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Colony-Stimulating Factor 1 Expression Is Down-Regulated during the Adipocyte Differentiation of H-1/A Marrow Stromal Cells and Induced by Cachectin/Tumor Necrosis Factor

AKIHIRO UMEZAWA,[1]* KOUICHI TACHIBANA,[2] KENICHI HARIGAYA,[3] SATOSHI KUSAKARI,[1] SHINGO KATO,[2] YONOSUKE WATANABE,[1] AND TOSHIYA TAKANO[2]

Departments of Pathology[1] and Microbiology,[2] Keio University School of Medicine, Shinanomachi, Shinjuku-ku, Tokyo, and 1st Division, Department of Pathology, Chiba University, Inohana, Chiba,[3] Japan

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We isolated clonal sublines of the established mouse marrow stromal cell line, H-1. These clonal sublines underwent differentiation into adipocytes in various degrees. One subline, H-1/A, underwent adipocyte differentiation after confluence, while another subline, H-1/D, did not differentiate. In H-1/A cells, the 4.5- and 2.5-kb major mRNA species of colony-stimulating factor 1 (CSF-1) were expressed before differentiation and were down-regulated at a posttranscriptional level during the differentiation of H-1/A cells. The down-regulation of the CSF-1 gene was not a result of arrested cellular growth, because no down-regulation was detected in the nondifferentiating sister line, H-1/D. This down-regulation appeared to be an early event in differentiation. Cachectin/tumor necrosis factor transiently induced the expression of CSF-1 and inhibited the differentiation of H-1/A cells into adipocytes. This induced expression of CSF-1 was due to an increased rate of transcription.

Colony-stimulating factors (CSFs) are a family of glycoproteins required for the survival, proliferation, and differentiation of the hematopoietic cells. Murine CSFs have been molecularly cloned and characterized on the basis of their specificity for the direction of hematopoietic differentiation (9, 12, 25, 34, 48). CSF-1, one of the CSFs, is secreted by certain organs or cells (17, 18, 34–37, 42) and by marrow stromal cell lines in vitro (10), and it stimulates the differentiation of certain hematopoietic stem cells into a monocytic lineage (14, 45, 46). The CSF-1 receptor belongs to a single class of high-affinity receptors and appears to be identical to the product of the c-*fms* proto-oncogene (41), which exhibits tyrosine kinase activity (28), as does the *src* gene product (20). Cotransfection of NIH 3T3 cells with CSF-1 and the c-*fms* gene induces cell transformation by an autocrine mechanism (38–40).

On the other hand, cachectin/tumor necrosis factor (TNF), one of the cytokines, is reported to induce the expression of granulocyte-macrophage CSF (GM-CSF) and granulocyte CSF (G-CSF) in lung fibroblasts (22, 29) and endothelial cells (3) and to repress the expression of lipogenic enzymes (47). The effects of cachectin/TNF seem to reflect one of the physiological bases for cachexia in patients with certain infections and malignancies (31, 43).

A marrow stromal cell line, H-1/A, cloned from the bone marrow of C57/B1 mice, produces CSFs and differentiates into adipocytes (15). We previously reported that colony-stimulating activity of H-1/A cells stimulated the growth and differentiation of granulocytes (30). Colony-stimulating activity of H-1/A cells decreases when the cells differentiate into adipocytes. Thus, this marrow stromal cell line seems to play important roles in the regulation of hematopoiesis by the secretion of CSFs and by their down-regulation in adipocyte differentiation. However, the regulatory mechanism of CSF production remains obscure in the marrow stromal cell. This study reports that the down-regulation of CSFs was controlled at a posttranscriptional level and preceded the switch-on of the glyceromonophosphate dehydrogenase (GPD) gene during the adipocyte differentiation of H-1/A cells. We also demonstrate that cachectin/TNF inhibited both adipocyte differentiation and the switch-on of a lipogenic enzyme gene and induced the expression of CSF-1 at a transcriptional level in the H-1/A cells.

MATERIALS AND METHODS

Cell culture. Sublines were cloned from H-1 cells by dilution plating and then routinely cultured with Fischer medium (GIBCO, Grand Island, N.Y.) supplemented with 10% horse serum (Irvine Scientific, Santa Ana, Calif.), penicillin, and streptomycin. The cultures were incubated at 33°C in an atmosphere of 5% carbon dioxide. In certain cases, the growth medium was supplemented with 10^{-6} M hydrocortisone (Upjohn Co., Kalamazoo, Mich.) or with 1 mM dibutyryl cyclic AMP (dbcAMP) (Sigma, St. Louis, Mo.) and 1 mM of caffeine. H-1/A cells were also treated with 200 U of cachectin/TNF (Genzyme Co., Boston, Mass.) per ml at confluence. To estimate adipocyte differentiation, dishes were fixed with 10% buffered neutral Formalin and stained with 0.5% oil red O in propylene glycol. Four photomicrographs were randomly taken for each dish, and obvious fat droplets in the cytoplasm were designated as markers of differentiation (13, 16, 49, 50). About 2,000 cells were then counted in each dish, and the percentage of adipocytes was calculated.

