Aproliferin—A Human Plasma Protein That Induces the Irreversible Loss of Proliferative Potential Associated With Terminal Differentiation

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Cellular proliferation is regulated not only by the action of growth factors and growth inhibitors whose effects are reversible but also by factors that induce the irreversible loss of proliferative potential associated with the terminal event in cellular differentiation. The authors have employed 3T3 T mesenchymal stem cells as a model system to study the terminal event in cellular differentiation because in these cells' distinct nonterminal and terminal states of differentiation can be identified and because transition from the nonterminal to the terminal states of differentiation can be induced by human plasma. In this paper is reported the 20,000-fold purification of a component of human plasma that induces the terminal event in differentiation. This factor is shown to have an apparent molecular weight of approximately 45,000 and an isoelectric point of approximately 7.6. It is trypsin-sensitive, acid and heat-labile, and is resistant to treatment with dithiothreitol and alkali. The ability of this human plasma protein to induce the irreversible loss of proliferative potential associated with the terminal event in differentiation serves as the basis for its designation "aproliferin." The data in this paper in addition show that no other pharmacologic or physiologic agents have been identified that can mimic the biologic effect of aproliferin. Therefore, aproliferin appears to be a functionally distinct protein in human plasma. (Am J Pathol 1986, 125:546–554)

NONTERMINAL and terminal states of differentiation have been identified in a variety of cell types. These include 3T3 T mesenchymal stem cells,[1-3] myoblasts,[4] mast cells,[5] lymphocytes,[6] melanoma cells,[7] erythroleukemia cells,[8,9] and neuroblastoma cells.[10] In these studies the transition from a nonterminal to a terminal state of differentiation has been established to be a critical regulatory process that is associated with the irreversible loss of proliferative potential. In addition, modulation in the control of this process has been identified and has been proposed to be involved in cellular senescence and transformation.[3,11,12]

In this regard, our studies have employed 3T3 T mesenchymal stem cells that can be induced to differentiate into adipocytes as a model system for the study of the process of terminal differentiation.[1-3,13,14] More specifically, we have 1) developed procedures to purify nonterminally differentiated cells, 2) established that such cells retain the ability to proliferate or to dedifferentiate, and 3) demonstrated that nonterminally differentiated cells can be induced to undergo terminal differentiation in association with loss of proliferative potential when exposed to human plasma.

The goal of the current studies was to identify factor(s) that can induce transition from the nonterminal to the terminal state of differentiation. In this regard, we have partially purified and characterized a polypeptide from human plasma that indices the terminal event in adipocyte differentiation, and we have designated this protein "aproliferin." We have also established that none of more than 20 physiologic and/or pharmacologic agents can mimic the biologic effect of aproliferin.

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Materials and Methods

Cell Culture

The Balb/c 3T3 T mesenchymal stem cell line[15,16] was used in all studies. Stock cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal calf serum (FCS) at 37 C in a 5% CO_2 atmosphere as previously described.[1,2,14,16] All stock cultures of cells were maintained at low density in 75-sq cm flasks by passage with 0.1% trypsin and 1 mM EDTA in phosphate-buffered saline (PBS) when cells were less than 60% confluent to prevent the selection of transformed cells that can grow at high cell densities. Cell cultures were also periodically assayed and proven to be free of mycoplasma contamination by the method of Chen.[17]

Preparation of Human Plasma and the CEPH and BaPH Plasma Fractions

Preparation of human plasma and the plasma fractions designated CEPH and BaPH is described in detail elsewhere.[1,2,13,14] Briefly, platelet-poor citrate-anticoagulated plasma was obtained from blood drawn by venipuncture from normal volunteers or patients undergoing plasmapheresis as therapy for autoimmune disorders. (Comparable results were obtained with each type preparation.) Plasma lots were frozen at −70 C; when needed, they were thawed at 4 C and centrifuged at 1000*g* for 10 minutes at 4 C prior to use. Such whole human plasma specimens, designated HP, contain factors that induce both nonterminal and terminal differentiation.

