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Polypeptide Changes Associated With Loss of Proliferative Potential During the Terminal Event in Differentiation

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The differentiation of murine mesenchymal stem cells occurs in nonterminal and terminal phases. In previous reports we established the characteristics of nonterminally differentiated cells and showed that transition from the nonterminal to the terminal state of differentiation can be induced by human plasma. We also showed that this transition is blocked by protein synthesis inhibitors and other pharmacological agents. In this paper, we have employed two-dimensional gel electrophoresis to evaluate changes in specific polypeptides that are induced when cells lose proliferative capacity associated with the terminal event in differentiation. Using silver staining procedures for analysis of electrophoretograms, we detected only seven major polypeptide differences between nonterminally differentiated and terminally differentiated cells. Six polypeptides were expressed only in preparations of terminally differentiated cells; these included two polypeptides identified in cytosolic fractions and four polypeptides identified in nuclear fractions. One polypeptide was also found to be selectively expressed only in nuclear fractions of nonterminally differentiated cells. Based on these observations we conclude that the loss of proliferative potential that occurs during the terminal event in mesenchymal stem cell differentiation is associated with changes in the composition of a limited number of specific polypeptides. We suggest that one or more of these polypeptides may be important in the regulation of cellular proliferation.

Key words: 2D gel electrophoresis, 3T3 T mesenchymal stem cells

The terminal event in cellular differentiation is associated with irreversible loss of proliferative potential [1]. This process has been previously studied with only limited success because it has not been possible to isolate purified populations of nonterminally differentiated cells that can be induced to parasynchronously undergo the terminal event in differentiation [2–6]. In this regard, most previous studies have compared rapidly growing cells with terminally differentiated cells. Such studies have therefore not been able to distinguish between changes associated with acquisition of

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the differentiated phenotype per se from changes associated specifically with loss of proliferative potential during the terminal event in differentiation.

The 3T3 T mesenchymal stem cell system, however, provides an excellent model to study the terminal event in the process of differentiation [7–9]. This is so because we have established that differentiation of 3T3 T mesenchymal stem cells occurs in distinct nonterminal and terminal phases [1,9,10]. Cells at both states exhibit a comparable differentiated phenotype, but nonterminally differentiated cells can be induced to reinitiate DNA synthesis and undergo clonal proliferation with or without loss of the differentiated phenotype, whereas terminally differentiated cells cannot [1]. In previously published papers we described methods to isolate highly enriched populations of nonterminally differentiated cells and methods to induce the terminal event in differentiation by incubating such cells in medium containing human plasma [8]. We have also isolated clones of 3T3 T mesenchymal stem cells that are selectively defective in their ability to undergo the terminal event in the differentiation process [11]. In addition, we have established that the terminal event in differentiation can be blocked by protein synthesis inhibitors [1]. This latter observation suggests that terminal differentiation may be associated with the synthesis/degradation of specific cellular proteins.

Using the model system in the current studies, we have employed two-dimensional (2D) gel electrophoresis to examine the polypeptide composition of nonterminally and terminally differentiated cells. The results establish that cells in the nonterminal and terminal states of differentiation are distinguished by a very limited number of differences in the composition of major proteins. We suggest that one or more of these proteins may function in a significant role in the control of cellular proliferation.

METHODS

General Methods

The 3T3 T mesenchymal stem cell line was primarily used for these studies [1]. These cells possess the potential to differentiate into numerous cell types under specific experimental conditions. In our studies, their differentiation into adipocytes was employed as a model system. Ninety-five percent enriched populations of 3T3 T cells at the nonterminal state of differentiation were routinely prepared by culture in medium (DME) containing 25% of the human plasma fraction designated CEPH (DME/CEPH) followed by their purification on a bovine serum albumin (BSA) gradient [1,7,9,10]. Similarly enriched populations of cells at the terminal state of differentiation were prepared by the continuous culture of undifferentiated stem cells in heparinized medium containing 25% platelet-poor human plasma (DME/HP) for 8–12 days or by the culture of nonterminally differentiated cells (purified on gradients) for an additional 4 days in DME/HP [1]. In selected studies nonterminal differentiation was also induced by culturing undifferentiated 3T3 T stem cells in medium containing whole platelet-poor human plasma (HP) and theophylline (1 mM). The latter method was less efficient than the former method in generating nonterminally differentiated cell populations [1], but it does permit isolation of nonterminally differentiated cells following culture in medium containing the identical plasma proteins that are employed to induce terminal differentiation. In this regard, theophylline inhibits the terminal event in differentiation.

Additional studies were performed on clones of 3T3 T stem cells that express selective defects in their ability to undergo the terminal event in differentiation. These clones, designated NTD, 4, 5, 7, and 11 [11] express a markedly reduced ability to undergo the terminal event in differentiation even when they are cultured in medium containing 25% human plasma.

