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Regulation of Vimentin Expression and Protease-mediated Vimentin Degradation during Differentiation of Human Monocytic Leukemia Cells

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Terminal differentiation of human monocytic leukemia THP-1 cells is induced *in vitro* by 12-*O*-tetradecanoylphorbol-13-acetate (TPA). We investigated the effects of TPA on the expression of vimentin during the differentiation of THP-1 cells at both the mRNA and the protein level. On northern blotting analysis, a 2.1 kb vimentin mRNA was up-regulated by TPA. On western blotting, small vimentin molecules with a molecular mass of approximately 40 kDa were observed in the soluble fraction and increased with TPA-induction of cellular differentiation. Since larger, including intact, vimentin molecules were detectable at a high TPA dose, we assessed the possible existence of protease activity directed against vimentin in THP-1 cells. With incubation of the cellular lysates of THP-1 cells, the endogenous vimentin became increasingly smaller over time, suggesting the presence of a vimentin-degrading protease. Phenylmethylsulfonyl fluoride inhibited this apparent protease activity against vimentin, suggesting the enzyme involved to be a serine protease. Interestingly, the protease activity was down-regulated by TPA treatment. TPA-treated THP-1 cells were found to express a vimentin-filament network based on immunocytochemical analysis using an anti-vimentin monoclonal antibody, V9. Taken together, these observations suggest that post-translational mechanisms work in cooperation with transcriptional regulation to maintain the vimentin-intermediate filament structure in differentiated THP-1 cells.

Key words: Intermediate filament — Macrophage — Phorbol ester — Protease — THP-1

Several lines of human leukemia cells are blocked at certain steps of the differentiation process, and can be induced to differentiate into a committed lineage in response to a variety of stimuli.[1] As an example, human promyelocytic leukemia cells, HL-60 and U937, differentiate into macrophage-like cells when treated with a phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (TPA).[2,3] The THP-1 cell line, isolated from a patient suffering from acute monocytic leukemia,[4] is also induced to differentiate into a monocyte/macrophage lineage by TPA,[5] and the differentiation of THP-1 cells is accompanied by changes in the expression of several genes.[6] Compared to other human myeloid cell lines, such as the HL-60, U937, KG-1 and HEL cell lines, differentiated THP-1 cells behave more like native monocyte-derived macrophages.[6]

Vimentin is the major intermediate filament protein in the cytoplasm of cells of mesenchymal origin.[7,8] *In vivo* studies using normal hematopoietic precursors,[9] as well as *in vitro* studies using leukemia cell lines,[10–12] have demonstrated that the expression of the vimentin intermediate filament is developmentally regulated, following cell lineage-specific patterns. The gene expression of vimentin has been demonstrated to increase upon treatment of HL-60 and U937 human leukemic cells with TPA.[13,14]

In the present study, we investigated the expression of vimentin during TPA-induced differentiation of THP-1 cells at both the mRNA and the protein level. Herein we demonstrate that vimentin expression is regulated not only by a transcriptional mechanism, but also by a post-translational mechanism.

MATERIALS AND METHODS

Materials Vimentin was purified from bovine eye lenses as described previously.[15] An anti-vimentin monoclonal antibody, V9,[16] was purchased from Dako (Denmark). The V9 antibody reacts with human and bovine vimentins.[17]

Cell culture THP-1 cells were cultured in a serum-free medium, Cosmedium 001 (Cosmo Bio, Tokyo) containing insulin and transferrin. Cell viability was estimated by means of the Trypan-blue-exclusion test and was greater than 90% throughout the study.

RNA probes and northern blotting analysis A full-length cDNA fragment of human vimentin was amplified by reverse transcriptase-polymerase chain reaction of total RNA from TPA-treated THP-1 cells using synthetic oligonucleotide primers corresponding to bases 41–61 (sense) and bases 1424–1426 (antisense) of human vimentin,[18] containing *Eco*R I and *Bam*H I linkers, respectively, and subcloned into pBluescript KS (Toyobo, Tokyo). The sequence of the insert was consistent with

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that of human vimentin.[18] A digoxigenin-labeled RNA probe was synthesized with a DIG RNA Labeling kit with T7 RNA polymerase (Boehringer Mannheim, Germany) using the T7 promoter sequence of the pBluescript vector. An RNA probe for human GAPDH was generated as described previously.[19]