Fractionation of whole human plasma by barium adsorption[18] as previously described[1,2,13,14] was employed to generate CEPH, which is a plasma fraction that does not adsorb to barium and that is enriched in factors that induce nonterminal differentiation. Some CEPH preparations, however, not only contain factors that induce nonterminal differentiation but also factors that induce the terminal event in differentiation. To reduce residual terminal differentiation-inducing activity in such preparations, one of two additional procedures was typically used. First, medium containing CEPH was conditioned by culturing in the presence of adipocytes for 4 days followed by sedimentation by centrifugation at 1000*g* for 10 minutes. Second, CEPH was processed by heparin–agarose affinity chromatography as previously described.[13,14] CEPH prepared by these procedures is designated "processed" CEPH, and it is significantly depleted in terminal differentiation-inducing activity.

The fraction of human plasma that does adsorb to barium was designated BaPH. This fraction is depleted of factors that induce nonterminal differentiation but does contain factors that induce the terminal event in differentiation. The BaPH fraction was used as the source of terminal differentiation-inducing activity for purification procedures even though other blood specimens, including serum, also contain this activity. The BaPH fraction was employed for purification of aproliferin because it was available in large quantities as a by-product of preparing CEPH that was used to prepare nonterminally differentiated cells. The use of BaPH for aproliferin purification did, however, present some problems because such preparations can contain significant mitogenic activity that can interfere with the process of terminal differentiation. Such mitogenic activity was partially inhibited in some experiments by incubation of BaPH with 100 mM DTT for 2 hours at 22 C followed by dialysis against 6.8 mM sodium citrate buffer for 48 hours at 4 C prior to use.

Isolation of Nonterminally Differentiated Adipocytes

Cells were induced to undergo nonterminal differentiation by culture in medium containing CEPH or "processed" CEPH. Nonterminally differentiated adipocytes were then further purified by use of a simple BSA/PBS gradient that was previously described in detail.[13,14] These cells were replated is tissue culture plastic flasks to produce cultures that were >95% adipocytes of which ≥80% are nonterminally differentiated. These cells were employed in all bioassays.

Assays for Terminal Differentiation-Inducing Activity

Purified cultures of nonterminally differentiated adipocytes were incubated in medium containing regular or "processed" CEPH or HP with or without other additives (see below). After culture therein for 2–4 days the specimens were assayed for their ability to reinitiate proliferation or to dedifferentiate. The inability to respond to such signals was employed as the basis for the designation of a cell to be terminally differentiated.

Proliferation assays involved treatment of purified populations of adipocytes with DMEM containing 30% FCS ± 50 μg/ml insulin for a 48-hour interval, during which time the rate of incorporation of ^{3}H-thymidine was assayed or by culture of cells in 30% FCS ± 50 μg/ml insulin for 10–16 days, after which time the extent of clonal proliferation was determined.[13,14]

Dedifferentiation, that is, loss of the differentiated phenotype, was assayed by phase microscopic quantitation of the percentage of adipocytes that lost the differentiated phenotype after treatment with 2×10^{-4}

M methyl isobutyl xanthine (MIX) or 100 ng/ml phorbol myristate acetate for 3 days while maintaining the cells in differentiation-promoting medium.[13,14]

Purification of Terminal Differentiation-Inducing Activity in Human Plasma

A series of biochemical procedures was employed to purify the active component from human plasma that induces the terminal event in differentiation. The first successful procedure we employed to purify aproliferin involved adsorption of human plasma with barium salts[1,2,13,14,18] as described above. Next, the BaPH plasma fraction was subjected to ammonium sulfate precipitation at various concentrations as described in detail elsewhere.[13] All fractions were then dialyzed extensively against 0.01 M sodium citrate, pH 7.4; they were concentrated by dialysis against polyethylene glycol and sterilized by Millipore filtration (0.2μ) prior to bioassay. The protein content of each fraction was determined by the Bio-Rad method.[19] A similar procedure for dialysis, concentration, sterilization, and protein quantitation was employed in subsequent procedures.

Since one of the major difficulties in fractionation of human plasma is the need to dissociate an active component from albumin, which is the major plasma protein, Affi-Gel Blue affinity chromatography (Cibacron Blue) was used; this matrix has a high affinity for albumin.[20,21] More specifically, ammonium sulfate fractions containing terminal differentiation-inducing activity were chromatographed on Affi-Gel Blue coupled to agarose (Affi-Gel Blue, Bio-Rad). The methods to prepare such columns have been described in detail elsewhere,[20,21] as have the procedures we employed to perform chromatography using Affi-Gel Blue.[13] In general, proteins that did not adhere to the column were removed with at least two column volumes of starting buffer. Proteins that adhered to the column were then eluted with step gradients of NaCl, that is, 0.3 M NaCl, 2.0 M NaCl, and finally 2.0 M NaCl and 6 M urea. Fractions within each peak were combined and analyzed.

