and the amount of incorporation was quantitated by scintillation spectroscopy after TCA precipitation of DNA by a standard procedure (3). Autoradiographic analysis was also used to determine the percentage of a cell population that underwent DNA synthesis during a given interval. For these studies cells were exposed to [<sup>3</sup>H]thymidine for 24 or 48 h and then processed for light microscopic autoradiography as previously described (3), except that the specimens were fixed in formaldehyde-glutaraldehyde instead of methyl alcohol. The concentration of [<sup>3</sup>H]thymidine added to the cell culture medium for autoradiographic analysis was 5  $\mu$ Ci/ml, whereas for scintillation spectroscopic analysis 1  $\mu$ Ci/ml was used. The specific activity of [<sup>3</sup>H]thymidine was 52.2 Ci/mmol (New England Nuclear, Boston, MA).

### Flow Microfluorimetric Analysis of Cell Cycle Distribution

Cell monolayers were trypsinized to produce a single cell suspension. To this suspension were then added DNAase, RNAase, and soybean trypsin inhibitor as previously described (24). Treated cells were sedimented by centrifugation, resuspended in a small volume of saline, and fixed in ethanol. They were stained with mithramycin (100  $\mu$ g/ml) and analyzed in a Becton-Dickinson fluorescence activated cell sorter (FACS-IV, Becton, Dickinson & Co., Rutherford, NJ). The percentage of cells in G<sub>1</sub>, S, or G<sub>2</sub>/M was determined as previously described (24).

## RESULTS

### Differentiation of Low Density 3T3 T Proadipocytes

#### Culture of low density 3T3 T proadipocytes in heparinized

DME containing 25% human plasma without other additives induces the differentiation of ~90% of the cell population. Figs. 1 and 2 illustrate the morphology of undifferentiated and differentiated low density proadipocytes, respectively. Differentiated adipocytes contain large droplets in the cell cytoplasm, which displaced the nucleus in some cells to give a "signet ring" morphology.

The kinetics of differentiation induced by heparinized DME containing human plasma are shown in Fig. 3. Within 2 d after addition of this medium to low confluence rapidly growing cells, growth arrest occurs as demonstrated by autoradiographic and spectroscopic analysis showing markedly decreased [<sup>3</sup>H]thymidine incorporation into DNA. Greater than 90% of such cells also have a 2 N content of DNA as determined by flow microfluorimetric analysis, and are therefore arrested in the G<sub>1</sub> phase of the cell cycle (Table I). We have designated this predifferentiation G<sub>1</sub> growth arrest state G<sub>D</sub> and we have previously shown that G<sub>D</sub> is a distinct G<sub>1</sub> arrest state that precedes adipocyte differentiation (26). Approximately 2 d after G<sub>D</sub> arrest, the first expression of the adipocyte phenotype developed, as evidenced by the accumulation of intracellular lipid droplets and increased activity of lipoprotein lipase (Figs. 3, 4). The data in Fig. 3 show that once the expression of the differentiated phenotype is initiated, it proceeds rapidly in a parasynchronous manner so that near maximum differentiation is observed ~4 d after G<sub>D</sub> arrest (Fig. 4).

These results establish that parasynchronous differentiation of 3T3 T proadipocytes occurs in low density cell cultures where few cell-to-cell contacts exist. In fact, we found that adipocyte differentiation occurred most rapidly and to the greatest extent in very low density cells cultured in heparinized

DME containing human plasma. For example, 80–100% differentiation occurred in cultures at a density of  $\sim 1 \times 10^4$  cells/cm<sup>2</sup>, whereas at a density of  $2\text{--}4 \times 10^4$  cells/cm<sup>2</sup> maximum differentiation was reduced to between 40 and 60%. The data also show that prolonged culture is not required for adipocyte differentiation.