Total RNA was extracted from THP-1 cells treated with TPA under various conditions by the AGPC method.[20] A 2.5 μg quantity of RNA per lane was electrophoresed on a 1% agarose gel supplemented with 0.66 *M* formaldehyde, blotted onto a nylon membrane and cross-linked by UV irradiation. The membrane was incubated at 68°C for 4 h in a prehybridization buffer containing 50% formamide, 5× saline sodium citrate (SSC), 2% blocking reagent (Boehringer Mannheim), 0.1% *N*-laurylsarcosine, and 0.02% sodium dodecyl sulfate (SDS). Hybridization was carried out at 68°C overnight in a prehybridization buffer containing 1 ng of digoxigenin-labeled RNA probe per cm^2 of membrane, and the detection procedure was carried out with a DIG-Luminescent Detection kit (Boehringer Mannheim) according to the manufacturer's instructions. For reprobing, the membrane was incubated with 0.1× SSC, 0.1% SDS at 100°C for 10 min to remove the preceding probe.

Western blotting analysis THP-1 cells (5×10^5/ml) were cultured in the presence or absence of TPA for various incubation times in a 150-mm dish and harvested with a rubber policeman. The cells were washed twice with phosphate-buffered saline (PBS), suspended in 0.5 ml of PBS containing 1 m*M* EDTA and sonicated. The cellular lysates were then centrifuged at 10,000*g* for 15 min. The supernatant was termed the "soluble fraction" and the precipitate was dissolved in SDS-polyacrylamide gel electrophoresis (PAGE) buffer and termed the "particulate fraction." After protein determination using a BCA Protein Assay kit (Pierce Chemical, Rockford, IL), 10 μg of each protein was subjected to SDS-PAGE on a 12.5% slab gel, transferred onto a nitrocellulose membrane and incubated with the V9 antibody and peroxidase-conjugated rabbit antibody to anti-mouse IgG (Cappel, Durham, NC).

Fluorescence microscopy THP-1 cells (2.0×10^5/ml) were grown in a Lab-Tek chamber slide (Nunc, Naperville, IL) in the presence of 10^{-8} *M* TPA for two days. Cells were fixed in 4% paraformaldehyde in PBS and permeabilized for 10 min in 0.1% Triton X-100 in PBS. Permeabilized cells were blocked with 1% bovine serum albumin in PBS, and incubated with the V9 antibody followed by FITC-conjugated goat anti-mouse IgG antibody (Zymed Laboratories, San Francisco, CA). The incubation time was 45 min in each case. Labeled cells were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA). A Zeiss Axiophot with epi-illumination for fluorescence was used for fluorescence and phase microscopy.

Protease activity against vimentin To assay protease activity against endogenous vimentin, the soluble fraction (5 μg protein) of TPA-treated THP-1 cells (1×10^{-8} *M* TPA, 24 h) was incubated at 37°C for various incubation times in a 10 μl solution of 30 m*M* Tris-acetate (pH 7.5), 100 m*M* KCl, 3 m*M* EDTA in the presence or absence of 10 m*M* $CaCl_2$. The reaction was terminated by the addition of 10 μl of 2× SDS-PAGE buffer (125 m*M* Tris-HCl, pH 6.8, 4% SDS, 14% glycerol), and the whole solution was boiled for 5 min then applied to a 12.5% SDS-PAGE gel. After electrophoresis, proteins were transferred to a nitrocellulose membrane and intrinsic vimentin was detected with the V9 antibody.

To assay the protease activity against bovine lens vimentin, the cellular lysate of TPA-treated THP-1 cells (1×10^{-8} *M* TPA, 24 h) as an enzyme source was preincubated at 37°C for 2 h in a solution of 30 m*M* Tris-acetate (pH 7.5), 100 m*M* KCl, 3 m*M* EDTA and 6 m*M* β-mercaptoethanol, in order to degrade the endogenous vimentin. The protease reaction was carried out with 350 ng of bovine lens vimentin and the preincubated enzyme source (200 ng protein) in the presence or absence of various reagents in a final volume of 10 μl. After incubation for various periods of time, the reaction was terminated by the addition of 40 μl of 1×SDS-PAGE buffer (62.5 m*M* Tris-HCl, pH 6.8, 2% SDS, 7% glycerol), the solution was boiled for 5 min and then 10 μl was subjected to SDS-PAGE followed by western blotting with the V9 antibody.