To establish that the cell populations that were employed for 2D gel electrophoretic analysis were at the desired differentiated states, all preparations were examined for their ability to reinitiate proliferation when cultured in medium containing 30% fetal calf serum (FCS) ± 50 μg/ml insulin. These assays employed measurement of ^{3}H-thymidine into DNA during a 48-hr interval followed by autoradiography or measurement of colony formation after plating cells at low density in the presence of 30% FCS ± 50 μg/ml insulin [1]. In most experiments the biological characteristics of such cell preparations were also established by measurement of their ability to be induced to lose the differentiated phenotype when treated with 1–5 × 10^{-4} M methyl isobutyl xanthine (MIX) for 72 hr. The significance of these biological characteristics in distinguishing nonterminally and terminally differentiated cells were described in detail in a previous paper [1]; they are summarized in Table I.

Preparation of Cell Fractions

Purified adipocytes at either the nonterminal or terminal states of differentiation that were attached to culture plates were rapidly lysed by treatment with ice-cold 0.01 M ammonium acetate buffer (pH 7.4) containing the serine protease inhibitor 1 mM phenylmethylsulfonylfluoride (PMSF) and/or other protease inhibitors, such as leupeptin and pepstatin. Cell lysates prepared from approximately 10^7 cells per specimen were further processed in some experiments by either homogenization in a Dounce apparatus or by repeated freeze thawing; comparable results were obtained by all these procedures. Lysed cells were then fractionated. The nuclei were recovered by centrifugation at 4,000 rpm for 10 min at 4°C. This nuclear pellet was saved and further processed (see below). The postnuclear supernatant (also designated the cytosol/microsomal fraction) was also further processed in selected studies to isolate a crude microsomal fraction. This was accomplished by sedimentation of the postnu-

TABLE I. Biological Characteristics of 3T3 T Cells Prepared for 2D Gel Electrophoresis

		Relative response in biological assays			
		Mitogenesis assays[a]			
Cell designation	Culture media	% Labeled nuclei	Colony formation	Dedifferentiation assays[b]	Differentiated state
3T3 T[c]	HP[d]	–	–	–	Terminal
	CEPH[d]	+	+	+	Nonterminal
	HP + theophylline	+	+	+	Nonterminal
TD-defective clones[c]	HP	+	+	+	Nonterminal

[a]Mitogenesis assays used DMEM containing 30% FCS ± μg/ml insulin as the mitogen. Mitogenesis was assayed by evaluation of ^{3}H-thymidine incorporation into DNA by autoradiography (% labeled nuclei) and by colony formation assays.

[b]Dedifferentiation assays employed treatment of cells with 1–5 × 10^{-3} M methyl isobutyl xanthine and the subsequent evaluation of expression of loss of the differentiated phenotype.

[c]References 1 and 11 describe these cell preparation procedures. TD, terminal differentiation.

[d]HP, platelet poor human plasma; CEPH, citrate eluate of barium adsorbed human plasma components.

clear supernatant for 90 min at 130,000g that yielded a microsomal pellet. After preparation, these specimens were frozen at −70°C and/or lyophilized. To isolate nuclear proteins, the nuclear pellet was washed twice in 10 mM Tris HCl, pH 7.5, containing 0.15 M NaCl and 1 mM PMSF. Nuclei were further homogenized in 10 mM Tris HCl containing 0.25 M sucrose, 5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 0.5 Nonidet P-40 (NP-40), and 1 mM PMSF and centrifuged at 3,000g for 10 min. The resulting pellet was then homogenized in 10 mM Tris HCl, pH 7.9, containing 1 mM EDTA and centrifuged at 12,000g for 10 min. The final pellet was next solubilized and processed for 2D gel electrophoretic analysis. This procedure, as described and discussed by Marushige and Marushige [12], preferentially extracts nonhistone nuclear proteins. Therefore, the 2D gels of our nuclear preparations are relatively histone free.

Two-Dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis was performed using the ISO DALT procedure [13] as modified by Tracy et al [14]. Lyophilized samples were resuspended in 40 μl of homogenization buffer containing 8 M urea and 5% (v/v) 2-mercaptoethanol. Five-microliter aliquots were used for protein assays. Samples were further prepared for isoelectric focusing by addition of 10 μl of 10% sodium dodecyl sulfate (SDS) and 33 μl of a buffer containing 8 M urea, 8% (v/v) NP-40, and 5% ampholytes (75% pH 3.5–10, 25% pH 5–7) followed by centrifugation at 100,000g for 1.5 hr in a Beckman Airfuge to remove insoluble debris.