Optimum differentiation was typically obtained in DME containing 15–30 U/ml heparin and 20–25% human plasma. To determine the dose-response effect of heparin and human plasma on proadipocyte differentiation, a series of studies were performed. If the heparin concentration was reduced to the minimum required for anticoagulation, i.e., 1–5 U/ml, a significant reduction in differentiation resulted. If the concentration of heparin was increased to >75 U/ml, decreased differentiation resulted. The latter was associated with increased

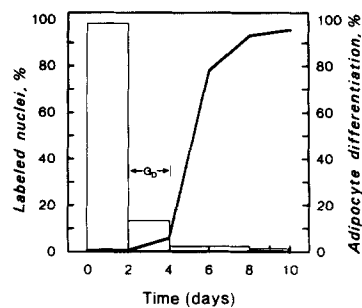
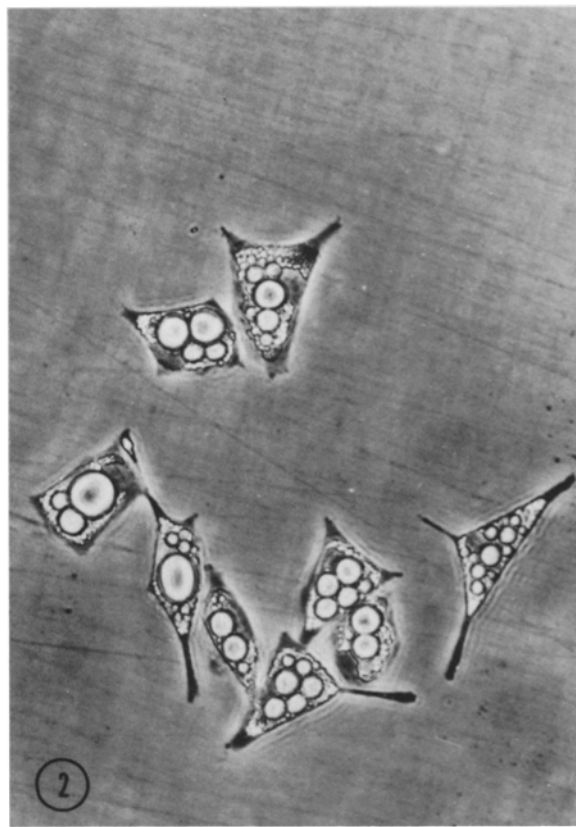
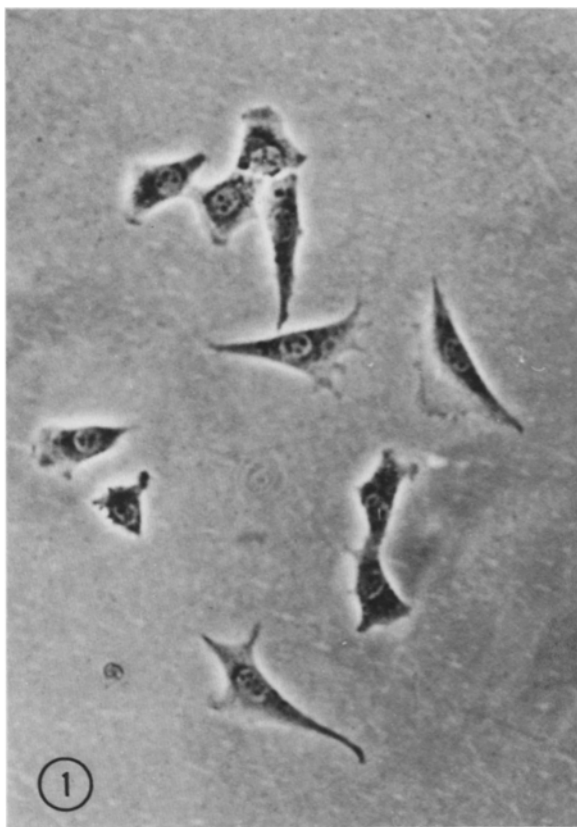


FIGURE 3 Kinetic analysis of growth arrest and differentiation of 3T3 T proadipocytes cultured in heparinized DME containing 25% human plasma. Within 2 d after addition of this medium, cell growth is markedly decreased, as evidenced by the small fraction of cells that incorporate <sup>3</sup>H-thymidine into

DNA (bar). Such predifferentiated arrested cells are designated to be in the G<sub>D</sub> state. After G<sub>D</sub> arrest, adipocyte differentiation typically begins within 2 d and reaches a near maximum extent within 4 d thereafter (—).