RESULTS

Vimentin expression in THP-1 cells The effects of TPA on the expression of vimentin during the differentiation of THP-1 cells were investigated at both the mRNA and the protein level. TPA has been shown to induce the differentiation of THP-1 cells into a monocytic lineage at concentrations in the range of 1.6×10^{-9} to 1.6×10^{-7} *M* (5). In preliminary experiments, we found that THP-1 cells became adherent to the plastic substrate and were nonproliferative when exposed to this dose range of TPA (data not shown). On northern blotting, vimentin mRNA was detected in untreated THP-1 cells and was dose-dependently up-regulated by TPA at concentrations in the range of 5×10^{-10} to 1×10^{-8} *M* (Fig. 1A). The level of vimentin mRNA increased over the course of 24-h incubation (Fig. 1B). When the cells were treated with 1×10^{-8} *M* TPA for 24 h, the relative intensity of the vimentin mRNA increased 5- to 7-fold as compared with that in untreated cells (Fig. 1, C and D). The size of the vimentin mRNA in THP-1 cells was 2.1 kb, the same as that reported in other cells.[13, 14, 21]

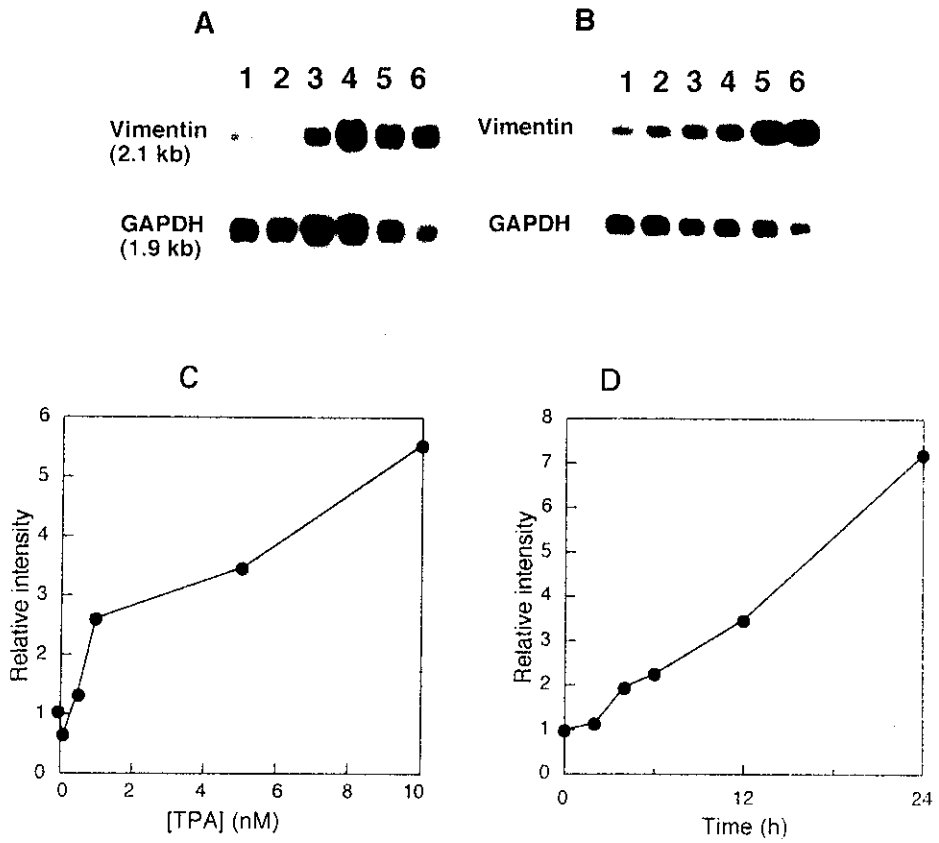

Fig. 1. Vimentin mRNA levels during the course of TPA-induced differentiation of THP-1 cells. A and B are northern blots of vimentin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in TPA-treated THP-1 cells. A, Cells were treated with various concentrations of TPA for 24 h. Lane 1, none; lane 2, 1×10^{-10} *M*; lane 3, 5×10^{-10} *M*; lane 4, 1×10^{-9} *M*; lane 5, 5×10^{-9} *M*; lane 6, 1×10^{-8} *M*. B, Cells were treated with 1×10^{-8} *M* TPA for various incubation periods. Lane 1, 0 h; lane 2, 2 h; lane 3, 4 h; lane 4, 6 h; lane 5, 12 h; lane 6, 24 h. C and D, Quantitation of vimentin mRNA levels in A and B, respectively. The intensity of vimentin mRNA, measured by densitometry, was normalized with respect to that of cognate GAPDH mRNA. The ratios of vimentin to GAPDH without TPA (lane 1 of A) and 0 time (lane 1 of B) were defined as 1.0 in C and D, respectively.

