Regulation of the Expression of Vimentin Gene during the Differentiation of Mouse Myeloid Leukemia Cells

Akio Tsuru, Nobuhiro Nakamura, Eiji Takayama, Yasuhiro Suzuki,* Kazunori Hirayoshi, and Kazuhiro Nagata

Department of Cell Biology and * Department of Molecular Pathology, Chest Disease Research Institute, Kyoto University, Sakyo-ku, Kyoto 606, Japan

Abstract. We have examined the expression of vimentin during the differentiation of mouse myeloid leukemia cells (M1), which were induced to differentiate into macrophages by exposure to conditioned medium (CM) obtained from rat embryo fibroblasts. The synthesis of vimentin, which was examined by two-dimensional gel electrophoresis, increased after 12–24 h of incubation of M1 cells in CM and the elevated level of synthesis continued up to 96 h. A macrophage cell line (Mml) that was derived from spontaneously differentiated M1 cells constantly synthesized much higher levels of vimentin. The amount of vimentin, which was revealed by immunoblot analysis using an mAb against human vimentin, also increased after differentiation by a factor of 7 when compared on the basis of constant protein and by a factor of 17 on the basis of constant cell numbers.

Mml cells contained >12- and 45-fold more vimentin compared with undifferentiated M1 cells on the bases of constant protein and constant cell numbers, respectively. Northern blot analysis using vimentin cDNA as a probe revealed increases in vimentin mRNA in the differentiated M1 cells and Mml cells. Nuclear run-on assay showed that the expression of vimentin gene during the differentiation of M1 cells was transcriptionally regulated.

Observations in indirect immunofluorescence microscopy and EM clearly showed that vimentin bundles were rarely observed in undifferentiated M1 cells, and increased amounts of and large-size vimentin bundles were easily observed in differentiated M1 and Mml cells. These results suggest the participation of increased amounts of vimentin filaments in the maldistribution of nuclei in M1 cells during differentiation.

INTERMEDIATE filaments represent a family of cytoskeletal structures whose members are expressed in developmentally regulated and tissue-specific patterns in vertebrate cells. There appear to be at least six distinct classes of proteins making up the various intermediate filaments (37). Although the physiological function of intermediate filaments remains obscure, these filaments have been thought to play a role in nuclear centralization, organelle movement, and the maintenance of cell shape (9, 17, 38). Among them, vimentin is present in the majority of cells of mesenchymal and nonmesenchymal origin, and its filaments are associated with both the nuclear and plasma membranes (9, 17).

Vimentin mRNA was reported to be increased after stimulation by the addition of serum or growth factor, such as PDGF, but not by the addition of epidermal growth factor nor insulin (34). Vimentin mRNA was not detected in Burkitt's lymphoma cells, whereas it was detected in normal lymphocytes and fibroblasts (19). The synthesis of vimentin was also reported to be decreased in monkey kidney cells transformed by SV-40 and in rat myoblast cells which express the v-*mos* gene (2, 36).

There are many reports that show the regulation of vimentin gene expression during differentiation. Although vimentin mRNA is found at low levels in immature, mitotic primitive erythroid cells of chicken embryo, and accumulates to increasingly higher levels during terminal differentiation of the definitive erythroid lineage, vimentin mRNA levels decline during the differentiation of human erythroid cells and murine erythroleukemia (MEL)[1] cells (3, 31).

Mouse myeloid leukemia (M1) cells were induced to differentiate into macrophages by exposure to conditioned medium from rat embryo fibroblasts that contained differentiation–inducing factors (14). Recently, two cytokines possibly contained in these conditioned medium, IL 6 and leukemia inhibitory factor (LIF), have been shown to induce the differentiation in M1 cells (7, 8, 20, 23). Differentiated M1 cells are no longer leukemogenic. During this differentiation, M1 cells acquire phagocytic and locomotive activities rendering concomitant quantitative and qualitative changes in actin and actin-binding proteins (15, 25, 26, 28, 29, 39). In addition to the functional changes, M1 cells display mor-

---

1. *Abbreviations used in this paper:* CM, conditioned medium; GAPDH, glyceraldehyde 3 phosphate dehydrogenase; MEL, murine erythroid leukemia cell line; M1, mouse myeloid leukemia cell line; Mml, macrophage cell line; rhIL 6, recombinant human interleukin 6; rhLIF, recombinant human leukemia inhibitory factor; TCA, trichloroacetic acid.

phological differences such as enlarged cytoplasm, increased filopodia, nuclear maldistribution, and so forth (12). Hayashi et al. quantified the difference in nucleus/cytoplasm ratios before and after differentiation of M1 cells (11).

We describe here marked changes in the expression of vimentin gene during the differentiation of M1 cells in terms of synthesis, amount, and mRNA levels. We also show the appearance of vimentin bundles in differentiated M1 cells by indirect immunofluorescence microscopy and EM.

Materials and Methods

Cell Line, Culture, and Metabolic Labeling

The M1 cell line was established from a myeloid leukemia of an SL strain mouse and has been maintained in vitro in our laboratory since 1969 (14). M1 cells were cultured in DME (Gibco Laboratories, Grand Island, NY) with 10% heat-inactivated horse serum (Cell Culture Laboratories, Cleveland, OH), were morphologically myeloblasts, and were neither motile nor phagocytic. When incubated for several days with various differentiation inducers, they differentiated into macrophage-like cells, and acquired motility and phagocytic activity, accompanied by the induction of other differentiated functions such as adherence to the substratum and the loss of mitotic activity (13, 24, 35). Conditioned medium (CM) was obtained from rat embryo fibroblasts as described previously (26), and used at a concentration of 10% (vol/vol) to induce the differentiation in M1 cells. Recombinant human IL 6 was kindly donated from Dr. J. Sagara (TOSO Co., Tokyo, Japan), and recombinant human LIF was a kind gift from Dr. M. Hozumi (Saitama Cancer Center Research Institute, Saitama, Japan).

The Mm1 line was derived from the M1 line and was maintained in vitro as macrophage-like cells (22). This line showed very active phagocytosis without CM incubation. The D⁻ line was also derived from the M1 line (25) and was not induced to differentiate into macrophages when treated with CM, IL 6, or LIF.

For metabolic labeling, 1×10^7 cells were incubated in 1 ml of methionine-free DME for 1 h, and then labeled with 0.1 mCi/ml of [³⁵S]methionine, (660.9 Ci/mmol sp act, New England Nuclear, Boston, MA) for 2 h at 37°C. Cells were washed with PBS three times after labeling and lysed in a urea-containing sample solution described by O'Farrell (33).

Gel Electrophoresis

One-dimensional SDS-PAGE was performed according to the method of Laemmli (16). Cells were washed with PBS and lysed in Laemmli's SDS-

sample buffer. Aliquots were resuspended in PBS and the protein concentration was determined (21).

Two-dimensional gel electrophoresis was performed according to the method described by O'Farrell (33). The gels for the first dimension contained 1.4% pH 5-8 Ampholine (LKB Instruments; Gaithersburg, MD) and 0.6% pH 3.5-10 Ampholine. The concentration of the SDS-polyacrylamide gels used for the second dimension was 4% polyacrylamide for the stacking gels and 10% for the resolving gels. Samples containing equal TCA-precipitable radioactivities were analyzed by two-dimensional gel electrophoresis. After being fixed in 50% TCA, the gels were immersed in 1 M sodium salicylate, dried, and exposed to x-ray film for fluorography (Fuji RXO-H film; Fuji Photo Film Co. Ltd., Kanagawa, Japan).

Immunoblot Analysis with Anti-vimentin Antibody

SDS-polyacrylamide slab gels were blotted onto nitrocellulose membranes as described by Towbin et al. (40). After incubation in a 10% skim milk solution (Yukijirushi Milk Co., Sapporo, Japan), blotted filters were incubated with mouse mAb against human vimentin (IgM class; Bioyeda, Rehovot, Israel). Guinea pig antibody against mouse μ and κ chains was labeled with Na¹²⁵I by the chloramine T method, and used as the second antibody. After autoradiography, the corresponding vimentin bands were cut out, and the radioactivities were quantitated using a gamma counter (LKB Instruments).