The active fraction from Affi-Gel Blue chromatography was next electrophoresed on 7.5–13.0% polyacrylamide gels containing 1% sodium dodecyl sulfate (SDS). The gels were sliced in small fractions, and the proteins were eluted from each slice by incubation in 0.1 M phosphate buffer (pH 7.4) for 16 hours at 4 C. For removal of SDS, these fractions were mixed with Dowex I-S for 1 hour at 22 C, and then the activity of each fraction was assayed.

The active fraction from Affi-Gel Blue chromatography was next chromatographed on Bio-Gel P60. Bio-Gel P60 columns were run under dissociating conditions in 0.05 M Tris HCl, pH 7.6, containing 3 M urea and 0.01 M sodium citrate. The column was calibrated with the use of transferrin, ovalbumin, and cytochrome C. Sample eluate fractions were dialyzed against 0.01 M ammonium acetate, then lyophilized prior to reconstitution in culture medium for bioassay.

Chromatofocusing[22] was the last chromatographic method used to purify terminal differentiation-inducing activity from human plasma. Chromatofocusing was performed in a variety of pH ranges. Definitive studies employed the pH range 7.3–8.3 using the polybuffer exchanger PBE 94 (Pharmacia) as described in detail elsewhere.[13,22] The active fractions prepared by Affi-Gel Blue chromatography or by P60 chromatography were employed as the starting material. After chromatography but prior to bioassay, the polybuffer was removed by ammonium sulfate precipitation.

Assays for Terminal Differentiation-Inducing Activity of Commercially Available Pharmacologic and Physiologic Agents

Purified populations of nonterminally differentiated adipocytes were employed as the test system for assay of whether a variety of commercially available pharmacologic or physiologic agents could induce the terminal event in differentiation. More specifically, these agents were added to nonterminally differentiated adipocytes in medium containing CEPH or "processed" CEPH and cultured therein for 4 days. The cells were assayed for their proliferative potential as described above for determining whether they had been induced to undergo the terminal event in differentiation.

Results

The objective of the current studies was to characterize the factor(s) that regulate the terminal event in mesenchymal stem cell differentiation. Two approaches were taken. First, the purification and characterization of a terminal differentiation-inducing factor from human plasma was undertaken. Second, the ability of commercially available pharmacologic and physiologic agents to induce the terminal event in adipocyte differentiation was evaluated.

In order to clearly characterize the potentialities of 3T3 T adipocytes at nonterminal and terminal states of differentiation and the effect of aproliferin in inducing transition from the former to the latter state, we present in Table 1 a brief summary of previously published data.[1,2,13,14] It describes nonterminal cells as fully differentiated adipocytes that have the potential to reinitiate proliferation and to undergo dedifferentiation responses. By contrast, it describes terminally differen-

Table 1—Resume of the Biological Characteristics of Differentiated Adipocytes at the Nonterminal and Terminal States

	State of differentiation	
	Nonterminal state	Terminal state
Morphology	Adipocyte phenotype	Adipocyte phenotype
Proliferation potential*	≥80%	≤10%
Dedifferentiation potential	≥80%	≤5%

* Results expressed as the approximate percent of cells responding to either proliferation or dedifferentiation signals.

tiated adipocytes as cells that lack significant proliferative potential and lack the potential to dedifferentiate. In this regard, aproliferin induces the transition from the nonterminal to terminal state of differentiation.

Purification of Aproliferin From Human Plasma

We have designated the factor in human plasma that induces the terminal event in differentiation aproliferin because its biologic effect is to cause the irreversible loss of proliferative capacity. The procedures we employed to purify aproliferin included barium adsorption, ammonium sulfate precipitation, chromatography on Affi-Gel Blue, molecular sieve chromatography on Bio-Gel P60, and isoelectric focusing using chromatofocusing chromatography. All fractions from these purification procedures were assayed for their ability to induce the terminal event in differentiation by their addition to nonterminally differentiated cells. After culture therein for 4 days, untreated and treated cells, together with appropriate controls, were then assayed either for their ability to proliferate and/or for their ability to dedifferentiate. Loss of the ability to respond to these agents was defined as evidence of terminal differentiation.