Isoelectric focusing was performed in 3.5% acrylamide gels containing 9 M urea, 2% NP-40, and 1.9% of pH 3.5–10 ampholytes. Gels were prefocused at 200 V for 1 hr, then focused at 600 V for 17 hr. Following electrofocusing the gels were removed into an equilibration buffer (10% glycerol, 2% SDS, in 0.1 M Tris HCl, pH 6.8, containing bromophenol blue), and incubated for 15 minutes. They were then frozen in a dry ice-ethanol bath, and either used immediately or stored at −70°C. The characteristics of the pH gradients were determined from pH readings on eluates of sections of several gels run in parallel. The second dimension employed gradients of 10–20% acrylamide prepared in 0.01 M Tris HCl buffer containing glycerol and SDS as described [13,14]. The DALT gels were run in the presence of Tris glycine buffer containing 3.5 mM SDS at 0.7 A.

Silver staining was performed by a modification of the method described by Wray et al [15]. The gels were first fixed in 50% methanol, rinsed in distilled water, and then soaked in 50% methanol containing 0.05% formaldehyde. The gels were then stained for 15 min in 20% $AgNO_3$, 0.09% NaOH, and 20% NH_4OH and developed in 0.05% (v/v) of 0.19% formaldehyde. Gels were finally incubated in 50% methanol for at least 1 hr, and they were then photographed.

Gel Comparisons and Analysis

The gels were analyzed by first identifying specific distinct reference spots that were present in all gels of a specific group of replicate specimens. These spots were used to align the gels and to form internal coordinates. A reference map consisting of all the spots observed in all the gels of a specific specimen group was prepared, and the spots on this map were numbered. Each individual gel preparation was then compared to the reference map and each spot was given a relative intensity value. A

spot was assigned a value of 2 if it was distinct and dark, 1 if the spot was faint but present, and 0 if there was no detectable staining at that point.

Multiple gels of different specimens were examined to eliminate variations intrinsic to 2D gel electrophoretic analysis. That is, at least three gels of each specimen type that was prepared from different samples and assayed at different times were employed. In all experiments care was taken to load identical amounts of protein onto each gel. Notwithstanding, some differences in staining efficiency and sample preparation occurred, and this resulted in some variability in the total number of spots observed on gels of different cell preparations. The problem of experimental variability was nonetheless overcome by analyzing specimens prepared from adipocytes at the same state of differentiation prepared by several different methods. For example, nonterminally differentiated cells were prepared by culture in medium containing CEPH or medium containing whole human plasma plus 1 mM theophylline. In addition, terminal differentiation-defective clones were used to prepare nonterminally differentiated cells following culture in medium containing whole human plasma (see Table I for reference).

Silver stained 2D gels of whole human plasma preparations were also analyzed and characterized. In this regard, any spot identified on 2D gels of cell fraction preparations that corresponded in location and configuration to major plasma proteins was eliminated from further evaluation (see Results). This approach was taken to eliminate the possibility of differential adherence or uptake of specific plasma proteins to nonterminally or terminally differentiated cells.

RESULTS

The goal of the studies reported in this paper was to determine if changes in protein composition could be detected in association with the terminal event in adipocyte differentiation. We therefore assayed preparations of nonterminally differentiated and terminally differentiated cells by 2D cell electrophoresis. Four populations of either nonterminally or terminally differentiated cells were studied, and the biological characteristics of each of these cell populations are summarized in Table I. In these studies, cytosol/microsomal, microsomal, or nuclear preparations were made from each type of cell population, and these were analyzed by 2D gel electrophoresis followed by silver staining. In this regard, the highly sensitive and semiquantitative silver staining procedure gives information about polypeptide/protein composition [13–15] and provides an excellent method to make comparative measurements on the composition of the amounts of specific proteins expressed in two related cell preparations.

Table II reports that 1,169 unique spots were identified in silver stained 2D gels of cytosol/microsomal fractions. These include two major and five minor differences that were consistently observed between nonterminally and terminally differentiated cell types. Table II also demonstrates that in crude microsomal preparations prepared from cytosol/microsomal fractions, 525 spots were detected, most of which were also present in the microsomal/cytosol preparations. Of these microsomal proteins, no consistent differences were observed between terminally differentiated and nonterminally differentiated cells. Therefore, the seven major protein differences observed in cytosol/microsomal preparations appear to be present selectively in the cytosol. Finally, proteins extracted from the nuclear pellet of nonterminally and terminally

TABLE II. Summary of Polypeptide Differences Between Nonterminally Differentiated and Terminally Differentiated Cells Detected on Silver Stained 2D Gel Electrophoretograms

Specimen designation	Total spots	Terminal differentiation specific		Nonterminal differentiation specific	
		Major	Minor	Major	Minor
Cytosol/microsomal proteins	1,169	2	5	0	0
Microsomal proteins	525	0	0	0	0
Nuclear proteins	821	4	15	1	1

differentiated 3T3 cells were examined, and a total of 821 spots (Table II) were identified. Of these, four major proteins were present only in terminally differentiated cells, while one protein was found only in nonterminally differentiated cells.