FIGURES 1 and 2 Photomicrographs of undifferentiated (Fig. 1) and differentiated (Fig. 2) low density 3T3 T cells. Differentiation of low density cultures was induced by culture in heparinized medium containing 25% human plasma. Differentiated cells (Fig. 2) contain multiple large cytoplasm lipid droplets that caused displacement of the nucleus.  $\times 400$ .

cytotoxicity. If the plasma concentration was reduced to 5% or less, no significant differentiation was observed. An intermediate level of differentiation was observed in the medium containing 10–15% plasma. Cytotoxicity resulted when the plasma concentration was increased to >30%. These results were obtained using freeze-thawed plasma that was less toxic than untreated plasma, and in the optimum concentration of the factor not being tested.

### Specificity of the Differentiation-promoting Activity of Heparinized DME Containing Human Plasma

Heparinized DME containing human plasma will only promote the morphological differentiation of cell lines previously shown to be capable of expressing the adipocyte phenotype *in vitro* under standard culture conditions. Results of studies not illustrated show that 3T3 T and 3T3 L1 proadipocytes differentiate in heparinized DME containing human plasma but that nontransformed BALB/c 3T3 and C3H/10T $\frac{1}{2}$  cells and chemically and virally transformed BALB/c 3T3 cells do not. L<sub>8</sub> myoblasts, human embryo lung cells (WI38, MRC-5, or HEL-299), and primary cultures of adult human and mouse skin fibroblasts also failed to morphologically differentiate into adipocytes in this medium. In these studies the concentration of plasma was 25% and the concentration of heparin in the culture medium was 30 U/ml.

These observations support the conclusion that plasma contains components that specifically induce the G<sub>0</sub> arrest and adipocyte differentiation of 3T3 T and 3T3 L1 proadipocytes.

### Differentiation-promoting Effect of Other Biological Fluids

Human amniotic fluids and ovarian cyst fluids were also

TABLE I

Flow Microfluorimetric Analysis of the Cell Cycle Distribution of Rapidly Growing Proadipocytes and Proadipocytes Cultured in Differentiation-promoting Medium

Cell growth characteristics*	Cell cycle distribution		
	G <sub>1</sub>	S	G <sub>2</sub> /M
	% cells		
Rapidly growing (DME-10% FCS)	55	35	10
G <sub>0</sub> arrested‡ (H-DME-25% HP)	95	2	3

\* Abbreviations: DME, Dulbecco's modified Eagle's medium; H-DME, heparinized DME; FCS, fetal calf serum; and HP, human plasma.

‡ Results obtained after culture in differentiation-promoting medium for 5 d. Comparable results were obtained after 10 d.

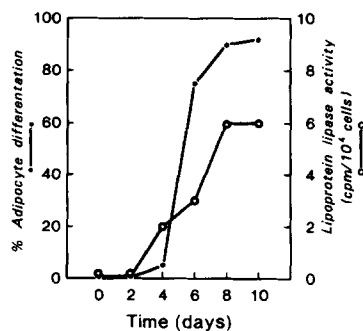


FIGURE 4 Correlation of the kinetics of differentiation of proadipocytes using a morphological assay (●) and an enzyme assay for lipoprotein lipase activity (○). The results show that, by both assays, differentiation is initiated approximately 2 d after G<sub>0</sub> arrest and near maximum differentiation occurs within 4 d after G<sub>0</sub> arrest.

TABLE II

Differentiation of Proadipocytes in Biological Human Fluids

Additives to heparinized DME	Adipocyte differentiation
Human plasma	90%
Human amniotic fluid:	
specimen 1	0%
specimen 2	0%
Human ovarian cyst fluid:	
specimen 1	0%
specimen 2	0%
specimen 3	0%

Heparinized DME containing 25% human plasma was used as the control differentiation-promoting medium. Amniotic fluid from two patients and ovarian cyst fluid obtained from three patients were used at concentrations of 10 and 25% in heparinized DME. All specimens were fed fresh medium at four-d intervals for 12 d and differentiation was assayed over an interval of three wk.