Isolation of adipocytes. Adipocytes were isolated by the procedure of Spiegelman et al. (44). Cells were harvested by treatment with trypsin and were centrifuged twice at 2,500 × *g* for 5 min to pellet the preadipocytes in 7 ml of phosphate-buffered saline (PBS) containing 1 mM of phenylmethylsulfonyl fluoride. The supernatant which contained adipocytes was gently mixed with monobromobenzene-saturated PBS and was recentrifuged in PBS saturated with bromobenzene.

* Corresponding author.

In order to harvest the cells from the dishes, the cells were scraped off with a rubber policeman.

DNA probes. The CSF-1 probe was a 3.9-kb *Eco*RI fragment from pGEM2MCSF-10 (a gift from J. S. Price, Cetus Co.) (25). pGEM2MCSF-10 contained 3.9-kb CSF-1 cDNA cloned from mouse L cells. The G-CSF probe was a 1-kb *Bam*HI-*Eco*RI fragment from pMG-3 (a gift from S. Nagata) (48). The interleukin-3 (IL-3) probe was a 0.6-kb *Pst*I-*Nco*I fragment from pILM-3 (a gift from I. G. Young) (9). The GM-CSF probe was a 0.75-kb *Bam*HI-*Eco*RI fragment from pGMG3.2 (a gift from A. R. Dunn) (12). The GPD probe was a 0.9-kb *Eco*RI fragment of pL10.9 (a gift from B. M. Spiegelman) (44). The actin probe was a 6.8-kb *Eco*RI fragment of pSP62-PL obtained from N. Davidson (19).

RNA blot analysis. Fresh growth medium or medium containing 200 U of cachectin/TNF per ml was added to semiconfluent or confluent layers of H-1/A cells. After incubation, cells were harvested for RNA blot analysis. In another experiment, fresh growth medium or medium containing 10^{-5} M l-epinephrine, 10 μg of phytohemagglutinin per ml, 5 μg of concanavalin A (Sigma) per ml, 10^{-6} M hydrocortisone, 0.5 mM dbcAMP, and 1 mM caffeine with 10 or 100 U of recombinant IL-1α, 1 or 10 U of recombinant IL-1β (Cistron Bio., Pine Brook, N.J.) per ml, or 10 μg, 1 μg, 100 ng, 10 ng, or 1 ng of lipopolysaccharide (Sigma) per ml was added to the semiconfluent layers of H-1/A cells. After incubation for 12 h, cells were harvested. Purified recombinant IL-1α (26) was a gift from P. Lomedico (Hoffman-La Roche, Nutley, N.J.).

RNA was prepared from cultured cells by homogenization in guanidinium isothiocyanate, followed by centrifugation over a cesium chloride cushion (4). The RNA was then electrophoresed in a 1.0% agarose gel, transferred to a Nylon filter (Du Pont Company NEN Products), and hybridized with cDNA inserts labeled with [^{32}P]CMP by random-primer method (8) at 65°C for 14 to 16 h in buffer containing 5× SSPE (1× SSPE is 0.75 M NaCl, 0.05 M NaCl, 0.004 M EDTA), 5× Denhardt solution, 0.02% poly(A), and 1% sodium dodecyl sulfate (SDS). The blots were washed with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 1% SDS at room temperature and 65°C. Final washings were done with 0.1× SSC containing 0.1% SDS at 65°C. The blots were exposed to X-ray film at −80°C, using an intensifying screen. The amounts of RNA hybridized to each probe were determined by densitometry of bands, using Quick Scan R & D (Helena Laboratories).

Bone marrow colony-forming units in culture (CFUc) assay. The medium was harvested from cachectin/TNF-treated or untreated H-1/A cells grown for 3 days in Fischer medium supplemented with 10% fetal bovine serum and antibiotics. It was filtered through a Millipore filter (pore size, 0.22 μm) and stored at −20°C until use. CFUc were allowed to proliferate in semisolid soft agar cultures as previously described (33). Fresh murine bone marrow cells were obtained from 8- to 12-week-old female C57/BL mice by flushing their tibias and femurs. Fresh marrow cells were inoculated in 1 ml of complete Iscove medium containing 0.3% agar (Difco Laboratories, Detroit, Mich.) and 10% appropriate conditioned medium in plastic petri dishes. After incubation for 6 days at 37°C in an atmosphere of 7.5% CO_2 in humidified air, the specimens were air-dried and stained by means of a double-staining technique using naphthol-ASD-chloroacetate and α-naphthyl butyrate as substrates (27).