In all these assays the activity of fractions being tested was expressed in terms of relative terminal differentiation-inducing potency. This approach was employed because in different assays using different cell preparations and/or different plasma preparations, the absolute extent of activity varied as much as 20%. To minimize this variation between experiments, control specimens were run in each assay; these controls included nonterminally differentiated cells cultured in medium containing regular or "processed" CEPH (see Materials and Methods) or terminally differentiated cells cultured in medium containing HP. The relative activity of specific fractions was thus expressed as % response to CEPH − % response to unknown ÷ % response to CEPH − % response to HP, wherein specimens cultured in medium containing HP were designated to be 100% terminally differentiated and specimens cultured in medium containing only CEPH were designated to be 0% terminally differentiated. All fractions and control specimens were tested at different dosages so that the specificity activity of each specimen could be determined.

Ion exchange chromatography was used in our initial attempts to purify aproliferin. The BaPH plasma fraction was chromatographed on DEAE and CM Sephadex at pH 7.4, and the protein fractions were eluted with a step NaCl gradient. Most protein and most activity eluted in the void volume from the CM-Sephadex column and in the 0.05 M NaCl fraction from the DEAE column. DEAE chromatography was also run at pH 7.8, 8.0, and 8.4 with increasing pH. Aproliferin activity bound more tightly to the column matrix, but so did most of the plasma protein. This procedure was therefore not useful for the purification of aproliferin. Heparin agarose chromatography was also used in initial attempts to purify aproliferin, but in these experiments aproliferin activity also did not bind.

We therefore fractionated BaPH using ammonium sulfate precipitation. The results of these procedures, presented in Table 2, show that maximum terminal differentiation-inducing activity is precipitated by 50% ammonium sulfate when the final protein concentration is approximately 25 mg/ml at pH 7.6. An important finding in these assays was that ammonium sulfate-

Table 2—Fractionation of Aproliferin Activity by Ammonium Sulfate Precipitation

Culture medium	Additives: Ammonium sulfate precipitated fraction of BaPH (% saturation)	Relative aproliferin activity*	
		Proliferation assay (%)	Dedifferentiation assay (%)
CEPH	–	0	0
BaPH	–	100	100
CEPH	0–35	53	100
	0–45	60	121
	0–50	100	160
	50–100	27	13

* Methods to determine relative aproliferin activity are described in the text.

Table 3—Fractionation of Aproliferin Activity by Affi-Gel Blue Chromatography

Culture medium	Additives: Fractions eluted under various conditions	Relative aproliferin activity*	
		Proliferation assay (%)	Dedifferentiation assay (%)
CEPH	–	0	0
HP	–	100	100
CEPH	0.5 M NaCl	0	0
	2.0 M NaCl	27	30
	2.0 M NaCl + 6 M urea	88	61

* Methods to determine the relative aprolifering activity are described in the text.

precipitated fractions could be assayed for aproliferin activity in the absence of anticoagulants because the active fractions did not clot in the absence of sodium citrate or heparin.

The "0–50% ammonium sulfate fraction" that contains essentially all aproliferin activity was next fractionated by Affi-Gel Blue chromatography. This procedure was performed in an attempt to dissociate aproliferin activity from albumin, which is the major protein in plasma. In this regard, Affi-Gel Blue has a high affinity for albumin.[20,21] Table 3 presents the results of this procedure. It shows that aproliferin activity binds to the Affi-Gel Blue matrix with very high affinity, in that 2 M NaCl-6 M urea is required to efficiently dissociate aproliferin activity from the column. Significant purification of aproliferin was obtained by this procedure; however, analysis of the active fraction by 2D gel electrophoresis still demonstrated that albumin was still present in this fraction, as were numerous additional polypeptides (data not shown).

To further purify aproliferin activity, we subjected the active fraction derived from chromatography on Affi-Gel Blue to molecular sieve chromatography using Bio-Gel P60. Figure 1 presents the results of a typical experiment. It demonstrates that most aproliferin activity eluted in the 30,000–50,000 molecular weight range and that this activity was dissociated from the major protein peak representing albumin that eluted in the higher molecular weight range of the column.