Detailed Analysis of Cytosol/Microsomal Polypeptides Using Silver Staining Techniques

Figure 1 shows typical examples of silver stained 2D gels of cytosol/microsomal preparations of nonterminally differentiated and terminally differentiated cells, respectively. A total of 1,169 spots were discriminated on analysis of multiple gels of these specimens. In any specific gel, not all of these spots were, however, detected as explained in the above section. That is, of the 1,169 spots only approximately 50% were of major intensity and could be detected in essentially all gels. In this regard, the areas where major and minor differences between specimens of nonterminally and terminally differentiated cells were observed are indicated by the two boxes.

Two major differences were found in areas comprising low molecular weight polypeptides, ie, box 1. Figure 2 shows this area in more detail. The two major differences are also indicated by arrows and numbers in a schematic map of the typical spots observed in this area (Fig. 2A). Polypeptides 264 and 727 were consistently present and dark in gels of terminally differentiated cells (Fig. 2B). In some specimens, spot 264 in fact appeared to consist of two subcomponents. In contrast, these two spots were absent or very faint in all gels from samples of nonterminally differentiated cells prepared in DME/CEPH (Fig. 2C) or in HP containing theophylline (DME/HP/theo) (Fig. 2D). In addition, these two polypeptides were faint or absent in all gels of nonterminally differentiated cells prepared from the four terminal differentiation-defective clones that were cultured in DME/HP (Fig. 2E).

Five minor high molecular weight polypeptides illustrated in Figure 1, box 2, were also present in most specimens derived from nonterminally differentiated cells. These spots appear to represent a family of related proteins or variations of a single protein. Since these spots were of the minor type, neither they nor comparable spots were studied in any greater detail in this paper.

Detailed Analysis of Nuclear Polypeptides Using Silver Staining Techniques

Typical silver stained 2D gels of polypeptides extracted from the nuclear pellet of nonterminally and terminally differentiated cells are illustrated in Figure 3A and B. In such specimens, five consistent major differences were observed. These were concentrated in two areas of the gels as indicated by the boxes and shown in more detail in Figure 4. Four spots that were of increased density in terminally differentiated cells relative to nonterminally differentiated cells were apparent in the area of