Nuclear run-off transcription assay. Run-off transcription assays were performed by using a modification of the method described by M. Groudine et al. (11). Nuclear RNA was radiolabeled by in vitro RNA elongation while reinitiation of RNA synthesis was prevented. Cells were harvested from four dishes by scraping with rubber policemen. Subsequent steps were performed at 4°C. The cells were washed in PBS and lysed by vortexing in Nonidet P-40 lysis buffer (10 mM Tris hydrochloride [pH 7.4], 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40). The lysates were incubated on ice for 5 min, resuspended in glycerol storage buffer (50 mM Tris hydrochloride [pH 8.0], 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA), and stored as a nucleus fraction at −80°C in 200-μl aliquots. The nucleus fraction was mixed with 200 μl of 2× reaction buffer (10 mM Tris hydrochloride [pH 8.0], 5 mM $MgCl_2$, 0.3 M KCl, 1 mM each ATP, CTP, and GTP, and 10 μl of [α-^{32}P]UTP [800 Ci/mmol]) and incubated for 30 min at 30°C with shaking. After incubation, nuclei were pelleted in a microfuge, resuspended in 300 μl of a solution consisting of 0.5 M NaCl, 50 mM $MgCl_2$, 2 mM $CaCl_2$, and 10 mM Tris hydrochloride (pH 7.4) with 200 U of DNase I (RNase-free, Pharmacia), and incubated for 30 min at 37°C. After the addition of 200 μl of a solution consisting of 5% SDS, 0.5 M Tris hydrochloride (pH 7.4), 0.125 M EDTA, and 10 μl of 20-mg/ml proteinase K, nuclei were vortexed thoroughly and incubated for 30 min at 40°C. The reaction mixture was extracted three times with phenol-chloroform, and the radiolabeled RNA was precipitated with an equal volume of 100% isopropanol. The precipitates were resuspended in 100 μl of a solution consisting of 10 mM Tris hydrochloride (pH 8.0), 1 mM EDTA, and 0.1 M NaCl and loaded onto the Sephadex G-50 column (Boehringer Mannheim) to remove unincorporated nucleotides. An equivalent amount of radiolabeled RNA (2×10^6 cpm) from each culture was hybridized to denatured DNA probes (>2 μg each), immobilized to Nylon filter paper in 2 ml of hybridization buffer [5× SSPE, 5× Denhardt solution, 0.02% poly(A), 1% SDS] for 36 h at 65°C after preincubation in hybridization buffer for 1 h at 65°C. The filters were washed in 2× SSC–1% SDS twice at 65°C. The filters were exposed to X-ray film at −80°C. The amounts of RNA hybridized to each probe were determined. The densitometry was done in the same way as in the blot analysis.

RESULTS

All the sublines of H-1 cells had a fibroblastlike morphology, and no adipocytes developed until they reached confluence. Sublines showed various degrees of differentiation into adipocytes at confluence. The manner of adipocyte differentiation of a subline H-1/A was described previously (30). In the H-1/A culture, 59% of the cells differentiated into adipocytes on the seventh day after confluence, while only 1% of the cells did so in subline H-1/D. It has been reported that hydrocortisone or dbcAMP and caffeine modulate adipocyte differentiation (16, 30). However, we found no effect on H-1/A cells (data not shown).

Blot hybridization of H-1/A RNA isolated from the semiconfluent culture was done with probes of mouse CSF-1, GM-CSF, G-CSF, and IL-3 cDNA (9, 12, 25, 48). Distinct 4.5- and 2.5-kb mRNA bands were detected in the semiconfluent H-1/A cells with the CSF-1 cDNA probe (Fig. 1a), while no bands were observed with the probes of GM-CSF, G-CSF, and IL-3 cDNA (data not shown). To characterize the difference between the 4.5- and 2.5-kb CSF-1 transcripts, the blots of H-1/A RNA were hybridized with different fragments of the CSF-1 cDNA. The two species of CSF-1 transcripts differed in their 3′ sequences (data not shown).

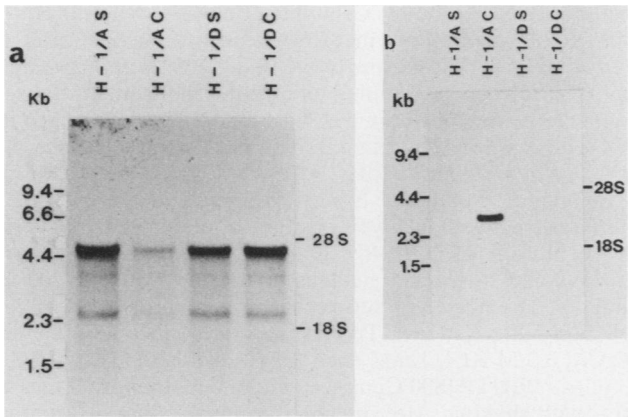

FIG. 1. Expression analysis of CSF-1 and GPD RNA during adipocyte differentiation of H-1/A cells. (a and b) Autoradiograms of RNA blot analysis from semiconfluent H-1/A cells (H-1/AS), H-1/A cells at 7 days postconfluence (H-1/AC), semiconfluent H-1/D cells (H-1/DS), and H-1/D cells at 7 days postconfluence (H-1/DC). Ten micrograms of the total RNA was electrophoresed in each lane. The blots were hybridized with a probe of the CSF-1 cDNA (a) or GPD cDNA (b), as described in Materials and Methods. The positions of the size markers and the 28S and 18S rRNA markers are indicated. The filters were exposed for 72 h at −80°C, using an intensifying screen.