Northern Blot Analysis and Dot Blot Analysis

Total RNAs were extracted from M1 cells with or without CM treatment and from Mm1 cells by guanidine thiocyanate/cesium chloride methods as described by Chirgwin et al. (4). Poly(A)-RNAs were purified by two cycles of oligo(dT)-cellulose column chromatography according to the methods of Aviv and Leder (1).

Total RNAs (10 μg/lane) or poly(A)-RNAs (1 μg/lane) were separated on 1% agarose gel containing formaldehyde (18), and were transferred onto nylon membrane filters (Gene Screen Plus, DuPont/New England Nuclear, Boston, MA). Blotted membranes were prehybridized overnight at 42°C in 5× SSC (1× SSC, 0.15 M NaCl, 15 mM sodium citrate, pH 7.0) containing 50% deionized formamide, 2× Denhardt's solution (1× Denhardt's, 0.02% polyvinylpyrolidone, 0.02% BSA, 0.02% Ficoll), 1% SDS, and 100 μg/ml sonicated-denatured salmon sperm DNA. A cDNA probe for vimentin, Eco R1-Bam H1 1.9-kb fragment of pCV96/1.9EB plasmid (kindly provided by Dr. E. Lazarides, California Institute of Technology), was labeled with ³²P by the multi-prime labeling method as described by Feinberg and Vogelstein (5). cDNA probes for β-actin (a kind gift from Dr. S. Sakiyama, Chiba Cancer Center Research Institute), and for glyceraldehyde 3 phosphate dehydrogenase (GAPDH; kindly provided by Dr. M. Hatanaka, Institute for Virus Research, Kyoto University) were also labeled for control experiment.
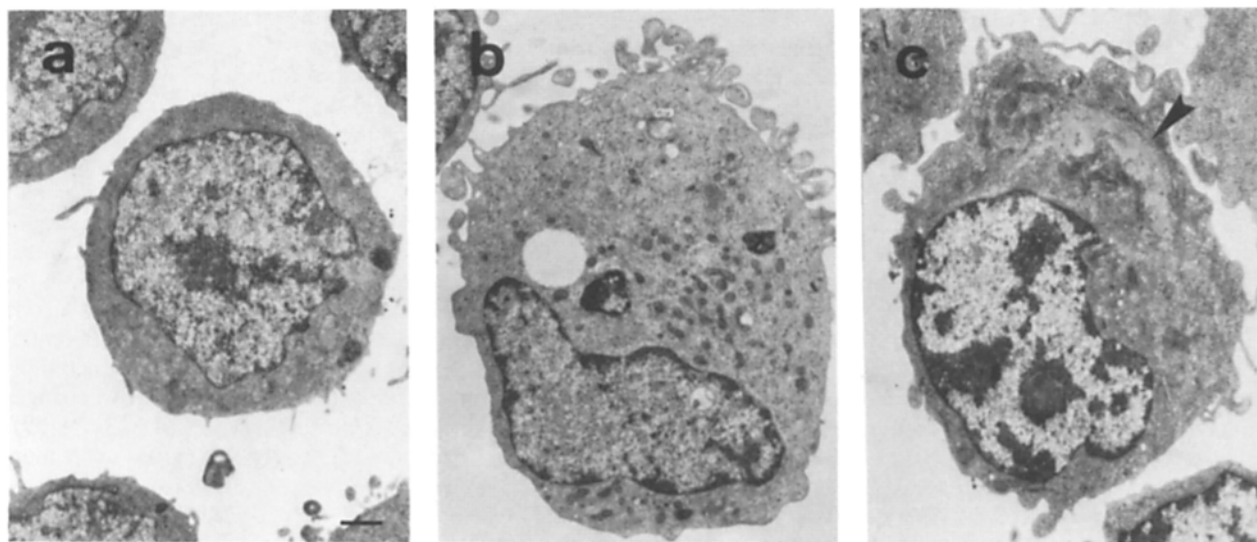

Figure 1. Low-power profiles by electron microscopy of M1 cells (*a*), M1 cells treated with 10% (vol/vol) CM for 3 d (*b*), and Mm1 cells (*c*). Cells were fixed, stained, and observed as described in Materials and Methods. Bar in *a*, 1 μm.

The filter was hybridized with ^{32}P-labeled cDNA ($1.2-2 \times 10^7$ cpm/ml) at 42°C for 16 h in 5× SSC containing 50% deionized formamide, 1× Denhardt's, 1% SDS, and 200 µg/ml sonicated-denatured salmon sperm DNA. After washing several times with 2× SSC at room temperature, 2× SSC containing 0.5% SDS at 65°C, and 0.1× SSC at room temperature, the filter was exposed at −80°C for 16 h using Fuji RXO-H film with Grenex 8 intensifying screens (Fuji Photo Film Co. Ltd.).

Dot blot analysis was performed as described above after the RNAs were blotted onto nitrocellulose membrane filters using a vacuum blot apparatus (Bio-Dot; Bio-Rad Laboratories, Richmond, CA).

Nuclear Transcriptional Analysis

M1 cells, M1 cells treated with 10% (vol/vol) CM for 3 d, and Mm1 cells were washed three times with ice-cold 1× SSC, and incubated in NP-40 lysis buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 0.5% (vol/vol) NP40) for 5 min on ice. After centrifugation at 500 g for 5 min, the nuclear pellets were frozen in 50 mM Tris-HCl pH 8.3, 40% (vol/vol) glycerol, 5 mM MgCl₂, 0.1 mM EDTA. Thawed nuclei were incubated in the reaction mixture containing 0.1 mCi of [α-^{32}P]UTP for the run-on assay described by Greenberg and Ziff (10). An equal amount of ^{32}P-labeled RNA, in terms of counts per minute, was hybridized with each cDNA immobilized on nitrocellulose membranes. For nitrocellulose binding, linearized plasmids by restriction enzyme digestion were denatured by NaOH treatment, and spotted onto nitrocellulose filters using the vacuum blot apparatus. 10 µg DNA was applied per dot.

Indirect Immunofluorescence Microscopy

Cells were attached to glass slides using cytospin, fixed with 4% paraformaldehyde in PBS at 4°C for 5 min, and washed in PBS three times. After brief permeabilization with 0.2% Triton X-100 in PBS for 10 min at room temperature, cells were incubated in anti-vimentin antibody for 1 h at room temperature. Cells were washed three times with PBS, incubated with FITC-labeled second antibody (rabbit anti-mouse IgM; Zymed Corp., San Francisco, CA) for 1 h, washed again, and then observed by means of fluorescence microscopy (Nikon, Tokyo, Japan).

Electron Microscopy

Cells were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at room temperature for 2 h, and then treated with 7% sucrose in 0.1 M phosphate buffer overnight at 4°C. After washing with phosphate buffer, the cells were postfixed with 1% osmium in 0.1 M phosphate buffer for 1 h at 4°C. Cells were dehydrated with serial concentrations of ethanol (50, 70, 90, and 100%) at 4°C, again with 100% ethanol at room temperature, and finally with propyrene oxide. After being embedded in Lubea K812 (Nacalai Tesque, Kyoto, Japan), thin sections were prepared using an ultramicrotome (Ultrotome 2088; Pharmacia LKB, Uppsala, Sweden), stained with uranyl acetate and lead actinate, and were observed using a JEM100CX type electron microscope (JOEL, Tokyo, Japan).