To further document the relative size of aproliferin, the active fraction from Affi-Gel Blue chromatography was subjected to polyacrylamide gel electrophoresis in 1% SDS, the gels were then sliced into fractions, and the polypeptides in each fraction were eluted with buffer. These fractions were then passed over Dowex I-S

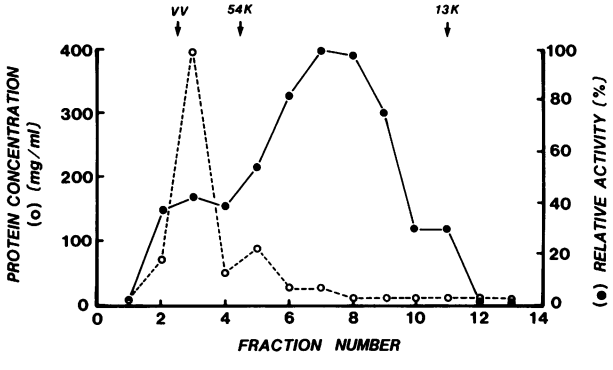

Figure 1—Purification of aproliferin by molecular sieve chromatography on Bio-Gel P60 in the presence of urea. Approximately 40 mg of active material prepared by Affi-Gel Blue chromatography was dialyzed against 0.05 M Tris-HCl, pH 7.6, containing 3 M urea and 1 M NaCl overnight, then chromatographed on Bio-Gel P60. Fractions of 15 ml were collected, extensively dialyzed, and concentrated. Fractions were tested for their relative activity in inducing the terminal event in differentiation assay described in Materials and Methods. The molecular weight markers employed to calibrate the column are presented as well.

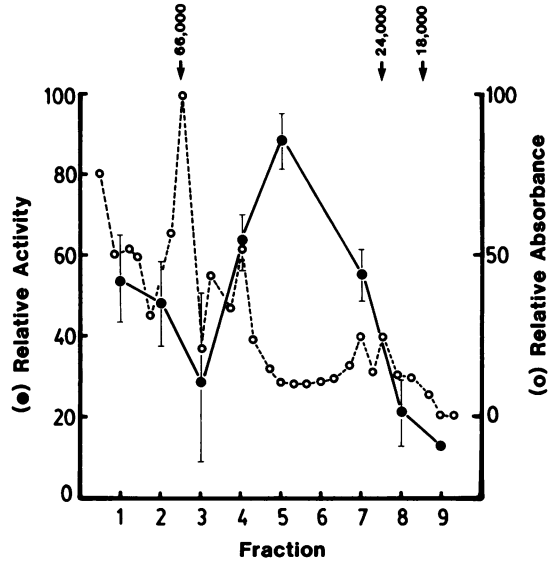

Figure 2—Characterization of the relative molecular weight of aproliferin by polyacrylamide gel electrophoresis in the presence of SDS. Samples of active fraction from Affi-Gel Blue chromatography (100 μg/gel) were loaded onto each of thirteen 1% SDS–7.5% polyacrylamide gels and electrophoresed as described in Materials and Methods. One of the gels was fixed and stained with Coomassie blue. This gel was scanned and the optical density plotted. The other gels were sliced, the proteins extracted, and the SDS removed as described. Fractions were added to cultures of purified adipocytes incubated in medium containing CEPH. After 4 days, the dedifferentiation response to MIX was tested for establishing the relative terminal differentiation-inducing activity. The results are the average of three experiments with the same fractions. Molecular weight standards were run and scanned at the same time as the experimental gels (see Materials and Methods).