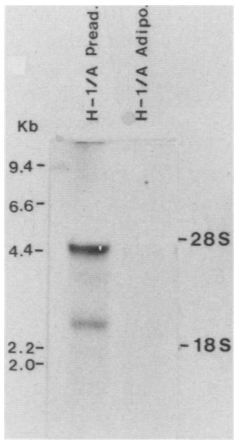

FIG. 2. Expression of CSF-1 RNA in preadipocytes and adipocytes of H-1/A. RNAs were extracted from H-1/A preadipocytes (Pread.) or isolated adipocytes (Adipo.). Ten micrograms of the total RNA was electrophoresed in each lane. The blot was hybridized with the CSF-1 probe as described in Materials and Methods. The positions of the size markers and the 28S and 18S rRNA markers are indicated. The filter was exposed for 72 h at −80°C, using an intensifying screen.

Even after 12-h treatments of H-1/A with various concentrations of phytohemagglutinin, concanavalin A, hydrocortisone, dbcAMP plus caffeine, l-epinephrine, recombinant IL-1α or IL-1β, or lipopolysaccharide, no detectable levels of hybridization were found with H-1/A RNA by G-CSF, GM-CSF, and IL-3 cDNA probes (data not shown).

H-1/A cells developed many fat droplets in their cytoplasm after reaching confluence, while few fat droplets were detected long after H-1/D cells reached confluence. Equal amounts of the total RNA from the semiconfluent and confluent cultures of H-1/A and H-1/D were subjected to blot hybridizations with the CSF-1 probe. In the confluent H-1/A culture in an adipocyte state, the CSF-1 mRNA levels decreased to approximately one-tenth of those of the semiconfluent H-1/A culture in a preadipocyte state (Fig. 1a). On the other hand, no reduction of the RNA levels was observed in H-1/D after confluence was reached (Fig. 1a). In contrast, the levels of GPD mRNA clearly increased in the confluent H-1/A cells compared with those in the semiconfluent culture (Fig. 1b). However, no detectable bands of GPD mRNA were observed in either semiconfluent or confluent H-1/D cells.

To obtain more accurate assessments of the change in the CSF-1 mRNA levels during differentiation, we purified adipocytes from the confluent H-1/A culture by using the procedure of Spiegelman et al. (44). The percentage of cells in the adipocyte preparation that were not adipocytes was found to be less than 1%. No detectable bands of CSF-1 mRNA were observed in the adipocyte preparation (Fig. 2). CSF-1 mRNA decreased as a result of differentiation of H-1/A cells into adipocytes rather than as a result of general RNA degradation, as similar amounts of rRNA were recovered from the preadipocytes and adipocytes.

We performed a study to follow the time course of CSF-1, actin, and GPD expression during differentiation (Fig. 3). The expression of CSF-1 and actin genes began to decrease just after the culture reached confluence, followed by a delayed increase of GPD expression.

H-1/A cells showed a fibroblastlike morphology, and no adipocytes were observed until they reached confluence. After reaching confluence, H-1/A cells differentiated efficiently into lipid-accumulating cells (Fig. 4a and 5). Cachectin/TNF is widely known to inhibit adipocyte differentiation (43, 47). By the treatment of the H-1/A culture at confluence with 200 or 1,000 U of cachectin/TNF per ml, the cells maintained their fibroblastoid morphology and did not accumulate any fat droplets for at least 10 days after reaching confluence (Fig. 4b and 5). Therefore, the lipid accumulation of the marrow preadipocytes, H-1/A, was prevented by

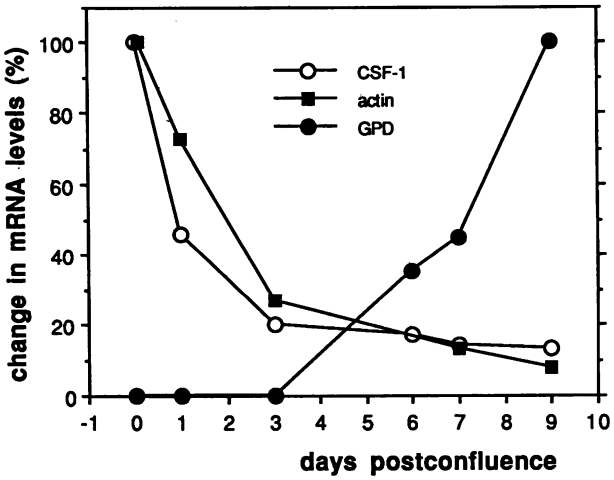

FIG. 3. Time course of CSF-1, GPD, and actin expression in differentiating H-1/A cells. CSF-1, actin, and GPD mRNA levels were determined densitometrically from autoradiograms of RNA blot analysis. At the indicated day postconfluence, RNA was isolated. Five micrograms of total RNA was electrophoresed in each lane. The blots were hybridized with each probe. The amounts of CSF-1 and actin mRNA at confluence (day 0) and GPD mRNA at day 9 postconfluence were regarded as equal to 100%.