Results

Morphological Changes in M1 Cells during Differentiation

Undifferentiated M1 cells are small leukemic myeloblasts with large and round nuclei when observed by EM (Fig. 1 a), or by microscopy after staining the cells with May-Grünwald-Giemsa solution (data not shown; see references 11 and 14). After incubation for 2 or 3 d in the CM from rat embryo fibroblasts containing the differentiation-inducing factor, M1 cells were induced to differentiate into macrophages. Differentiated M1 cells treated with CM for 2 or 3 d and Mm1 cells, which are macrophage-like cells originating from spontaneously differentiated M1 cells were also observed by electron microscopy (Fig. 1, b and c). In differentiated cells, the size of the cells increased and cells became irregular in shape with numerous villiary projections and blebs of the cytoplasm, philopods, and creeping pseudopods. The nuclei seemed to be deeply indented and polymorphic. The cytoplasm of CM-treated cells became more hypertrophic with an increase in the number of organelles. Mm1 cells seemed similar to CM-treated M1 cells, and large filamentous bundles were often found when observed even by low-power electron microscopy (Fig. 1 c, arrowhead). As typically seen in macrophages, nuclei in differentiated M1 and Mm1 cells were squeezed into one side of the cell.
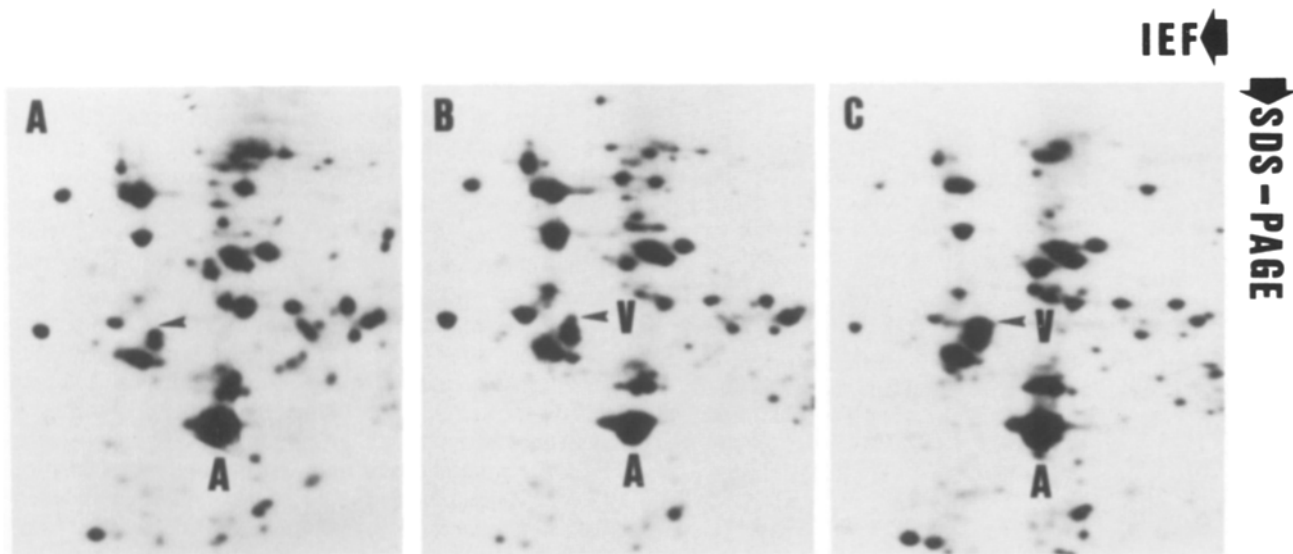

Figure 2. Two-dimensional analysis of the extract from M1 cells (A), M1 cells treated with CM (B), and Mm1 cells (C). M1 and Mm1 cells and M1 cells incubated with 10% (vol/vol) CM for 3 d were labeled for 2 h with 0.1 mCi/ml of [35S]methionine. Cells were lysed in the lysis buffer described by O'Farrell (33), and aliquots of the cell lysate containing equal amounts of TCA-precipitable radioactivity were analyzed by two-dimensional gel electrophoresis consisting of isoelectric focusing as the first dimension (right to left) and SDS-10% polyacrylamide slab gels as the second dimension (top to bottom). Arrowheads indicate the position of vimentin. A, actin.
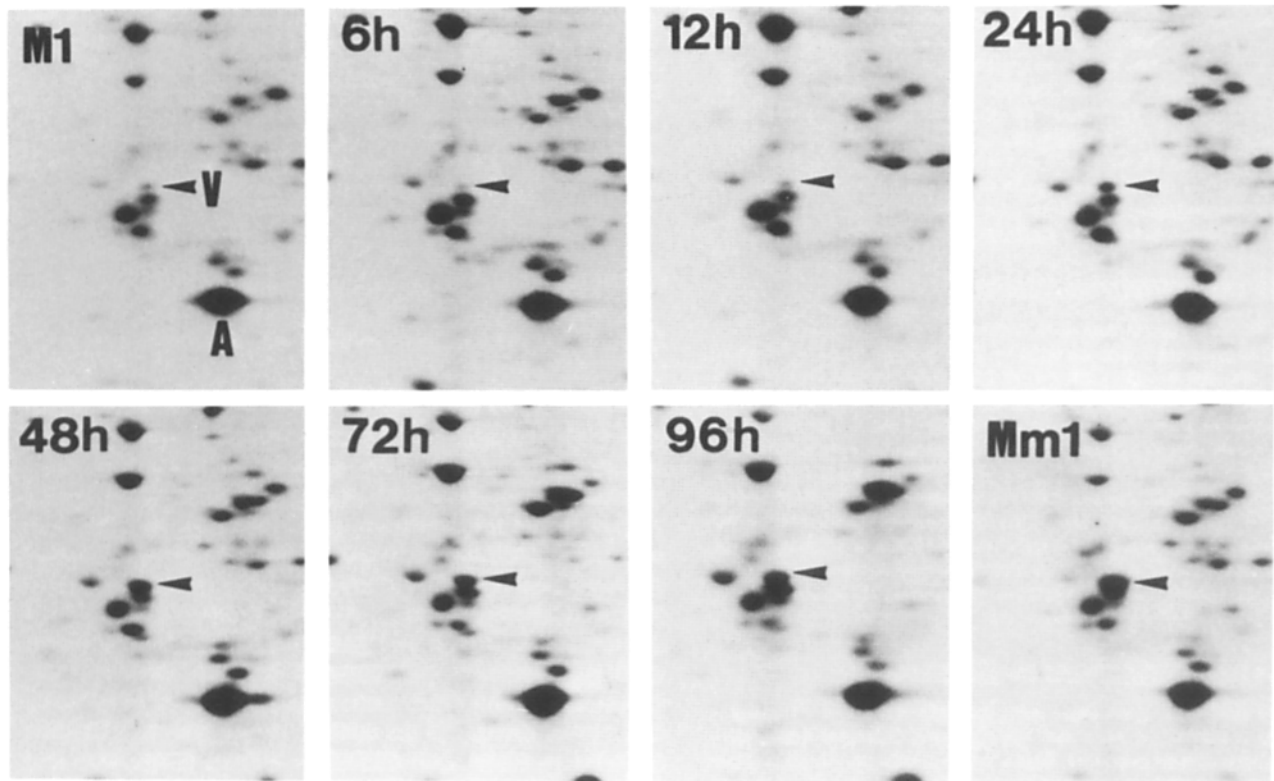
Figure 3. Kinetics of vimentin synthesis after the incubation of M1 cells with CM. M1 cells were labeled with 0.1 mCi/ml [^{35}S] methionine for 2 h at various times after the treatment with 10% (vol/vol) CM, and lysed in O'Farrell's lysis buffer. Aliquots containing equal amounts of TCA-precipitable radioactivity were analyzed as described in Fig. 2. Untreated M1 cells and Mm1 cells were also analyzed as controls. Arrowheads indicate the position of vimentin. *A,* actin.

Induction of Vimentin during Differentiation

M1 cells, CM-treated M1 cells, and Mm1 cells were labeled with [^{35}S]methionine, and the total cell lysates were analyzed by two-dimensional gel electrophoresis. In addition to several differences in the spots among these three cell lysates on two-dimensional gels, the synthesis of vimentin was clearly observed to increase after the differentiation of M1

Figure 4. Immunoblot analysis of vimentin induction. M1 cells (lane *1*), M1 cells treated with 10% (vol/vol) CM for 3 d (lane *2*), and Mm1 cells (lane *3*) were washed with PBS and lysed in Laemmli's SDS-sample buffer. Aliquots of the cells were suspended in PBS, sonicated for 20 s three times at the maximum setting of the ultrasonic generator (Kaijo Denki, Ltd., Tokyo, Japan), and the protein concentration of the lysate was determined by Lowry's method (21). After electrophoresis using SDS-10% polyacrylamide slab gels containing DTT, blotting of the protein was performed according to the method of Towbin et al. (41). Anti-human vimentin mAb (IgM; Bioyeda) was used at a dilution of 2,000-fold, and then detected by ^{125}I-labeled anti-mouse μ and κ chain antibody (guinea pig). Fuji RXO-H x-ray film was used for autoradiography.

cells (Fig. 2). The synthesis of vimentin in Mm1 cells was higher than that in CM-treated M1 cells. The kinetics of vimentin synthesis induction was examined by treating the cells with CM and by labeling with [^{35}S]methionine at different times after CM treatment (Fig. 3). Control M1 cells synthesized only a trace amount of vimentin, and the synthesis of vimentin increased at 12–24 h after CM treatment. After the synthesis reached a maximum level at 48 h, the high level of vimentin synthesis continued up to 96 h.