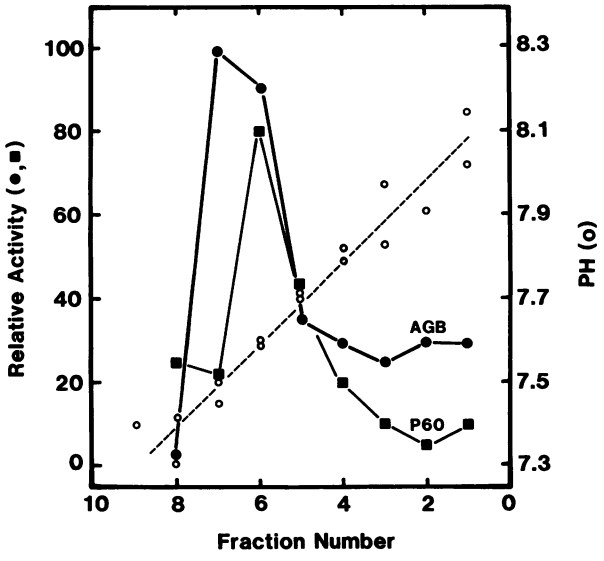

Figure 3—Purification of aproliferin by chromatofocusing. The results of two chromatofocusing assays of the relative terminal differentiation-inducing activity of fractions derived from Affi-Gel Blue (AGB) chromatography (●– – –●) or from Biol-Gel P60 chromatography (■– – – –■). Twenty and two milligrams, respectively, were chromatofocused with the use of Phamacia Polybuffer and PBE96. The columns were run in the presence of 3 M urea, and a gradient of 7.3–8.3 was employed. After fractions were collected, they were extensively dialyzed or subjected to reprecipitation with ammonium sulfate followed by extensive dialysis. They were then reconcentrated to their original volume prior to bioassay. The data for the Affi-Gel-Blue fraction was derived from a single experiment, whereas the data for the Bio-Gel P60 fractions reprsent the mean of three experiments.

minicolumns for removal of SDS as described in Materials and Methods, and they were then bioassayed. Figure 2 illustrates the results. The data confirm that aproliferin activity can be dissociated from albumin and that the active fraction migrates in the 30,000–50,000 molecular weight range with an estimated molecular weight of approximately 45,000.

Chromatofocusing was the final chromatographic procedure we employed to purify aproliferin. Chromatofocusing is the column chromatographic method that separates proteins primarily on the basis of their isoelectric points (pIs). Proteins with pIs higher than the initial pH of the gel elute in the void volume, whereas proteins with lower pIs are retained and can be eluted with a salt gradient. Chromatofocusing of the active fractions prepared by Affi-Gel Blue chromatography and by chromatography on Bio-Gel P60 was performed both in the presence and in the absence of 3 M urea. Initial assays employed a broad pH range, but more definitive studies employed a pH range from 7.3 to 8.3 because the results of initial experiments performed under dissociating conditions demonstrated that the vast majority of aproliferin activity was detected in this pH range. In this regard, it is important to stress that even under dissociating conditions some aproliferin activity elutes at a lower pH. This activity is thought to represent aproliferin bound tightly to albumin or other proteins and was therefore not analyzed further. Figure 3 presents the results, which demonstrate that the predominant peak of aproliferin activity is observed with a pI of approximately 7.5–7.6. This was found both when the active fraction from Affi-Gel Blue and Bio-Gel P60 chromatography was subjected to chromatofocusing. Table 4 summarizes the results of all purification procedures, including chromatofocusing, and demonstrates that aproliferin has been purified approximately 20,000-fold.

Biochemical Characteristics of Aproliferin

To establish the general biochemical characteristics of aproliferin, we assayed the effect of heat, pH, trypsin, and dithiothreitol on the activity of partially purified fractions of aproliferin prepared by barium adsorption and ammonium sulfate precipitation. The results of these assays are presented in Table 5. The data demonstrate that aproliferin's activity can be destroyed by trypsinization, by acidification, and by heating at

Table 4—Summary of the Purification of Aproliferin

Fractionation step*	Total protein (mg)	Specific activity $\left(\frac{1}{\text{mg/ml}}\right)$	Total units	Recovery (%)	Purification (fold)
Control	5600	1.25	7000	–	0.0
Barium adorption	4800	1.42	6857	98	1.1
$NH_4(SO_4)_2$ precipitation	1720	3.30	5733	81	2.6
AGB chromatography	380	13.00	5066	72	10.7
P60 chromatography	2	2000.00	4000	57	1600.0
CF chromatography	0.02	25000.00	500	7	20000.0

* Total protein refers to the total amount of protein in each fraction. Specific activities were determined from curves of concentration plotted against terminal differentiation-inducing activity. The specific activity is the inverse of the concentration of each fraction required to obtain 50% relative activity. The total activity is determined by multiplying the total protein by the specific activity, and the recovery is based on the amount of the total activity that is recovered at any step. Because of the variability of the bioassay, these results can only be considered to be approximate. AGB, Affi-Gel Blue; P60, Bio-Gel P60; CF, chromatofocusing.