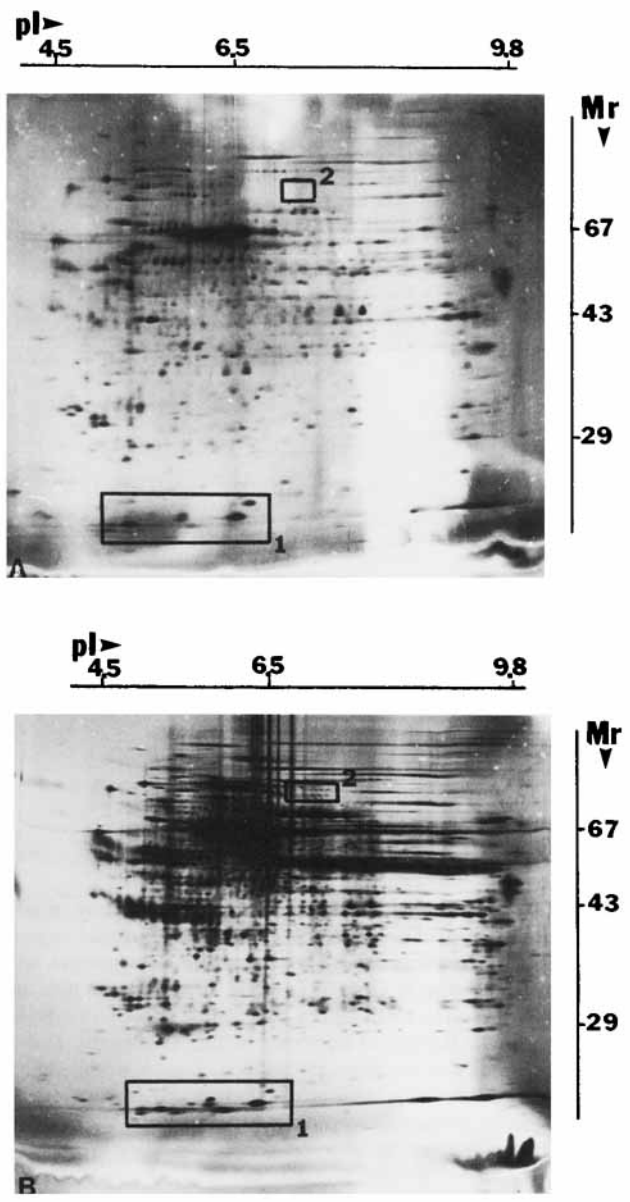

Fig. 1. Silver stained 2D gels of (**A**) nonterminally differentiated adipocytes and (**B**) terminally differentiated adipocytes. Cytosol/microsomal extracts were prepared from nonterminally differentiated and terminally differentiated cells and separated as indicated in the Methods and discussed in the text. Polypeptides were separated in the first dimension by isoelectric focusing in the pH range of 4 (left) to 10 (right) and in the second dimension by SDS polyacrylamide gels of approximate MW range of 10,000 (bottom) to 200,000 (top). The enclosed boxes indicate the areas where significant differences were found. Mr, molecular weight; pI, pH at which various polypeptides' isoelectric points exist.

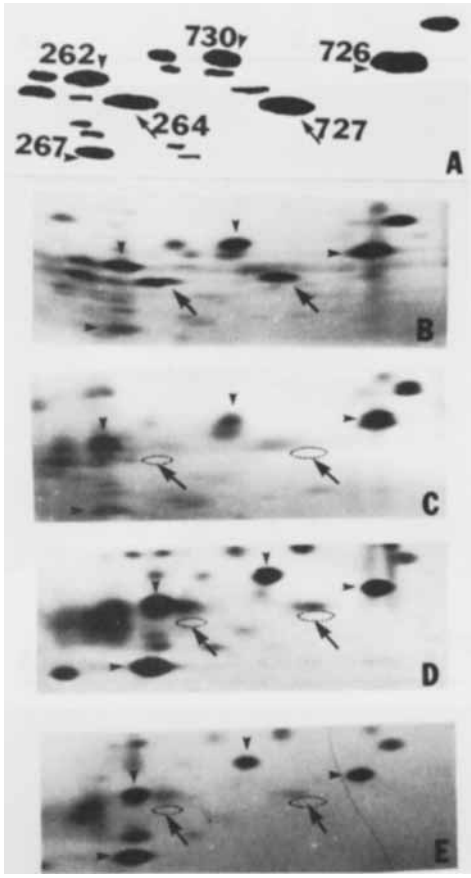

Fig. 2. Major polypeptide differences in cytosol/microsomal preparations of nonterminally differentiated and terminally differentiated cells. **Panel A** presents a schematic representation of the low molecular weight region in the gels presented in Figure 1 where major protein differences were found. The large arrows designate the two spots (264, 727) which differ between nonterminally differentiated and terminally differentiated cells. The small arrows indicate the reference spots. **B** presents a region of a 2D gel of a cytosol/microsomal fraction of terminally differentiated cells. It shows that all the spots identified in A can be localized in this specimen. By contrast, **C** presents the same region of a 2D gel of comparable preparation of a nonterminally differentiated cell; it shows that spots 264 and 727 cannot be identified. This is also true of nonterminally differentiated cells prepared by culture in DME/HP containing theophylline **(D)** and of nonterminally differentiated preparations of terminal differentiation-defective cell clone NTD-4 cultured in DME/HP **(E)**. The areas of gels C, D, and E where spots 264 and 727 are missing are designated by oval dotted lines.

the gel designated reigon 1. These include the spots 37, 38, 281, and 284. In region 2 of the gels is illustrated spot 459 that was preferentially expressed in nonterminally differentiated cells. Concerning the designation of these proteins as "nuclear," it must be stated that in crude cell fractions some cytosolic proteins may be present in nuclear fractions and vice versa. In summary, Table III is presented to review the characteristics of the seven distinct polypeptides described above.

Qualification of the Results of 2D Electrophoretic Analysis Evaluated by Silver Staining Techniques

A total of 7 major and 21 minor consistent differences have been detected between nonterminally and terminally differentiated cells. All seven major differences

TABLE III. Biochemical Characteristics of Major Polypeptides That Are Different Between Nonterminally and Terminally Differentiated Cells As Detected on Silver Stained 2D Gel Electrophoretograms

Specimen designation	Spot No.	pI	Molecular weight
A. Terminal differentiation specific			
1. Cytosol proteins	264	5.5–6.0	10,000–15,000
	727	6.0–6.5	10,000–15,000
2. Nuclear proteins	37	5.3–5.6	50,000–55,000
	38	5.3–5.6	50,000–55,000
	281	5.5–6.0	45,000–50,000
	284	5.5–6.0	45,000–50,000
B. Nonterminal differentiation specific			
1. Nuclear proteins	459	6.5–7.0	55,000–60,000

represent spots that were consistently present on gels of specimens of a given state of differentiation, and these spots were consistently absent or of very low intensity on all gels of specimens derived from cells at the contrasting state of differentiation. The 21 minor differences, however, represented faint or absent spots that show a significant degree of variability.