The amount of vimentin was examined by using immunoblot analysis with mAb against vimentin. Fig. 4 shows that the amount of vimentin was increased after differentiation of M1 cells while only a trace amount of vimentin was detected in undifferentiated M1 cells. The amount of vimentin in Mm1 cells was further increased as was the case with synthesis.

Time-course analysis of vimentin accumulation in the cytoplasm was also performed by immunoblot analysis at different times after CM treatment. Each band of vimentin on the filter detected by immunoblot analysis at different times was excised and the radioactivity was counted by a gamma counter to quantify the amount of vimentin. As seen in Fig. 5, the amount of vimentin was rapidly increased after 6–12 h of CM treatment, reached a peak at 48–72 h, and then decreased. The increase in CM-treated M1 cells at the peak was over sevenfold higher than nontreated control M1 cells when an equal amount of protein was applied to SDS-PAGE, and over 17-fold higher when compared on the basis of equal cell numbers. The amounts of vimentin in Mm1 cells were >12- and 45-fold higher compared with those in undifferentiated M1 cells on the bases of equal protein and equal cell
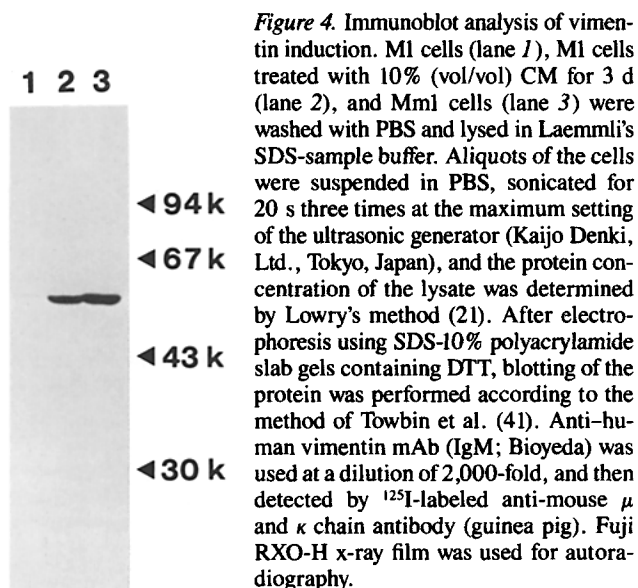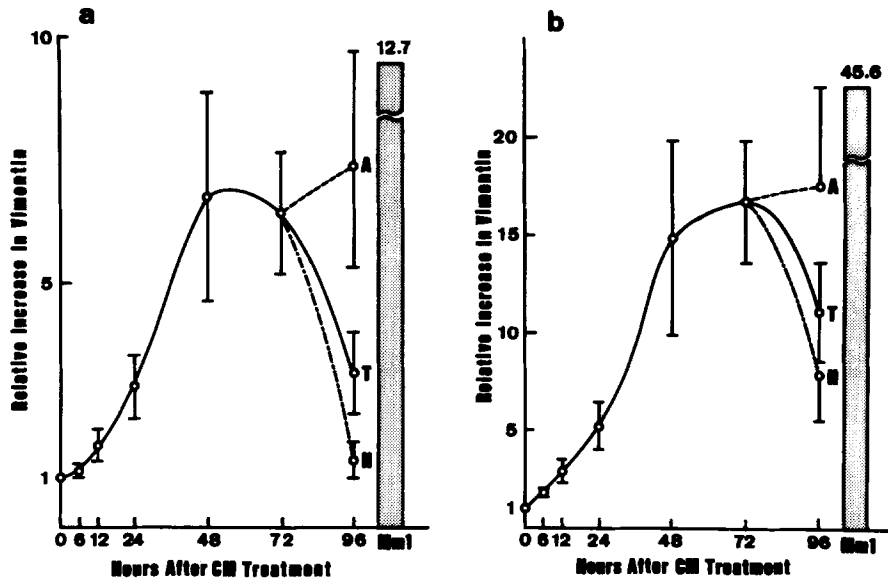
Figure 5. Kinetics of vimentin accumulation after the incubation of M1 cells with CM. M1 cells were lysed in Laemmli's SDS-sample buffer at various times after the treatment with 10% (vol/vol) CM and analyzed by immunoblotting as described in Fig. 4. Vimentin bands on the dried gel were cut out by superimposing the x-ray film, and the radioactivity was counted by a gamma counter (Pharmacia LKB). *A* is the result of electrophoresis by adjusting the amount of protein to be applied to the gels, and *b* is the result obtained by adjusting the cell numbers per lane. The abscissa indicates the relative increase in vimentin by normalizing the amount of vimentin in nontreated M1 cells to 1. The amounts of vimentin in Mml cells are indicated by bars at the right side of each figure. Adherent (*A*) and nonadherent (*N*) M1 cells were separately collected at the fourth and fifth day of incubation, and the amounts of vimentin were determined as above.

numbers, respectively (see bars in Fig. 5). The differences in induction between equal protein and equal cell numbers were due to the protein contents per cell. Differentiated M1 cells and Mml cells contained approximately two- and four-fold higher levels of protein per cell, respectively, when compared with nontreated M1 cells. The decrease in the amount of vimentin after 3 d of incubation with CM appeared to be due to cell death, because the vimentin content did not decrease when only the cells adhering to the dish were analyzed (Fig. 5). The viability and phagocytosis of nonadherent cells, consisting of both undifferentiated cells and dead cells, were lower than those of adherent cells. The content

of vimentin of nonadherent cells was markedly low at 96 h of incubation.

To exclude possible influence of unknown factor(s) in CM on the expression of vimentin regardless of differentiation, we examined the effect of IL 6 and LIF, two differentiation-inducing factors for M1 cells, on the expression of vimentin in M1 cells and also the effect of CM on that in D⁻ subline of M1 cells, which is resistant to IL 6 and LIF as well as in Balb/3T3 cells. As shown in Fig. 6, the level of vimentin in M1 cells treated with 1,000 U/ml rh (recombinant human) IL 6 for 3 d was similar to that of cells treated with CM. Recombinant hLIF also induced vimentin in M1 cells (data not shown). On the other hand, CM did not influence the amount of vimentin accumulated in D⁻ cells or Balb/3T3 cells.
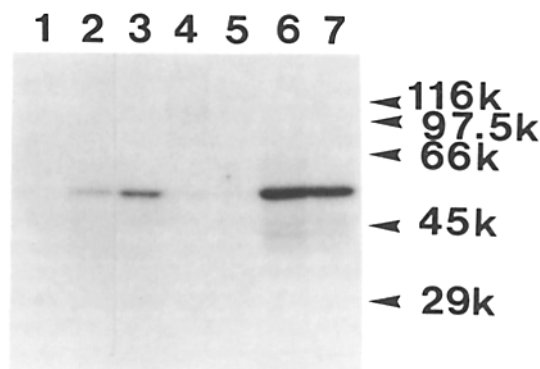

Figure 6. Effect of IL-6 on the amount of vimentin in M1 cells and effect of CM on that in D⁻ cells and Balb/3T3 cells. M1 cells were treated with 10% (vol/vol) CM or with 1,000 U/ml rhIL 6 for 3 d. D⁻ cells or Balb/3T3 cells were treated with 10% (vol/vol) CM for 3 d. The amount of vimentin was determined on the basis of equal protein concentration as described in Fig. 4. Lane *1*, M1 cells without treatment; lane *2*, M1 cells treated with CM; lane *3*, M1 cells treated with rhIL-6, lane *4*, D⁻ cells without treatment; lane *5*, D⁻ cells treated with CM; lane *6*, Balb/3T3 cells without treatment; and lane *7*, Balb/3T3 cells treated with CM.