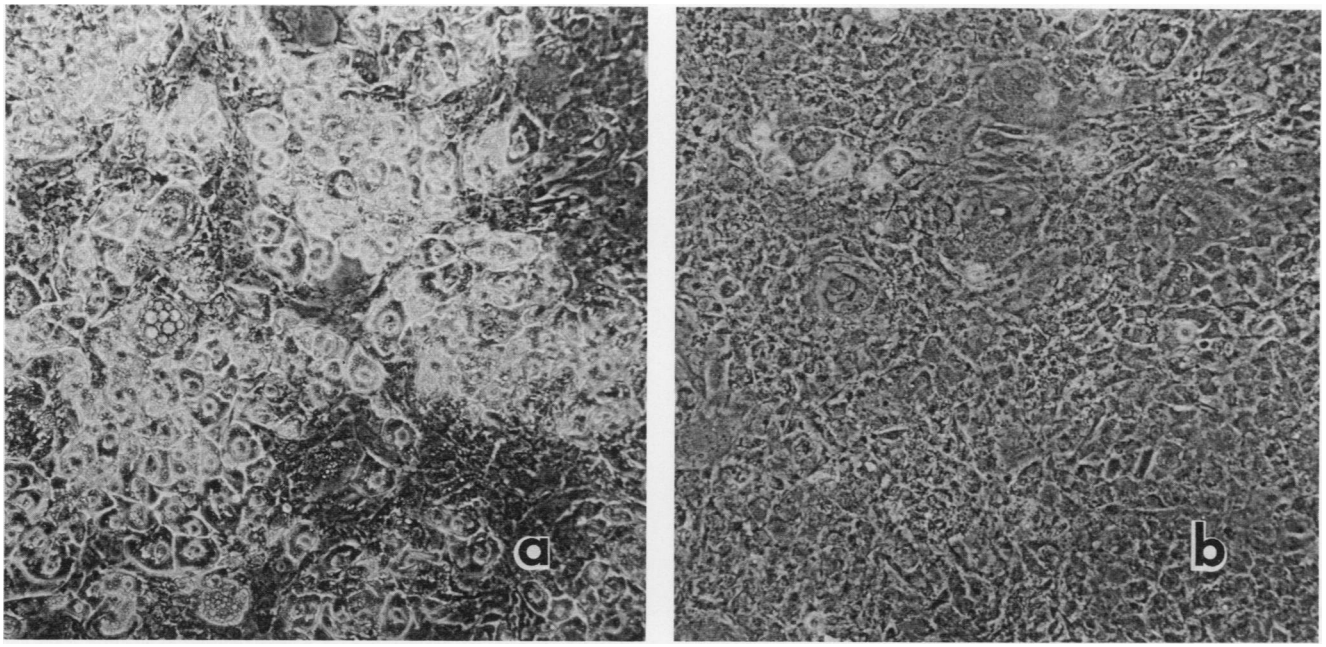

FIG. 4. Differentiation of untreated H-1/A cells and cachectin/TNF-treated H-1/A cells. (a) H-1/A adipocytes at 6 days after confluence. Obvious fat droplets in the cytoplasm were observed, which are an indication of differentiation. (b) H-1/A cells at 6 days after treatment with 200 U of cachectin/TNF per ml. Cachectin/TNF was added to the growth medium when cells reached confluence. The culture medium containing cachectin/TNF was changed every 3 days.

cachectin/TNF, as also found for TA 1 adipocytes (47). At confluence, the CSF-1 expression was significantly enhanced at 12 and 24 h after treatment with 200 U of cachectin/TNF per ml (Fig. 6a). However, GPD was not expressed in either the control cultures or the cachectin/TNF-treated cultures. For comparison, we analyzed the expression of the actin gene on the same blot. The level of actin mRNA did not change with cachectin/TNF treatment. To see whether cachectin/TNF increases CSF-1 expression transiently, we exposed the H-1/A cells to cachectin/TNF just at confluence. The culture medium containing 200 U of cachectin/TNF per ml was changed every day in order to avoid the effect by the degradation of cachectin/TNF in the medium. The CSF-1 transcripts increased and after reaching a peak on the second day began to decrease (Fig. 6b). On the ninth day after the treatment, the level of the CSF-1 was lower than that in the untreated H-1/A cells. We also saw the effects of cachectin/TNF on completely differentiated adipocytes. A 12-h exposure to 200 U of cachectin/TNF per ml was toxic to the differentiated H-1/A adipocytes.

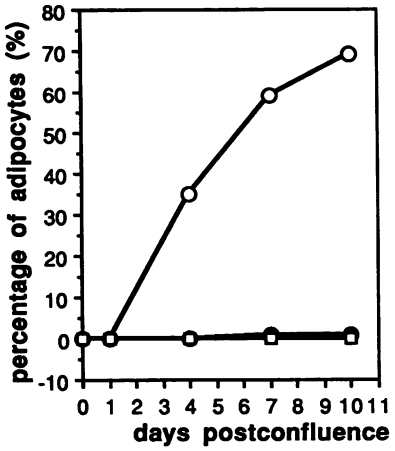

FIG. 5. Time course of cell differentiation in H-1/A cells, as measured by lipid-accumulating cells after the treatment with cachectin/TNF. H-1/A cells reached confluence at day 0. Cells were treated with 200 (●) or 1,000 (□) U of cachectin/TNF per ml at day 0. Cachectin/TNF was not added to the growth medium of a control culture (○). To estimate adipocyte differentiation, four random photomicrographs were taken for each dish, and about 2,000 cells were then counted in each dish. The mean percentage of adipocytes was calculated from four dishes.