On western blotting using an anti-vimentin mAb, V9,[16] the immunoreactive material was faintly detected as a doublet at approximately 40 kDa in untreated THP-1 cells. The intensity of the doublet increased, however, as the TPA dose and incubation time were increased (Fig. 2, A and B). Nonetheless, the doublets were smaller in molecular size than vimentin in eye lens[22] and Ehrlich ascites tumor cells,[23] which appear at positions corresponding to approximately 57 kDa on SDS-PAGE. When THP-1 cells were treated with more than 10^{-9} *M* TPA, larger vimentin molecules were also detected (lanes 3–5 in Fig. 2A). Furthermore, vimentin expression was more evident in adherent than in suspended cells after TPA treatment and a large molecule corresponding to the intact 57 kDa molecule was identified in adherent cells (Fig. 2C). As the small vimentin molecules were seen even when the cells were immediately lysed with 2% SDS in the presence of EDTA and phenylmethylsulfonyl fluoride (PMSF) (Fig. 2D), they were not likely to have been an artifact of the *in vitro* procedures employed. These results suggested the occurrence of limited proteolysis of vimentin and the existence of protease(s) directed against vimentin in the leukemic cells. The observed hydrolysis of vimentin could be due to the action of multiple proteases and the 40 kDa molecule could be a product of secondary degradation. However, the 40 kDa band is not simply a reflection of the limitation of antibody reactivity, since this species has also been isolated from THP-1 cells using ganglioside-immobilized affinity chromatography (unpublished results by K. Honke and Y. Wada). In THP-1 cells, most vimentin molecules were recovered from the soluble fraction (Fig. 2, A and C), in