Transcriptional Regulation of Vimentin Expression

Next, we examined the induction of vimentin at the mRNA level. Total RNAs isolated from undifferentiated M1, CM-treated M1 and Mml cells were electrophoresed under denatured conditions with formamide, blotted onto nylon membranes, and hybridized with cDNA for chick vimentin. RNAs were also hybridized with cDNA for mouse β-actin and GAPDH as controls. Fig. 7 *C* clearly shows the induction of mRNA for vimentin in differentiated M1 cells and Mml cells. In differentiated M1 cells and Mml cells, mRNA levels for β-actin were 1.3–2.0-fold higher than that in undifferentiated M1 cells, which coincides with our previous results at the protein level (25). Fig. 7 also shows that mRNA levels for GAPDH did not change during the differentiation. Because the size of vimentin mRNA was ~2.1 kb, which was equal to that of 18S ribosomal RNA, we further checked the induction of vimentin mRNA by using oligo(dT) cellulose-purified mRNAs for Northern blot analysis to exclude the possibility of cross-reaction with 18S ribosomal RNA. The induction of vimentin mRNA after differentiation of M1 cells
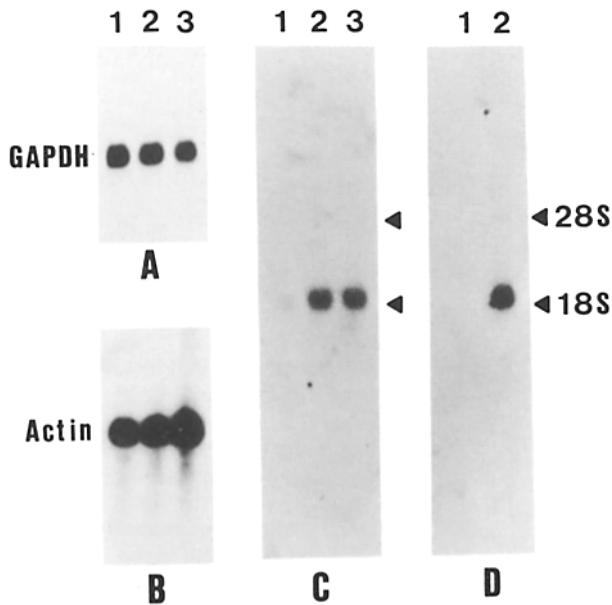
Figure 7. Detection of vimentin mRNA in M1 cells, M1 cells incubated with CM, and Mm1 cells. (*A*, *B*, and *C*) Total RNA was isolated by the methods of Chirgwin et al. (4) from M1 cells (lane *1*), M1 cells treated with 10% (vol/vol) CM for 3 d (lane *2*), and Mm1 cells (lane *3*). (*D*) Poly(A)-RNA was further purified by two cycles of oligo(dT)-cellulose column chromatography according to the procedure of Aviv and Leder (1). Lane *1*, poly(A)-RNA from M1 cells; lane *2*, poly(A)-RNA from CM-treated M1 cells. Total RNA and poly(A)-RNA were separated on 1% agarose gel containing formaldehyde, transferred onto nylon membrane filters, and hybridized with ^{32}P-labeled cDNAs for GAPDH (*A*), β-actin (*B*), and vimentin (*C* and *D*) as described in Materials and Methods. Arrowheads indicate 28S and 18S ribosomal RNA bands.

was confirmed by Northern blot analysis using purified mRNAs (Fig. 7 *D*).

The kinetics of vimentin mRNA accumulation were examined by using total RNAs isolated from M1 cells at different times after CM treatment by Northern blot analysis (Fig. 8, *A* and *B*) and by dot blot analysis (data not shown). Control M1 cells and Mm1 cells were also assayed as negative and positive controls, respectively. As shown in Fig. 8, mRNA levels for vimentin increased after 12 h of CM treatment, with the peak occurring after 48 h. When both adherent and nonadherent cells were collected together, the levels of vimentin mRNA were found to have decreased after 48 h of incubation. This decrease might be due to the cell death because of prolonged culture of differentiated M1 cells without medium change. Thus, we collected only adherent cells whose viability was >90%. The level of vimentin mRNA in these cells decreased only slightly after 72 or 96 h.

The amount of vimentin mRNA in Mm1 cells was also examined by Northern blot analysis and quantitated by dot blot analysis (Fig. 8). When Mm1 cells, which are adherent macrophage-like cells, were collected after reaching confluence, their level of vimentin mRNA was higher than that of undifferentiated M1 cells, but significantly lower than the maximum level in CM-treated M1 cells. However, the level of vimentin mRNA in Mm1 cells collected from the sparse culture was slightly higher than the maximum level in CM-treated M1 cells.

To examine whether the expression of vimentin mRNA is regulated at the transcriptional level, we performed a nuclear run-on assay by labeling the nucleus with α-[^{32}P]-UTP, and by hybridizing the labeled mRNA in the nucleus with cDNA
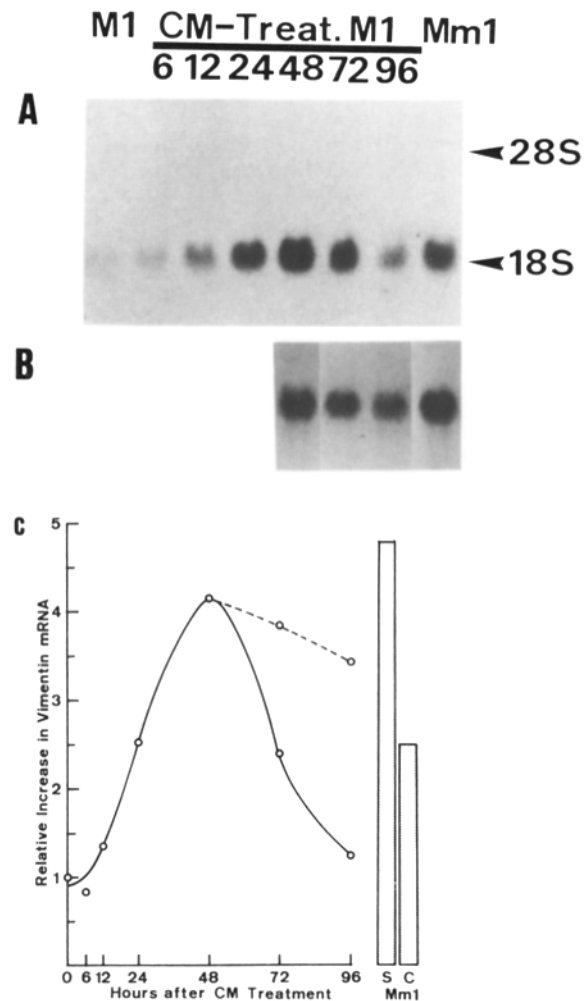

Figure 8. Kinetics of vimentin mRNA induction during the differentiation of M1 cells. Total RNAs prepared from the cells at various times of incubation with 10% (vol/vol) CM were separated on 1% agarose gel as described in Fig. 7, and the amount of vimentin mRNA was detected with a ^{32}P-labeled cDNA probe for vimentin (*A*). Total RNAs were also prepared from only adherent cells obtained on the fourth and fifth day of incubation with CM, and the amount of vimentin mRNA was detected as above (*B*). Mm1 cells were inoculated at the density of 3×10^6 cells/dish (9 cm in diameter), and collected at the third day (Mm1 in Fig. 8 *B*), or at the sixth day (Mm1 in Fig. 8, *A*). The medium of the latter culture of Mm1 cells was changed at the third day. Total RNAs were serially diluted and blotted onto nylon membrane filters, then hybridization was performed using a ^{32}P-labeled cDNA probe for vimentin. Each spot on the filter was excised and the radioactivity was counted. By using the linear portion of the radioactivity in the serially diluted sample, the induction kinetics were determined (*C*). *M1* indicates the RNA isolated from nontreated M1 cells, and *Mm1* from Mm1 cells. The dotted line in Fig. 8 *C* indicates the amount of vimentin mRNA in the adherent cells. In Fig. 8 *C*, the abscissa indicates the relative increase in vimentin mRNA where the amount of vimentin mRNA of nontreated M1 cells was normalized to the value 1.
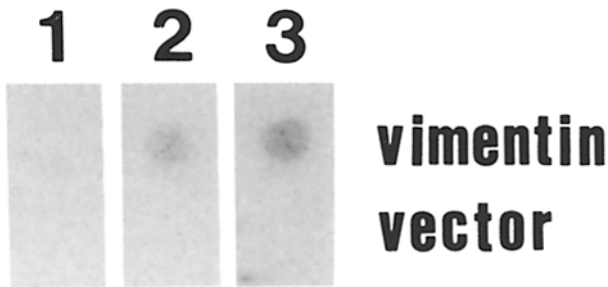
1 2 3

vimentin

vector

Figure 9. Nuclear transcriptional analysis of vimentin gene during differentiation. Vimentin cDNA bound to nitrocellulose filters was hybridized with ^{32}P-labeled run-on transcripts in nuclei isolated from nontreated M1 cells (*1*), CM-treated M1 cells (*2*), and Mm1 cells (*3*). Vector DNA (pSP65) was blotted onto nitrocellulose filters, and analyzed similarly for control experiment.

for vimentin which was blotted onto nitrocellulose membrane filters. The transcripts were clearly increased in CM-treated cells and Mm1 cells (Fig. 9), suggesting that the induction of vimentin during the differentiation of M1 cells was transcriptionally regulated.