In our analysis, we chose to focus our evaluation selectively on the major observed differences between nonterminally and terminally differentiated cells because they were consistent and could be adequately analyzed by repeated visual inspection. This approach was taken because of the fact that with the silver staining procedure [15] variation in the staining characteristics of individual spots can occur, and this variation is most significant in spots that contain minor proteins that are present in a concentration at the lower limit of detection using the silver staining assay, ie, approximately 0.02 ng/spot [16].

In this regard, several additional factors that could influence the observed results were evaluated. For example, cell protein preparations could be contaminated by plasma proteins, different proteins might show altered susceptibility to proteolysis, differential carbamylation artifacts owing to exposure to urea could result, and noncovalent or covalent modification of proteins, such as phosphorylation or glycosylation, could occur selectively. In order to limit the potential significance of these problems, we followed a series of specific procedures. First, cells were extensively washed prior to lysis to remove most plasma proteins. In addition, any spot on any gel that corresponded to an identifiable plasma protein was eliminated by evaluation (see Methods). Nonterminally and terminally differentiated cell populations were also processed identically and a variety of protease inhibitors were employed during the preparation of samples. In preliminary studies, we also compared the relative phosphorylation of proteins in different preparations of cells and found no differences (Sparks and Scott, unpublished observations).

Another very important approach we used to assure that the observed differences were valid involved isolation of cells at different states of differentiation by several procedures. For example, to prepare specimens at the nonterminal state of differentiation the three media listed in Table I were used. In some of these latter studies both nonterminally and terminally differentiated cell samples that had been exposed to the same human plasma lots were also evaluated and still showed distinct differences.

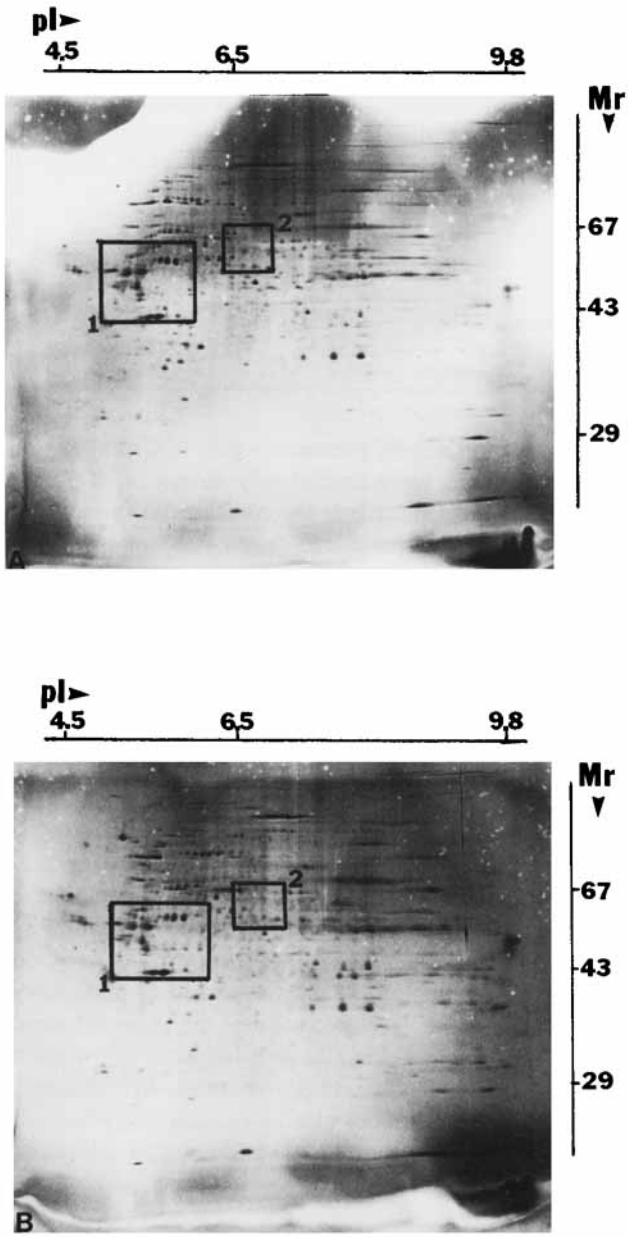

Fig. 3. Silver stained 2D gels of nuclear proteins of nonterminally **(A)** and terminally **(B)** differentiated cells. The proteins were extracted and analyzed by 2D gel electrophoresis as discussed in the Methods and the text. The areas showing major protein differences are indicated by two boxes. Mr, molecular weight; pI, pH at which various polypeptides' isoelectric points exist.