TABLE III

Comparative Effect of Plasma from Different Species on Adipocyte Differentiation

Plasma additives to heparinized DME	Adipocyte differentiation
Human	95%
Fetal calf	85%
Baboon	5%
Guinea pig	0%
Rabbit	5%
Calf	5%

Mouse and cat plasma preparations were also tested, but both were markedly cytotoxic to 3T3 T proadipocytes. Proadipocytes incubated in heparinized DME containing 5, 10, or 20% cat plasma died within 12–24 h after exposure without evidence of differentiation. Most proadipocytes cultured in heparinized DME containing 10–20% mouse plasma also died within 24–30 h, but such cells did show evidence of some lipid accumulation. The morphology of such cells was, however, significantly different than that observed in human and fetal calf plasma. Heparinized DME containing mouse plasma induced only the formation of multiple small fine oil red "O" staining granules rather than signet-ring type granules. Furthermore, mouse plasma induced similar toxic granulation in a variety of other cell lines and strains including L<sub>8</sub> myoblasts, human embryonic lung cells, and primary cultures of human skin fibroblasts. Mouse serum also nonspecifically induced toxic granulation in many cell types.

tested for their ability to induce adipocyte differentiation of 3T3 T proadipocytes. The data in Table II show that no evidence of adipocyte differentiation was observed when two different amniotic fluids and three different ovarian cyst fluids were tested. These data suggest that heparin is not the sole component required for differentiation and that components that induce adipocyte differentiation are not present in all biological human fluids.

To determine if plasma derived from other animals also promotes adipocyte differentiation, we tested plasma obtained from the following animals: baboon, guinea pig, fetal calf, calf, rabbit, cat, and mouse. The data in Table III show that of these, only heparinized DME containing human or fetal calf plasma promotes adipocyte differentiation. Because of the extreme difficulty in obtaining heparinized fetal calf plasma, all subsequent studies used human plasma preparations.

### Properties of the Differentiation-promoting Component(s) of Human Plasma

Studies were next performed to establish the characteristics of the differentiation-promoting components of human plasma. Fig. 5 shows that the activity of human plasma is destroyed by

clotting induced during the preparation of serum. In particular, heparinized and nonheparinized DME containing 10–30% human serum are shown to be ineffective differentiation-promoting agents. Comparable results were obtained when heparinized and nonheparinized DME containing 10–30% fetal calf serum were tested. Variation in the concentration of heparin (5–75 U/ml) in the medium did not influence the ability of proadipocytes to differentiate in either human or fetal calf serum (data not illustrated). Clotting that occurs during the preparation of serum, therefore, either destroys differentiation-promoting components of plasma or causes the generation of inhibitors of differentiation.

The differentiation-promoting activity of human plasma was also markedly effected by heat. Heating heparinized DME containing human plasma or heating citrate anticoagulated plasma prior to addition to heparinized DME at 60°C for intervals as brief as 1 h inactivates ~70% of its differentiation-promoting capacity. Heat inactivation for 3 h at 60°C destroyed >99% of the differentiation-promoting activity. By contrast, freeze-thawing human plasma either had no effect or slightly augmented its differentiation-promoting activity.

The data in Fig. 6 and in the following paper (27) suggest that the differentiation-promoting components of human plasma are proteins or polypeptides. In this paper we show that the differentiation-promoting plasma components are removed by barium adsorption performed by a slight modification of a previously described method (20). Three fractions can be obtained by this procedure (see Materials and Methods): barium-adsorbed plasma (BaP), a citrate eluate of barium precipitated plasma proteins (CEP), and an eluate of barium-precipitated plasma proteins not soluble in citrate but soluble in ethylenediaminetetraacetic acid (EDTAP).

Fig. 6 also shows that neither BaP, CEP, nor EDTAP fractions alone induced significant differentiation when they were added to low density, rapidly growing proadipocytes in heparinized DME. Even when cells were fed these fractions repeatedly and observed over a 21-d interval, no significant differentiation was observed. Increasing the protein concentra-

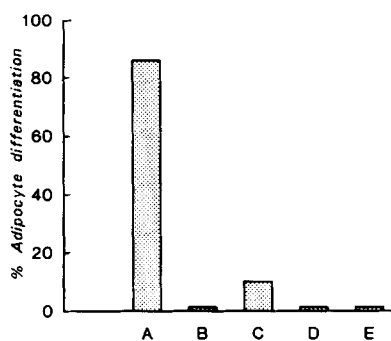


FIGURE 5 Comparison of the differentiation-promoting effect on BALB/3T3 T proadipocytes of: heparinized DME containing human plasma (fetal calf plasma gave similar results) (A), DME containing human serum (B), heparinized DME containing human serum (C), DME containing calf or fetal calf serum (D) or heparinized DME containing calf or fetal calf serum (E).