Appearance of Vimentin Bundles after Differentiation

Indirect immunofluorescence observation was performed by using an mAb against human vimentin for undifferentiated M1, CM-treated M1, and Mm1 cells. Whereas M1 cells were

not stained or very weakly stained if any, CM-treated M1 cells contained strongly stained filamentous structures (Fig. 10). The intensity of the staining was drastically increased in Mm1 cells. The nuclei in some Mm1 cells seemed to be surrounded by networks consisting of thick bundles of vimentin, and others showed strong staining of vimentin at the one side of the cytoplasm (Fig. 10 *f*).

Electron microscopic observations at high magnitudes revealed the differences in vimentin bundles among these three cells. We hardly detected vimentin bundles in undifferentiated M1 cells. Fig. 11 *a* shows a small cluster of wavy filaments, the diameter of which was ~10 nm, in undifferentiated M1 cells. However, it was very hard to find such clusters in undifferentiated M1 cells, and the possibility of detecting such clusters of vimentin was below 1 out of 50 thin section pieces of the cells searched. During differentiation, it became very easy to detect vimentin bundles in the cells. Fig. 11 *b* shows bundles of vimentin in cells treated with CM for 3 d. The sizes of the bundles were larger than those in M1 cells. The sizes and the frequency of vimentin bundles became more prominent during the prolonged incubation with CM (Fig. 11 *c*, 4 d incubation). In Mm1 cells, large parts of the cytoplasm were occupied by vimentin bundles (Fig. 11 *d*). Almost all thin section preparations of Mm1 cells had such prominent bundles.

These biochemical and morphological observations described above were consistent, and clearly indicated that vimentin synthesis was induced and that thick bundles were
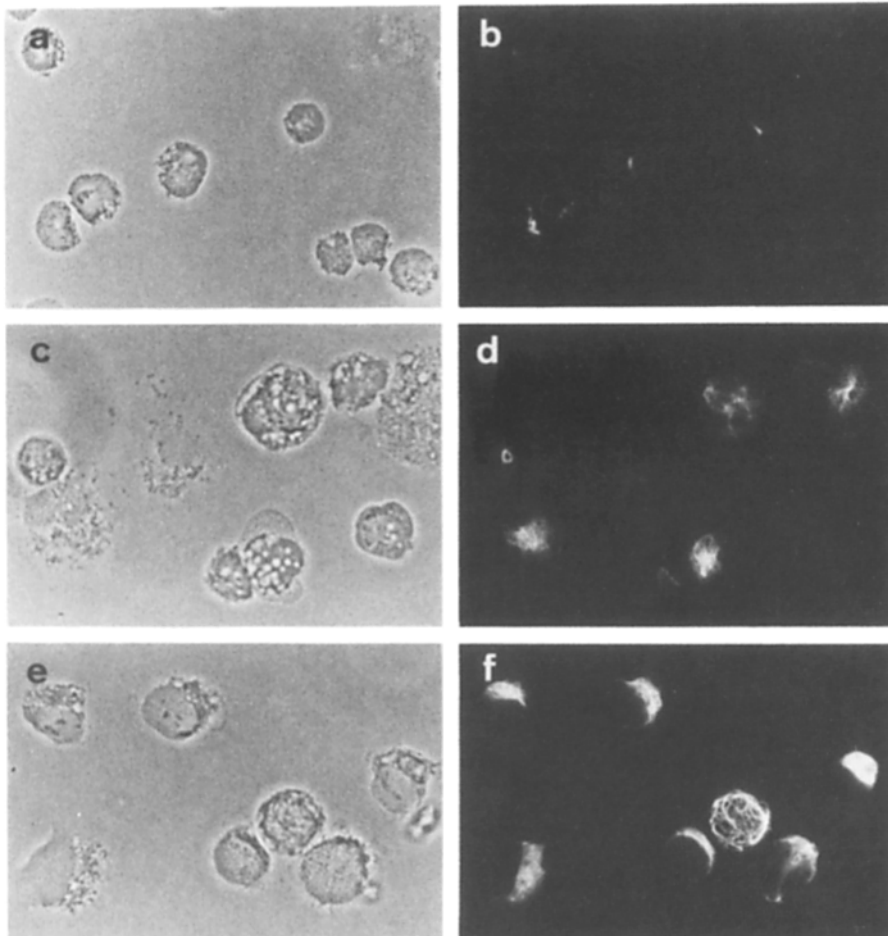

Figure 10. Immunofluorescence microscopic observation of vimentin. M1 cells (*a* and *b*), M1 cells treated with 10% (vol/vol) CM for 3 d (*c* and *d*), and Mm1 cells (*e* and *f*) were attached to the glass slides by cytospin centrifugation, and fixed with 4% paraformaldehyde. After brief permeabilization, cells were incubated with anti-vimentin antibody, then with FITC-labeled anti-mouse IgM. (*a, c,* and *e*) Phase contrast. (*b, d,* and *f*) Indirect immunofluorescence microscopy.
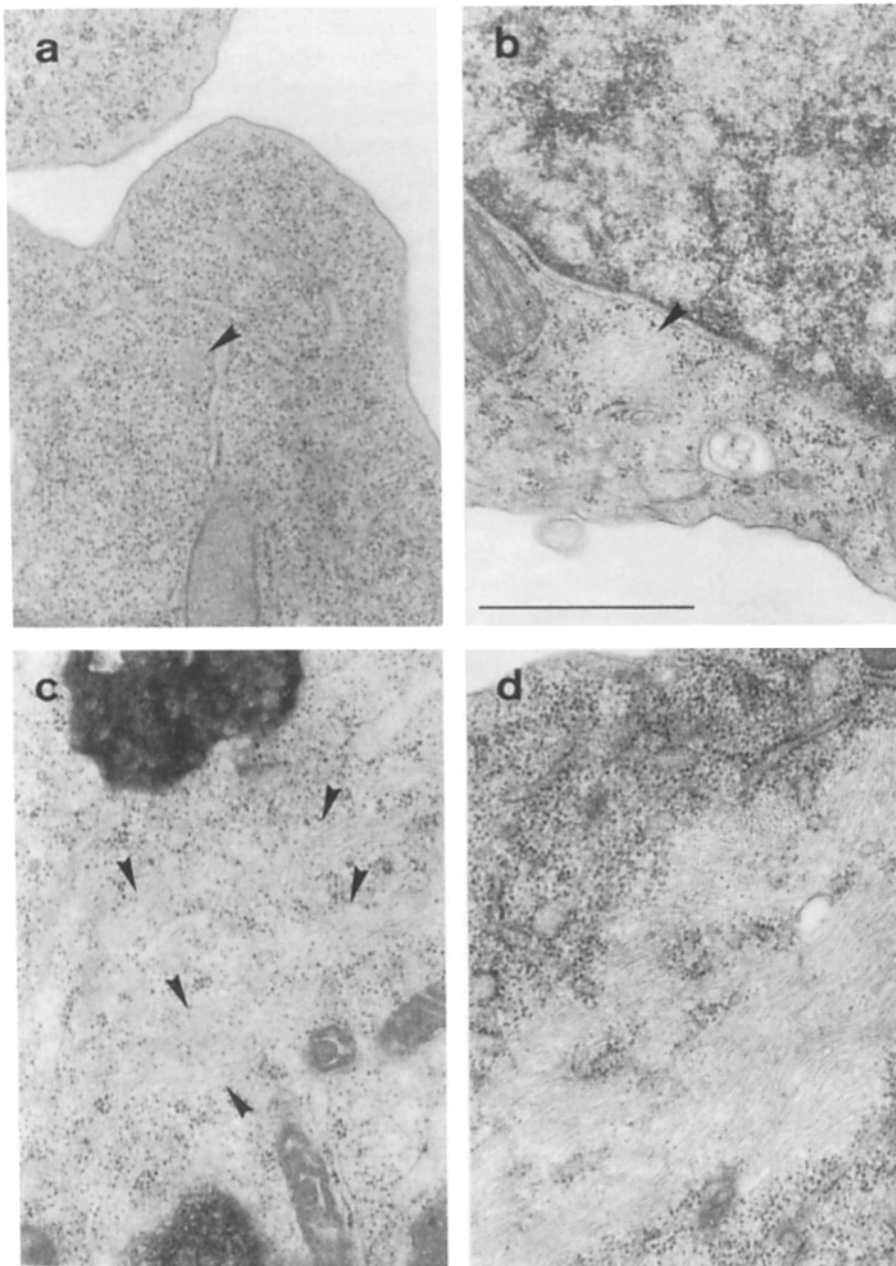
Figure 11. Transmission electron micrograph of M1 cells. (*a*) Nontreated M1 cells; (*b*) M1 cells treated with 10% (vol/vol) CM for 3 d; (*c*) M1 cells treated with CM for 4 d; and (*d*) Mm1 cells. Bar, 1 μm.