To determine whether cachectin/TNF induced the production of active CSF-1, the colony assay was performed by using conditioned medium from the cachectin/TNF-treated H-1/A cells (Table 1). Cachectin/TNF increased CSF-1 activity in H-1/A culture medium. Neither cachectin/TNF nor growth medium alone stimulated the clonal proliferation of macrophages. The results suggest that cachectin/TNF induced CSF-1 at both mRNA and protein levels.

To determine whether these changes in the amounts of CSF-1 RNA were due to transcriptional or posttranscriptional events, we performed nuclear run-off transcription assays. When cells differentiated into adipocytes, the transcription rates of CSF-1-specific RNA did not change significantly, while those of actin RNA decreased by 60 and 70% in two independent experiments, respectively (Fig. 7a). From these results, we conclude that the change of RNA stabilization or any other posttranscriptional events contributed to the decrease of CSF-1-specific cytoplasmic RNA during adipocyte differentiation. In contrast, the induction of CSF-1 by cachectin/TNF exposure in the H-1/A cells at confluence occurred at the transcriptional level (Fig. 7b), although cachectin/TNF did not affect the transcription rate

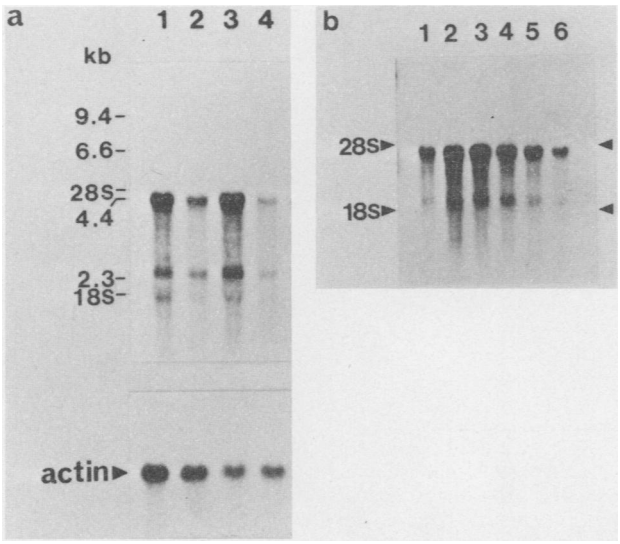

FIG. 6. Expression of CSF-1 (a and b) and actin (a) genes in H-1/A cells with exposure to cachectin/TNF. (a) H-1/A cells were treated with 200 U of cachectin/TNF per ml when cells reached confluence. RNAs were extracted from H-1/A cells at 12 (lane 1) and 24 (lane 3) h after treatment. Lanes 2 and 4 were the control cultures (without cachectin/TNF) for lanes 1 and 3, respectively. Ten micrograms of total RNA was electrophoresed in each lane. The blot was hybridized with the CSF-1 probe (upper gel) first. Then, the blot was dehybridized with a solution of boiled 0.01% SDS and 0.01× SSC 5 times and rehybridized with the actin probe (lower gel). (b) H-1/A cells at confluence were treated with 200 U of cachectin/TNF per ml. The culture medium containing cachectin/TNF was changed every day. RNAs were extracted from untreated H-1/A cells at confluence (lane 1) and H-1/A cells at 1 (lane 2), 2 (lane 3), 3 (lane 4), 6 (lane 5), and 9 (lane 6) days after the cachectin/TNF treatment. Ten micrograms of total RNA was electrophoresed in each lane. The blot was hybridized with the CSF-1 probe. The conditions of hybridization were described in Materials and Methods (a and b). The positions of the size markers and the 28S and 18S rRNA markers are indicated to the left of the gels. The filters were exposed for 48 (upper gel in panel a), 8 (lower gel in panel a), or 24 (panel b) h at −80°C, using an intensifying screen.

of the actin gene. The increased amounts of CSF-1-specific mRNA in response to cachectin/TNF exposure may also have been caused by a prolonged half-life of CSF-1 RNA. We isolated RNAs from the H-1/A cultures at confluence at different times after the addition of dactinomycin and determined the amounts of CSF-1-specific RNA. The quantitative evaluation of the decay of CSF-1 RNA by densitometry revealed that the half-life of CSF-1 RNA was about 2 h in both the cachectin/TNF-treated and untreated cultures (Fig. 8). The actin RNA used as an internal control was stable for more than 8 h. On the basis of these findings, we conclude that the changes in the transcription rates significantly contribute to the increased level of CSF-1 RNA in response to cachectin/TNF treatment.

DISCUSSION

Marrow stromal cells, H-1/A, accumulate fat droplets in their cytoplasm after confluence was reached. This morphological change represents adipocyte differentiation rather than uptake of fat from the culture medium, because expression of the GPD gene was induced after confluence was reached. GPD, which provides the glycerophosphate backbone for acylation, is one of the key enzymes for synthesis of triglyceride, a marker for adipocyte differentiation (44). Neutral triglyceride is synthesized from acetates in H-1/A cells (16).