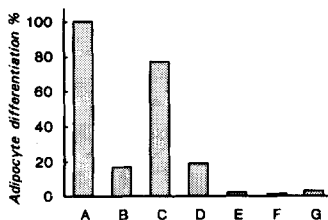


FIGURE 6 Analysis of the differentiation-promoting capacity of human plasma or plasma fractions prepared in heparinized DME for 3T3 T proadipocytes: (A) complete human plasma; (B) barium adsorbed plasma (BaP); (C) BaP and CEP fractions; (D) BaP and EDTAP fractions; (E) CEP fraction only; (F) EDTAP fraction only; (G) CEP and EDTAP fractions.

tion of these fractions twofold higher than that calculated to be present in heparinized DME containing 25% human plasma was also ineffective. By contrast, if heparinized medium containing both the BaP and CEP fractions was used, proadipocytes differentiated to a degree comparable to that observed in heparinized medium containing unfractionated plasma. This suggests that barium adsorption does not destroy the activity of plasma components and that at least two different components are required for adipocyte differentiation. One is present in barium-adsorbed plasma and one adsorbs to the barium precipitate and can be eluted with sodium citrate.

## DISCUSSION

The culture of rapidly growing, low density proadipocytes for 2 d in heparinized medium containing only human plasma induces their growth arrest at a distinct state in the  $G_1$  phase of the cell cycle,  $G_D$  (26). Thereafter, adipocyte differentiation in the absence of cell growth occurs in 80–100% of the cell population within ~4 d. Differentiation under these culture conditions is evidenced by the appearance of large cytoplasmic fat droplets and by elevation of lipoprotein lipase activity.

Use of this culture system to induce proadipocyte growth arrest and differentiation offers many advantages over those used in previous studies. Most importantly, predifferentiation growth arrest at the  $G_D$  state and subsequent adipocyte differentiation occurs rapidly and parasynchronously in the majority of cells cultured at low density. By contrast, differentiation in previous studies typically occurs only in high density cultures maintained for prolonged intervals in high concentrations of serum and/or insulin or other hormones (5, 10–12).

The data suggest that human and fetal calf plasma either contain higher concentrations of differentiation-promoting components compared with other biological fluids or that human and fetal calf plasma contain unique plasma components. The components in human plasma that promote  $G_D$  arrest and differentiation are shown to be active only in the presence of heparin and to be inactivated by blood clotting and by heating to 60°C for 1–3 h. The differentiation-promoting activity of human plasma is also removed by barium adsorption. The data show that addition of barium chloride to citrate-anticoagulated human plasma results in formation of a barium citrate precipitate and that specific plasma components adsorb to this precipitate so that the resultant barium adsorbed plasma is ineffective in promoting differentiation. The data further show that it is possible to elute components from the barium citrate precipitate and that these components reconstitute the differentiation-promoting activity of BaP. In the following paper (27), data will be reported that show that BaP promotes the  $G_D$  growth arrest of adipocytes but does not induce adipocyte differentiation, whereas CEP contains components that promote adipocyte differentiation but only in cells that have previously been  $G_D$  arrested.

Few previous *in vitro* studies have analyzed the effect of heparin on cell proliferation in culture. Hatcher et al. (14) showed that addition of high concentrations of heparin (333 U/ml) to medium containing optimum growth-promoting concentrations of serum slows the growth rate of rat smooth muscle cells and fibroblasts, whereas lower concentrations of heparin promote proliferation. Comparable data were reported by Hoover et al. (15). The effects of heparin were shown to require the presence of an acid labile serum factor. It was suggested that a complex of heparin and serum factor(s) modified the protease activity in the culture medium and thereby modified cellular

growth rate (14). Although no in vitro studies have been reported concerning the effect of heparin on cell differentiation, in vivo studies suggest that heparin may affect the control of cell proliferation and cell differentiation and the control of tumor cell metastasis (1, 6, 13, 17, 21, 28).

If the physiological events that control cell proliferation and cell differentiation in normal and malignant cells are to be identified, it is most important to establish the mechanisms that control the coupling of growth arrest and differentiation. We have, therefore, used the culture system described in this paper to obtain experimental evidence that there are at least five phases in the coupling process. In the following paper we present a model that illustrates the coupling process and we present the hypothesis that carcinogenesis may be associated with defects in specific phases of the coupling of growth arrest and differentiation (27).

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