formed during the differentiation of myeloblastic leukemia cells.

Discussion

When incubated for 2 or 3 d with CM containing differentiation-inducing factor, M1 cells were induced to differentiate into macrophages, and acquired the locomotive and phagocytic activities (14, 15). The differentiation inducing factor was purified from the CM of L929 cells (40), and recently, the cDNAs have been cloned from murine and human cells (7, 8, 20). IL 6 has also been reported to induce the differentiation in M1 cells (23).

The acquisition of these activities might be attributable to changes in the actomyosin system. We reported quantitative and qualitative changes in actin during the differentiation of M1 cells where the qualitative changes in actin such as poly-

merization and the β/γ ratio of cytoplasmic actin, were marked rather than the quantitative changes (25, 26). We have identified three actin binding proteins in M1 cells; one inhibits the polymerization of actin (27), and the two others cause the monovalent-cation sensitive gelation of actin filaments (28, 39). These three factors have all been purified.

Besides these changes, differentiated M1 cells reveal characteristic morphological changes. The sizes of cells are enlarged, and the ratio of nucleus to cell gradually decreases during the differentiation of M1 cells (12), which was quantitized by Hayashi et al. (11). The nuclei are squeezed into one side of the cytoplasm in differentiated cells (CM-treated M1 cells and Mm1 cells) which was observed by May-Grünwald-Giemsa staining or by electron microscopy (Fig. 1). Because this maldistribution of nuclei is now thought to be sustained by intermediate filaments (for a review, see Lazarides [17]), it should be interesting to examine changes in the expression

and distribution of vimentin during the differentiation of M1 cells.

In this paper, we reported that vimentin expression was enhanced after the differentiation of M1 cells at the levels of protein synthesis, protein content, and mRNA accumulation. This induction of vimentin was regulated at the level of transcription. The maldistribution of the nuclei in differentiated M1 cells, which have been reported previously (11, 12), might be partly explained by this induction and accumulation of vimentin, and the formation of vimentin bundles after differentiation. Although the increases in vimentin compared on the basis of equal protein were >7-fold higher after 48–72 h of incubation with CM and >12-fold higher in Mml cells, the increases were >17-fold higher in CM-treated M1 cells and >45-fold higher in Mml cells when compared on the basis of equal cell numbers. The differences in these increases were due to differences in total protein content before and after differentiation, which we have already reported (25).

The increase in the amount of vimentin was not caused only by culturing M1 cells with CM, but also by the treatment with IL 6 and LIF, which induce the differentiation in M1 cells (7, 8, 20, 23). The amount of vimentin in D⁻ cells and Balb/3T3 cells was not increased by the treatment with CM. These findings suggest that the increase in vimentin is the result of M1 cell differentiation, and not the direct effect caused by unknown factor(s) in CM.

We found that the level of vimentin mRNA in Mml cells collected from confluent culture was lower than that from sparse culture. This observation suggests that the expression of vimentin is partly associated with growth conditions, which is consistent with the finding that the steady-state levels of cytoplasmic vimentin mRNA in 3T3 cells are increased by addition of serum (6). Although the level of vimentin mRNA in confluent Mml cells was lower than that of the maximum level of CM-treated M1 cells, the level of vimentin mRNA in Mml cells collected from sparse culture is a little higher than that.

When all cells were collected for the analysis of vimentin content by immunoblot and of vimentin mRNA by Northern blot, the amount of vimentin and the level of vimentin mRNA were decreased at the fourth day of incubation with CM. These decreases may be attributable to the cell death caused by prolonged culture without medium change because neither the amount of vimentin nor that of vimentin mRNA decreased when assayed by collecting only adherent cells, and because the amount of vimentin of nonadherent cells was much lower than that of adherent cells. When the medium was changed at the third day of incubation, and adherent cells were further cultured without CM, the amount of vimentin increased nearly 40-fold of that of undifferentiated M1 cells at the fifth day (data not shown). These results suggest that the induction of vimentin during the differentiation of M1 cells is not a transient phenomenon, but is closely related to cell differentiation.

The increase in the amount of vimentin after CM-treatment seemed to precede those in the induction of vimentin mRNA and the synthesis of vimentin (Figs. 3, 5, and 8). These results suggest either the stabilization of vimentin or the decrease in vimentin degradation activities occurred immediately after the incubation of M1 cells with CM. Recently, we found Ca²⁺-independent protease activity in undifferentiated M1 cells in addition to a Ca²⁺-dependent protease called calpain or CANP (manuscript in preparation). Interestingly, only Ca²⁺-dependent protease activity was detected in differentiated M1 and Mml cells. We are now characterizing the nature of this new type of protease.

The size of mRNA in M1 cells was ∼2.1 kb, which comigrated with 18S ribosomal RNA bands, and did not change before or after differentiation (Fig. 7). The band detected by vimentin cDNA hybridization was not an artifact due to cross-reactivity with ribosomal RNA, because poly(A)-RNA purified by two cycles of oligo(dT)-cellulose affinity chromatography was also detected by the Northern blotting method at the same position and at the same density. The nuclear run-on assay revealed that the increase in the amount of vimentin mRNA was not due to a decrease in the turnover rate, but was due to increased transcription of vimentin mRNA after CM-treatment.

The regulation of vimentin expression has been reported to be closely related to differentiation (3, 31), growth (5) and transformation (2, 19, 36) in various cells. Vimentin expression is inhibited in monkey kidney cells infected with SV-40 (2), and vimentin mRNA is not detected in Burkitt's lymphoma by Northern hybridization using vimentin cDNA (19). Treatment of HL60 cells, human promyelocytic leukemia line cells, or K562 cells, human erythroleukemic line cells, with 12-O-tetradecanoyl-phorbol 13-acetate (TPA) is reported to cause the induction of vimentin mRNA, although the treatment of HL60 cells with retinoic acid has the opposite effect (6). Lazarides and his colleagues reported the species specific expression of vimentin genes using the differentiation system of chick and murine erythroid cells (3, 31). Vimentin synthesis is increased during the differentiation of chick erythroid precursors, whereas it decreases after the maturation of mammalian erythrocytes. This might reflect the phenomenon in which mammalian erythrocytes lose their nuclei during maturation. Interestingly, when chick vimentin gene is introduced into MEL cells, the expression of vimentin mRNA is enhanced during differentiation, whereas similarly transfected hamster vimentin genes are negatively regulated (32).