Table 5—Biochemical Characteristics of Aproliferin

Treatment	Relative terminal differentiation-inducing activity
None	100
DTT (65 mM × 1 hour)	100
Heat (56 C × 1 hour)	58
pH 5 (1 hour)	55
pH 9 (1 hour)	96
pH 10 (1 hour)	85
Trypsin (50 μg/ml × 2 hours at 37 C)	5
Trypsin + soybean trypsin inhibitor	85

The active fraction from 0–50% ammonium sulfate precipitation of the BaPH plasma fraction was used in these studies. For pH treatments, the plasma was maintained at the indicated pH for 2 hours at 4 C, then brought to pH 7.4. Trypsin treatment was performed for 2 hours at 37 C, after which time it was inhibited by the addition of 100 μg/ml of soybean trypsin inhibitor. Experiments were also performed wherein plasma was incubated in the presence of soybean trypsin inhibitor for 1 hour at 4 C prior to the addition of trypsin and subsequent incubation for 2 hours at 37 C. Relative activity was determined by dedifferentiation assays of the loss of adipocyte phenotype in response to treatment with MIX (3×10^{-4} M) for 72 hours as assessed morphologically (see Materials and Methods).

56 C for 1 hour. In contrast, aproliferin's activity is alkaline-resistant and is resistant to treatment with dithiothreiotol. The latter observation suggests that aproliferin is a protein whose activity is not dependent on the presence of disulfide bonding.

Effect of Pharmacologic and Physiologic Agents As Potential Inducers of the Terminal Event in Differentiation

The data presented above establish that human plasma contains a protein that promotes the terminal event in mesenchymal stem cell differentiation. Even though this protein, designated aproliferin, has been purified approximately 20,000-fold, it is still necessary to determine whether it is a unique protein and whether any other factors can mimic its biologic activity. Complete purification and partial sequencing of aproliferin will be required before we can conclusively prove aproliferin is unique. At the present time it is, however, possible to evaluate whether physiologic or pharmacoligic agents can mimic the effect of aproliferin. In this regard, we performed the studies described below.

Dexamethasone, insulin, fetuin III, fibronectin, tunicamycin, Na azide, and HMBA (N,N'-hexamethylene-bis-acetamide) were the pharmacologic agents we tested to determine whether they possess the potential to induce the terminal event in adipocyte differentiation. These agents were evaluated because some have been shown to influence the process of cellular differentiation and/or proliferation in different cell systems.[23] To assay the potential effects of these agents, nonterminally differentiated adipocytes were cultured in medium containing CEPH ± additives for 3 days. Such cultures were then refed medium containing 30% FCS ± 50 μg/ml insulin as a mitogen. The proliferative responses of these cells were assayed by their ability to undergo DNA synthesis within a 48-hour interval and/or by their clonogenic potential. Table 6 shows the dosages of the compounds used. None of these pharmacologic agents demonstrated significant terminal differentiation-inducing activity.

We also assayed the effects of a variety of plasma components on the process of terminal differentiation. These included commercially available and more purified preparations of thrombin, prothrombin, antithrombin III, fibrinogen, plasminogen, factors IX and IXa, and factors X and Xa. The dosages of these agents that were employed are presented in Table 6. These agents also failed to show significant activity in inducing the terminal event in adipocyte differentiation.

Finally, conditioned medium was prepared from five strains of human cells derived from patients that showed premature senescence, ie, progeria, Werner's syndrome, and Cockayne's syndrome. The reason we tested these media was to assay the possibility that senescence-prone cells might secrete a factor that promotes the terminal

Table 6—Pharmacological and Physiological Agents That Do Not Possess Activity to Induce the Terminal Event in Differentiation

Agent	Dosage range tested
Dexamethasone	10^{-5}–10^{-6} M
Insulin	1–50 μg/ml
Fetuin III	0.2–2.0%
Fibronectin in solution	1–10 μg/ml
Tunicamycin	0.1 μg/ml
Na azide	200–800 μM
HMBA	5×10^{-3}–5×10^{-5} M
Thrombin	10.0–0.02 units/ml
Human prothrombin	0.1–0.01 units/ml
Human antithrombin III	10.0–0.001 units/ml
Human fibrinogen	50–0.5 mg/ml
Human plasminogen	5×10^{-3}–5×10^{-5} units/ml
Bovine factor IX	0.1–0.001 units/ml
Human factor IXa	10.0–1.0 units/ml
Bovine factor X	0.1–0.001 units/ml
Equine factor Xa	0.1–0.001 units/ml
Conditioned medium from senescence prone human cells	
Progeria fibroblasts (AG 1972)	25%
Werner fibroblasts (AG 3141)	25%
Cockayne fibroblasts (AG 5012)	25%
Progeria lymphoblasts (AG 3344)	25%
Werner lymphoblasts (AG 3364)	25%