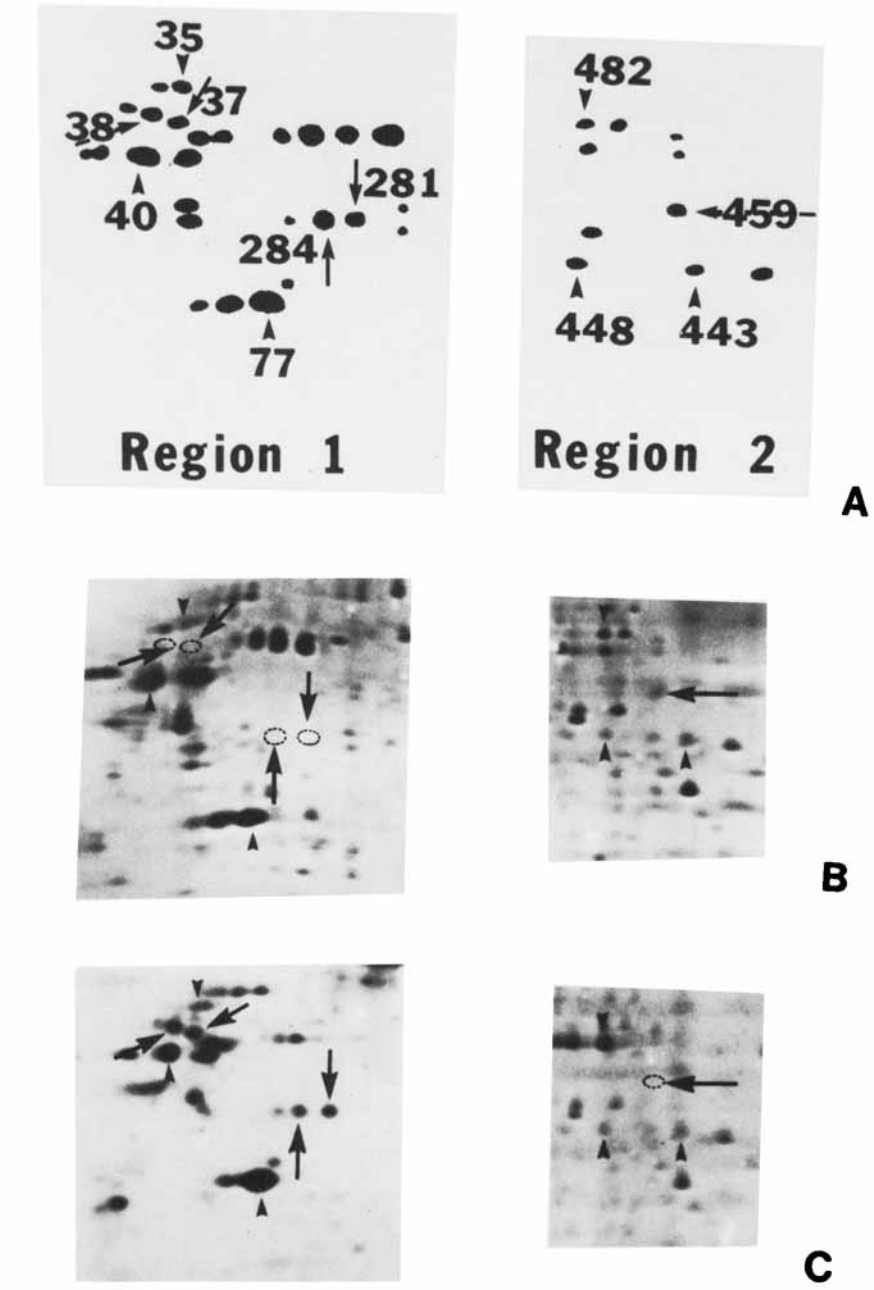

Fig. 4. Major polypeptide differences in nuclear protein preparations of nonterminally differentiated and terminally differentiated cells. The two regions illustrated in Figure 3 are shown in greater detail and for each region three different segments are presented: **(A)** a schematic representation of the spots in regions 1 and 2, **(B)** the 2D gel appearance of spots in regions 1 and 2 of nonterminally differentiated cells, and **(C)** the 2D gel appearance of spots in regions 1 and 2 of terminally differentiated cells. More specifically, these illustrations show that spots 37, 38, 281, and 284 (large arrows) are present in terminally differentiated cells but absent in nonterminally differentiated cells. By contrast, in region 2 the illustration shows that spot 459 (large arrow) is present in nonterminally differentiated cells but is absent in terminally differentiated cells. The areas of missing spots are circled with dotted lines; reference spots, 35, 40, 77, 482, 448, and 443 are designated with small arrows.

Because all the control and experimental procedures we performed gave consistent results, we suggest that the observations we have documented represent significant differences in the relative characteristics of nonterminally and terminally differentiated cell populations.