Various markers for detecting the differentiation of M1 cells have been reported including the induction of phagocytosis (15), motility (24), lysosomal enzymes (30), the decrease in tumorigenicity, and morphological changes (13, 35). The induction of vimentin expression additionally provides one of the clear markers for identifying differentiation of the M1 cell line at the protein and mRNA levels. We are now analyzing vimentin genes, especially its promoter region. The identification of cis-acting transcriptional elements and transcriptional factors may be important in understanding myeloid differentiation at the molecular level.

References

1. Aviv, H., and P. Leder. 1972. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. *Proc. Natl. Acad. Sci. USA.* 69:1408–1411.

2. Ben-Ze'ev, A. 1984. Inhibition of vimentin synthesis and disruption of intermediate filaments in simian virus 40-infected monkey kidney cells. *Mol. Cell. Biol.* 4:1880–1889.

3. Capetanaki, Y. G., J. Ngai, C. N. Flytzanis, and E. Lazarides. 1983. Tissue-specific expression of two mRNA species transcribed from a single vimentin gene. *Cell.* 35:411–420.

4. Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry.* 18:5294–5299.

5. Feinberg, A. P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132:6–13.

6. Ferrari, S., R. Battini, L. Kaczmarek, S. Rottling, B. Calabretta, J. K. de Riel, V. Philiponis, J. -F. Wei, and R. Baserga. 1986. Coding sequence and growth regulation of the human vimentin gene. *Mol. Cell. Biol.* 6:3614–3620.

7. Gearing, D. P., N. M. Gough, J. A. King, D. J. Hilton, N. A. Nicola, R. J. Simpson, E. C. Nice, A. Kelso, and D. Metcalf. 1987. Molecular cloning and expression of cDNA encoding a murine myeloid leukemia inhibitory factor (LIF). *EMBO (Eur. Mol. Biol. Organ.) J.* 6:3995–4002.

8. Gough, N. M., D. P. Gearing, J. A. King, T. A. Willson, D. J. Hilton, N. A. Nicola, and D. Metcalf. 1988. Molecular cloning and expression of the human homologue of the murine gene encoding myeloid leukemia-inhibitory factor. *Proc. Natl. Acad. Sci. USA.* 85:2623–2627.

9. Granger, B. L., and E. Lazarides. 1982. Structural associations of synemin and vimentin filaments in avian erythrocytes revealed by immunoelectron microscopy. *Cell.* 30:263–275.

10. Greenberg, M. E., and E. B. Ziff. 1984. Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene. *Nature (Lond.).* 311:433–438.

11. Hayashi, M., O. Gotoh, J. Okabe-Kado, and M. Hozumi. 1981. Mechanisms controlling the kinetics in proliferation and differentiation of populations of mouse myeloid leukemic cells in vitro. *J. Cell. Physiol.* 108:123–134.

12. Hirai, K., K. Nagata, M. Maeda, and Y. Ichikawa. 1979. Changes in ultrastructures and enzyme activities during differentiation of myeloid leukemia cells to normal macrophages. *Exp. Cell Res.* 124:269–283.

13. Hozumi, M. 1985. Established leukemia cell lines: their role in the understanding and control of leukemia proliferation. *CRC Crit. Rev. Oncol./Hematol.* 3:235–277.

14. Ichikawa, Y. 1969. Differentiation of a cell line of myeloid leukemia. *J. Cell. Physiol.* 74:223–234.

15. Ichikawa, Y. 1970. Further studies on the differentiation of a cell line of myeloid leukemia. *J. Cell. Physiol.* 76:175–184.

16. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

17. Lazarides, E. 1987. From genes to structural morphogenesis: the genesis and epigenesis of a red blood cell. *Cell.* 51:345–356.

18. Lehrach, H., D. Diamond, J. M. Wozney, and H. Boedtker. 1977. RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. *Biochemistry.* 16:4743–4751.

19. Lilienbaum, A., V. Legagneux, M. -M. Portier, K. Dellagi, and D. Paulin. 1986. Vimentin gene: expression in human lymphocytes and in Burkitt's lymphoma cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:2809–2814.

20. Lowe, D. G., W. Nunes, M. Bombara, S. McCabe, G. E. Ranges, W. Henzel, M. Tomida, Y. Yamamoto-Yamaguchi, M. Hozumi, and D. V. Goeddel. 1989. Genomic cloning and heterologous expression of human differentiation factor. *DNA (NY).* 8:351–359.

21. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.

22. Maeda, M., and Y. Ichikawa. 1973. Spontaneous development of macrophage-like cells in a culture of myeloid leukemia cells. *Gann.* 64:265–271.

23. Miyaura, C., K. Onozaki, Y. Akiyama, T. Taniyama, T. Hirano, T. Kishimoto, and T. Suda. 1988. Recombinant human interleukin 6 (B-cell stimulatory factor 2) is a potent inducer of differentiation of mouse myeloid leukemia cells (M1). *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 234:17–21.

24. Nagata, K., and Y. Ichikawa. 1984. Changes in actin during cell differentiation. *Cell Muscle Motil.* 5:171–193.

25. Nagata, K., J. Sagara, and Y. Ichikawa. 1980. Changes in contractile proteins during differentiation of myeloid leukemia cells. I. Polymerization of actin. *J. Cell Biol.* 85:273–282.

26. Nagata, K., J. Sagara, and Y. Ichikawa. 1982. Changes in contractile proteins during differentiation of myeloid leukemia cells. II. Purification and characterization of actin. *J. Cell Biol.* 93:470–478.

27. Nagata, K., J. Sagara, and Y. Ichikawa. 1982. A new protein factor inhibiting actin-polymerization in leukemic myeloblasts. *Cell Struct. Funct.* 7:1–7.

28. Nagata, K., J. Sagara, and Y. Ichikawa. 1983. Changes in actin-related gelation of crude cell extracts during differentiation of myeloid leukemia cells. *Cell Struct. Funct.* 8:171–183.

29. Nagata, K., K. Takagi, T. Hashida, and Y. Ichikawa. 1985. A monovalent cation-sensitive actin-binding factor in a myeloid leukemia cell line. *Cell Struct. Funct.* 10:105–120.

30. Nagata, K., E. Takahashi, M. Saito, J. Ono, M. Kuboyama, and K. Ogasa. 1976. Differentiation of a cell line of mouse myeloid leukemia. I. Simultaneous induction of lysosomal enzyme activities and phagocytosis. *Exp. Cell Res.* 100:322–328.

31. Ngai, J., Y. G. Capetanaki, and E. Lazarides. 1984. Differentiation of murine erythroleukemia cells results in the rapid repression of vimentin gene expression. *J. Cell Biol.* 99:306–314.

32. Ngai, J., V. C. Bond, B, J. Wold, and E. Lazarides. 1987. Expression of transfected vimentin genes in differentiating murine erythroleukemia cells reveals divergent cis-acting regulation of avian and mammalian vimentin sequences. *Mol. Cell. Biol.* 7:3955–3970.

33. O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007–4021.

34. Rittling, S. R., and R. Baserga. 1987. Functional analysis and growth factor regulation of the human vimentin promotor. *Mol. Cell. Biol.* 7:3908–3915.

35. Sachs, L. 1978. Control of normal cell differentiation and the phenotypic reversion of malignancy in myeloid leukemia. *Nature (Lond.).* 274:535–539.

36. Singh, B., R. Goldman, L. Hutton, N. K. Herzog, and R. B. Arlinghaus. 1987. The P55 protein affected by v-mos expression is vimentin. *J. Virol.* 61:3625–3629.

37. Steinert, P. M., and D. R. Roop. 1988. Molecular and cellular biology of intermediate filaments. *Annu. Rev. Biochem.* 57:593–625.

38. Steinert, P. M., J. C. R. Jones, and R. D. Goldman. 1984. Intermediate filaments. *J. Cell Biol.* 99:22s–27s.

39. Takagi, K., Y. Ichikawa, and K. Nagata. 1985. Changes in K+,Na+-sensitive actin gelation factor during the differentiation of myeloid leukemia cells. *J. Biochem. (Tokyo).* 97:605–616.

40. Tomida, M., Y. Yamamoto-Yamaguchi, and M. Hozumi. 1984. Purification of a factor inducing differentiation of mouse myeloid leukemic M1 cells from conditioned medium of mouse fibroblast L929 cells. *J. Biol. Chem.* 259:10978–10982.

41. Towbin, H., T. Staehlin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.

1664