The pharmacologic and physiologic agents listed above were purchased from Sigma Chemical Company (St. Louis, Mo). Conditioned medium from senescence prone cells were prepared by the culture of approximately 10^6 of such cells in 5 ml of DMEM containing CEPH for 24 hours, after which time the cells were removed by centrifugation. These strains designated parenthetically were purchased from the NIA Cell Repository (Camden, NJ). Highly purified preparations of thrombin, prothrombin, antithrombin III, and factor IXa were also assayed; they were kindly provided by Dr. David Fass, of the Mayo Clinic. HMBA, N,N'-hexamethylene-bis-acetamide.

event in differentiation. The results stated in Table 6 show that none of these cells produced detectable activity.

The observation that physiologic proteins, hormones, and various pharmacologic agents do not induce the terminal event in differentiation, whereas whole human plasma does, suggests that aproliferin is probably a unique factor that can induce nonterminally differentiated adipocytes to irreversibily lose proliferative capacity and thereby undergo the terminal event in differentiation.

Discussion

The control of cellular proliferation is a most important regulatory process. In normal stem cells the control of proliferation appears to be mediated by a balance of forces that either stimulate proliferation or stimulate nonterminal and terminal differentiation. In this regard, the majority of previous experimental studies on the control of cellular proliferation have involved attempts to establish the mechanisms by which DNA synthesis and cellular proliferation are initiated in quiescent cell populations.[24-26] Many growth factors that induce these events have in fact been characterized and purified. Several growth-inhibitory factors[27,28] have also been described; however, these factors generally inhibit initiation of DNA synthesis or cell growth in a transient, reversible manner. Many fewer studies have attempted to establish the mechanisms by which cellular proliferative capacity can be irreversibly limited in nonterminally differentiated cell populations. This is due in part to the fact that few, if any, adequate model cell systems have previously been described wherein nonterminally differentiated cells can be rapidly and parasynchronously induced to terminally differentiate and irreversibly lose their proliferative potential when exposed to a physiologic inducer.

The 3T3 T mesenchymal stem cell system we have developed, however, provides an excellent model for analyzing the biologic and molecular basis for the irreversible loss of proliferative capacity that is associated with the terminal event in differentiation. This is so because purified populations of these cells can be prepared in a nonterminal state of differentiation, and they can then specifically be induced to undergo the terminal event in differentiation, whereby they irreversibly lose their proliferative potential when exposed to human plasma or specific fractions of human plasma.[1-3,13,14] We have therefore employed this model system to evaluate factors that regulate the terminal event in differentiation.

In the current paper we report the partial purification and characterization of aproliferin from human plasma. More specifically, by use of a variety of biochemical methods, including barium adsorption, ammonium sulfate precipitation, Affi-Gel Blue chromatography, molecular sieve chromatography, and chromatofocusing, we report the 20,000-fold purification of aproliferin and show that it has a molecular weight of approximately 45,000 daltons and a pI of 7.5. We also report that the biologic activity of aproliferin can be destroyed by trypsinization, acidification, or heating, but not by alkalinization or treatment with dithiothreitol. Although our most purified preparations of aproliferin are still not homogeneous by 2D gel electrophoresis, the evidence we have presented, which establishes that biologic terminal differentiation-inducing activity co-purifies with aproliferin strongly suggests that aproliferin is a specific protein with a highly unique biologic function. In this regard, we also reported in this paper that none of approximately 20 other physiologic and/or pharmacologic agents showed aproliferin-like activity. We therefore suggest that aproliferin is a biochemically and functionally distinct human plasma protein.

The greatest significance of our discovery that aproliferin can induce the terminal event in mesenchymal stem cell differentiation may relate to studies on the mechanisms of carcinogenesis. This is so because the expression of aberrant control of the terminal event in differentiation and other manifestations of aberrant control of cellular proliferation are thought by many investigators to be most important basic characteristics of cancer cells.[3,11,12,29-33] We suggest in this regard that cancer could result at least in part in loss of a cell's ability to respond to aproliferin and/or related molecules and that such cells would therefore lack the potential to undergo the terminal event in differentiation. This abnormality would be manifest as the continued proliferation of terminal differentiation-defective cells that could result in the generation of neoplastic cell clones.

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