DISCUSSION

In this paper, we report that seven reproducible differences in the composition of major proteins can be detected in nonterminally and terminally differentiated mesenchymal stem cells by analysis of silver stained 2D gel electrophoretograms. Six of these proteins were identified only in terminally differentiated cells; two were localized to the cytoplasm and four were localized to the nucleus. One other distinct protein was only identified in the nucleus of nonterminally differentiated cells. These observations suggest that the terminal event in the process of mesenchymal stem cell differentiation is associated with large changes in the expression of a limited number of major cytosolic and nuclear proteins.

We also demonstrated that additional changes in the expression of less abundant proteins can be detected between nonterminally differentiated and terminally differentiated cells, but these changes are difficult to evaluate critically because of their low abundance. In this regard, it is possible that the terminal event in mesenchymal stem cell differentiation may be associated not only with the seven changes in abundant proteins we have documented but also with additional changes in other less abundant proteins. This possibility is important to consider because most cells express approximately 5,000 genes, and our 2D gel electrophoretic studies only evaluated the 1,250 to 1,500 polypeptides that were present in a high enough concentration to be reproducibly detected by the silver procedure.

Nonetheless, the results of the current studies establish that within the limits of detection of our experimental system, the terminal event in differentiation is associated with only a limited number of protein changes, some of which show preferential expression in terminally differentiated cells and some of which show preferential expression in nonterminally differentiated cells.

These results are most significant because we utilized highly enriched populations of nonterminally and terminally differentiated adipocytes in our analysis, and, furthermore, the cells we employed at the nonterminally/terminally differentiated state were prepared by several different methods and showed comparable results. Since the nonterminally and terminally differentiated populations we employed differed only in their proliferative capacity, ie, cells at each state exhibited an identical morphology, expressed similar levels of adipogenic enzymes, and were similarly growth arrested in the G_1 phase of the cell cycle, we suggest that the protein differences we have observed are important and are associated with the loss of proliferative potential associated with the terminal event in differentiation.

Previous studies on cell differentiation have also employed 2D gel electrophoresis, and such studies have employed many cell types including adipocytes [17–19], myeloid leukemia cells [20], erythroleukemia cells [21], melanoma cells [22], and muscle cells [23]. Essentially all previous studies have, however, compared rapidly growing or density arrested cells with their terminally differentiated counterparts. Therefore, the previously observed changes in protein composition may reflect changes in the expression of differentiation-associated proteins that are not specifically asso-

ciated with the terminal event in differentiation and loss of proliferative potential. In this regard, enzymes required for triglyceride and fatty acid metabolism are turned on and actin genes turned off during the general process of adipocyte differentiation, but neither event has been shown to be specifically associated with the terminal event in differentiation [18,24].

In contrast, we have specifically compared nonterminally and terminally differentiated populations of adipocytes that express similar phenotypes and which differ only in their proliferative potential. In our studies we have found a very limited number of specific protein differences between such cells. Only two of the proteins we demonstrated to be associated with the terminal event in differentiation may be similar to those reported in previous studies. That is, the two cytosolic proteins we found to be preferentially expressed in terminally differentiated cells, ie, polypeptides 264 and 724, may be comparable to the "late stage" adipocyte differentiation proteins previously described in a less well defined system [19,25].

Many important questions remain to be answered concerning the basis for the difference in expression of specific proteins in nonterminally and terminally differentiated cells. The differences could be due to primary changes in gene expression at the two states of differentiation. The differences could also be due to changes in the rates of protein synthesis/degradation, and at least one previous study has established that such changes are associated with differentiation [21]. The differences could be due to variations in post-transcriptional protein modification, such as glycosylation, phosphorylation, etc, which might influence a protein's biochemical characteristics and also its ability to be detected in silver stained 2D gel electrophoretograms. In this regard, we have evaluated the phosphoproteins in nonterminally and terminally differentiated cells and have not been able to detect significant differences (Scott and Sparks, unpublished observation).

In future studies it will also be important to determine if metabolic labeling methodologies can be employed to identify additional proteins whose expression is changed during the terminal event in differentiation. In this regard, preliminary data suggest that the terminal event in mesenchymal stem cell differentiation is also associated with the loss of expression of a specific 35,000 molecular weight cytosolic/microsomal protein that can be detected in ^{35}S-methionine labeled specimens (Wier and Scott, unpublished observations).

The most significant question that must now be resolved concerns whether any of these proteins specifically function to control cellular proliferation and what the mechanisms are that mediate the functions of these proteins. Notwithstanding the need to answer these important questions, the current results clearly establish that the terminal event in the process of mesenchymal stem cell differentiation is associated with extensive changes in the level of expression of less than 1% of the proteins that constitute nonterminally and terminally differentiated cells.

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