TABLE 1. CSF-1 production in H-1/A cells after exposure to cachectin/TNF for 3 days[a]

Growth factor	Concn (U/ml) of cachectin/TNF	No. of days exposed	No. of cells plated/ml (10^4)	No. of plates	No. of colonies
CM H-1/A[b]	0	3	3	4	57 ± 8
	200	3	3	4	195 ± 25
	1,000	3	3	4	282 ± 8
	0	3	1	4	34 ± 5
	200	3	1	3	71 ± 6
	1,000	3	1	3	169 ± 27
Cachectin/TNF[c]	0		3	4	0 ± 0
	200		3	4	0 ± 0
	1,000		3	4	0 ± 0
	0		1	4	0 ± 0
	200		1	4	0 ± 0
	1,000		1	4	0 ± 0

[a] H-1/A cells were cultured with either 200 or 1,000 U of cachectin/TNF per ml for 3 days in Fischer medium supplemented with 10% horse serum. The conditioned medium were collected and tested (10% vol/vol) for their ability to stimulate the clonal proliferation of normal murine monocytic stem cells (CSF-1 activity). Fresh bone marrow cells were obtained from 8- to 12-week-old female C57/B1 mice by flushing their tibias and femurs. 3×10^4 or 1×10^4 fresh marrow cells were cultured in 1 ml of Iscove medium containing 0.5% agar and 10% conditioned medium in plastic dishes. After incubation for 6 days at 37°C in an atmosphere of 7.5% CO_2 in humidified air, the specimens were fixed with absolute methanol, air-dried, and stained. Colonies with more than 100 cells were scored with the aid of a Nikon optical microscope.

[b] CM H-1/A, Conditioned medium of H-1/A cells.

[c] As a control, the culture medium containing cachectin/TNF was incubated in the absence of cells. The culture medium containing cachectin/TNF was then used for the colony assay in the same manner as for CM H-1/A.

In H-1/A cells, the CSF-1 gene is expressed, but the G-CSF, GM-CSF, and IL-3 genes are not. The expression of CSF-1 gene is reduced after the differentiation of H-1/A cells into adipocytes. Our RNA blot analysis clearly demonstrates that the decrease of colony-stimulating activity levels after the adipocyte differentiation of H-1/A (30) can be explained by changes in the CSF-1 mRNA levels, not by those in its secretion levels. The isolation of adipocytes helped to clarify these results. The down-regulation of CSF-1 gene seems to be linked with the processes of differentiation per se, rather than with the culture condition, since another differentiation-incompetent subline, H-1/D, did not show any similar alterations in the differentiation or CSF-1 regulation. In the human promyelocytic cell line HL-60, down-regulated expression of myeloblastin is reported to cause arrest of growth and differentiation (2). In the differentiation of the murine erythroleukemia cell, c-*myc* mRNA levels decrease dramatically and the decline of c-*myc* expression is required for the commitment to the differentiation (6, 23). In the present study, CSF-1 mRNA dramatically decreased before GPD expression increased. The induction of CSF-1 by treatment with cachectin/TNF was accompanied with the inhibition of GPD expression. This suggests that the down-regulated expression in CSF-1 mRNA may be a prerequisite for the commitment of the H-1/A cells to enter the differentiation program. Alternatively, the down-regulation of the

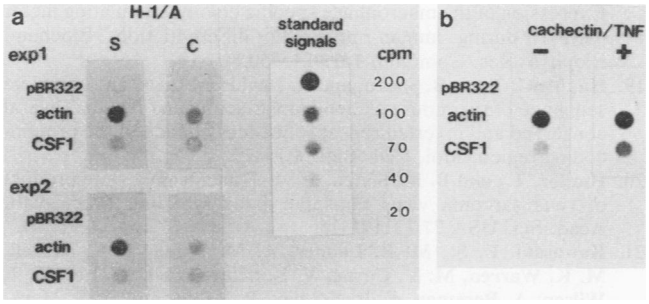

FIG. 7. Nuclear run-off transcription of H-1/A cells at semiconfluence and postconfluence (a) and cachectin/TNF-treated and untreated cells (b). (a) Semiconfluent H-1/A (H-1/AS) and H-1/A cells at 10 days postconfluence (H-1/AC) were harvested from four dishes, and nuclei were isolated. Almost 70% of the cells differentiated into adipocytes at 10 days postconfluence. The in vitro ^{32}P-labeled nuclear RNA was hybridized to the CSF-1 cDNA, and actin cDNA was immobilized to the nylon filter. The transcription rate of CSF-1 gene was estimated by measuring the amount of labeled RNA transcribed in nuclei isolated from the H-1/A cells. The amount of CSF-1-specific transcription was quantitated by hybridization to an excess of the CSF-1 cDNA bound to a Nylon filter. To demonstrate the specificity of observed signals, pBR322 DNA was used for nonspecific binding of the labeled nuclear RNA. pBR322 DNA gave weak signals. For the standard signals, ^{32}P-labeled DNAs of known radioactivity were dotted on the filter, as shown to the right of the gel. Spots on the autoradiograms were scanned with a densitometer. The areas of peaks for the standard signals on a recording sheet provide the standard curve. The intensity of sample signals was converted to counts per minute by using this standard curve. The value for the irrelevant DNA dot (pBR322 DNA) was subtracted from the values for the CSF-1 and actin signals. The ratios presented in the text were obtained by dividing the subtracted values for CSF-1 and actin of H-1/A cells at 10 days postconfluence by those of semiconfluent cells, respectively. The sample and signal-control filters were both exposed for 48 h at −80°C, using an intensifying screen. (b) H-1/A cells at confluence were treated with 800 U of cachectin/TNF per ml. After incubation for 15 h, cells were harvested for analysis. The ratio of the CSF-1 signal (cachectin/TNF-treated cells/untreated cells) was found to be 4.8, while that of the actin signal was found to be 1.0. The ratios were obtained as described above. The filter was exposed for 48 h at −80°C, using an intensifying screen.

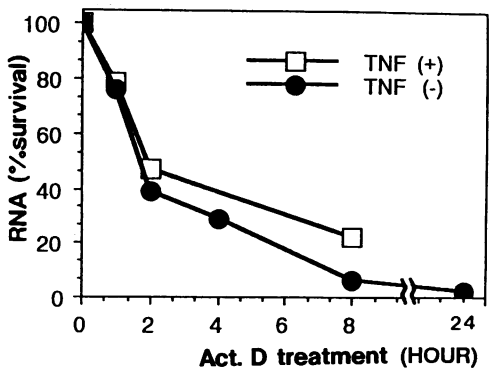

FIG. 8. Decay of CSF-1-specific mRNA in cachectin/TNF-treated (□) and untreated (●) H-1/A cells at confluence. H-1/A cells at confluence were treated with 200 U of cachectin/TNF per ml for 12 h followed by dactinomycin (Act. D) (5 μg/ml). The total RNA extracted at the times indicated after the addition of dactinomycin was analyzed by Northern blots, using a CSF-1 probe. Five micrograms of the total RNA was electrophoresed in each lane. The amount of the total RNAs was checked by 28S and 18S rRNAs stained with methylene blue. The decay rates of the CSF-1-specific RNA were determined from the band intensities from the autoradiograms of two or three independent RNA blots by densitometric scanning. The means for each set of data were plotted at the times indicated. The amount of RNA at the time of dactinomycin addition was regarded as equal to 100%. The cachectin/TNF-treated cells were killed with 24-h exposure to dactinomycin.

CSF-1 may result from the onset of differentiation. To address this question, we need to conduct further experiments to determine whether the constitutive expression of exogenous CSF-1 gene inhibits differentiation.

During the differentiation of H-1/A cells into adipocytes, the reduction of CSF-1 transcripts is due to a posttranscriptional event, such as the degradation of CSF-1 mRNA, rather than to a decreased rate of transcription. Posttranscriptional events are known to be one of the mechanisms which regulate cytoplasmic mRNA levels (7). In the terminal differentiation of erythroblasts, erythrocyte-specific mRNAs such as those of the globin gene appear to be conserved, while the other mRNAs are specifically destroyed (1). When the developmental cycle of the slime mold is interrupted, developmentally regulated mRNAs are specifically destroyed (5).

On the other hand, both CSF-1 protein and mRNA synthesis were induced by cachectin/TNF treatment in semiconfluent H-1/A cells. The induction of growth factors such as G-CSF and GM-CSF is reported in cachectin/TNF-treated lung fibroblasts and endothelial cells, although the transcription rates have not been studied (3, 22, 29). Recently, CSF-1 transcription has been investigated in human monocytes (17, 32, 42). In H-1/A cells at confluence, CSF-1-specific mRNA was induced at a transcriptional level by cachectin/TNF treatment. The half-life of the CSF-1 mRNA in the cachectin/TNF-treated H-1/A cells at confluence was almost the same as that in the untreated cells.

In the monocytes, the induction of CSF-1 by cachectin/TNF is transient and the level of CSF-1 transcripts returned to that of the control cells by 24 h (42). In H-1/A cells, the induction was observed for at least 3 days after treatment and the level of CSF-1 transcripts then returned to the level of the untreated cells. The possibility still remains that cachectin/TNF inhibited GPD expression only transiently. When cells were exposed to 200 and 1,000 U of cachectin/TNF per ml continuously, no adipocyte differentiation was observed for at least 10 days after confluence was reached and no GPD expression was detected for at least 9 days after confluence. Therefore, cachectin/TNF inhibits the expression of lipogenic enzymes, rather than delaying their expression.

The 4.5- and 2.5-kb transcripts of CSF-1 were expressed in H-1/A, while L cells express high levels of the 4.5-kb species as well as several other species of about 3.8, 2.3, and 1.4 kb (34). Multiple CSF-1 transcripts have been reported in a human pancreatic carcinoma cell line, MIA PaCa-2 (21), which arise from alternative splicing of a large primary transcript (24). In H-1/A, the 3′ sequence of the 4.5-kb transcript was not detected in the 2.5-kb species. The two major mRNA species may result from alternative splicing in H-1/A cells as well. The gene coding CSF-1 did not demonstrate any rearranged structure by Southern analysis of the H-1/A DNA with several restriction enzymes (data not shown).

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