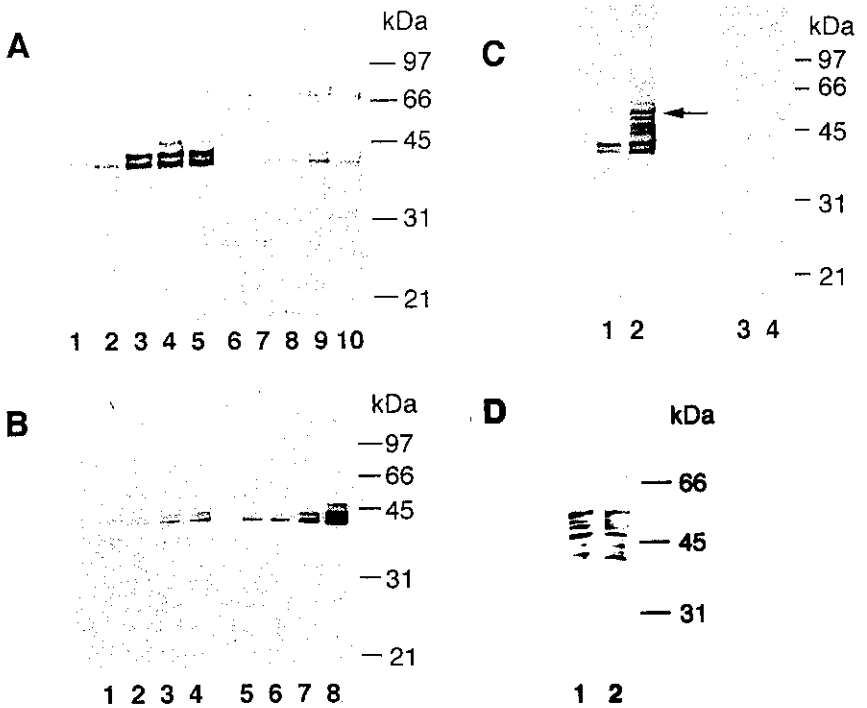

Fig. 2. Vimentin levels during the course of TPA-induced differentiation of THP-1 cells. A, Cells treated with various concentrations of TPA for 48 h were harvested and fractionated into a soluble fraction (lanes 1–5) and a particulate fraction (lanes 6–10). Ten micrograms of protein was subjected to SDS-PAGE and transferred to a nitrocellulose membrane as described in "Materials and Methods." Vimentin was visualized with an anti-vimentin monoclonal antibody, V9 (16). Lanes 1 and 6, none; lanes 2 and 7, 1×10^{-10} *M*; lanes 3 and 8, 1×10^{-9} *M*; lanes 4 and 9, 5×10^{-9} *M*; lanes 5 and 10, 1×10^{-8} M. B, Time course using 1×10^{-8} *M* TPA. A 10 μg quantity of protein from the soluble fraction was applied. Lane 1, 30 min; lane 2, 1 h; lane 3, 2 h; lane 4, 3 h; lane 5, 6 h; lane 6, 12 h; lane 7, 24 h; lane 8, 48 h. C, After being treated with 1×10^{-8} *M* TPA for 48 h, suspended cells and adherent cells were separated. Vimentin expression was examined as described above. Lanes 1 and 3, suspended cells; lanes 2 and 4, adherent cells; lanes 1 and 2, soluble fraction; lanes 3 and 4, particulate fraction. The positions of molecular size markers (Bio-Rad, low molecular weight standard) are shown on the right. Note that the 57 kDa intact vimentin is seen in the soluble fraction of adherent cells (arrow in lane 2). D, After treatment with 1×10^{-8} *M* TPA for 48 h, adherent cells were immediately lysed with PBS containing 1 m*M* EDTA (lane 1) and PBS containing 2% SDS, 5 m*M* EDTA and 0.5 m*M* phenylmethylsulfonyl fluoride (lane 2).

contrast to other cells, where vimentin is fractionated in detergent-resistant 'cytoskeleton.'[22, 23]

Next, to examine whether differentiation-induced THP-1 cells organize vimentin-filament networks, THP-1 cells were immunocytochemically visualized with the anti-vimentin mAb, V9. As shown in Fig. 3, a well-developed network of vimentin filaments was discernible throughout the cytoplasm under fluorescence microscopic observation. This morphological feature was consistent with the vimentin-filament structure observed in blood monocytes and HL-60 cells.[9, 11]

Protease activity against vimentin in THP-1 cells

During incubation of the cellular lysates of TPA-treated THP-1 cells at 37°C, the vimentin molecules became increasingly smaller, with accumulation of the 40 kDa product (Fig. 4). This degradation was enhanced in the presence of $CaCl_2$. Further incubation resulted in disappearance of the 40 kDa band (data not shown). The mAb V9 was unreactive with breakdown products smaller than 40 kDa. This observation indicated the existence of protease activity directed against endogenous vimentin. Next, protease activity was assayed using bovine eye-lens vimentin as a substrate. Before the addition of the exogenous substrate to the reaction mixture, THP-1-cell lysates used as an enzyme source had been preincubated for 2 h to degrade endogenous vimentin. During preincubation, the endogenous vimentin included in the enzyme source decreased to an undetectable level (data not shown). As shown in Fig. 5, the THP-1-cellular lysate dose-dependently degraded bovine lens vimentin. The proteolysis pattern generally resembled that of the endogenous vimentin, except that intact vimentin was a

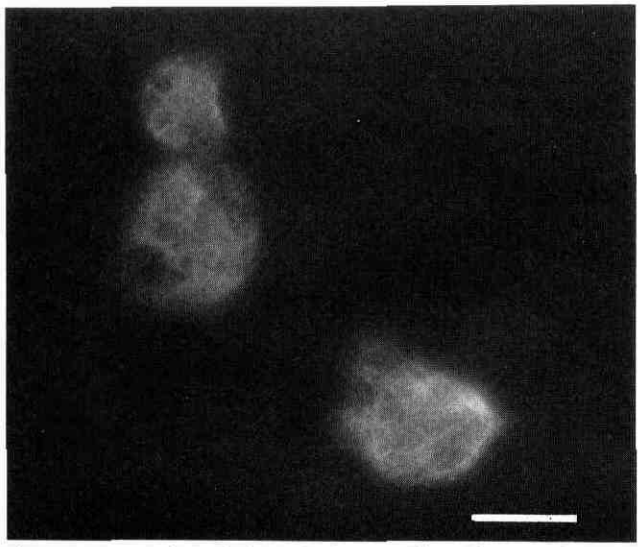

Fig. 3. Expression of vimentin intermediate filaments in TPA-treated THP-1 cells. Cells were treated with 1×10^{-8} *M* TPA in a LabTek chamber for 48 h and stained with an anti-vimentin monoclonal antibody, V9 (16), as described in "Materials and Methods." Bar = 10 μm.

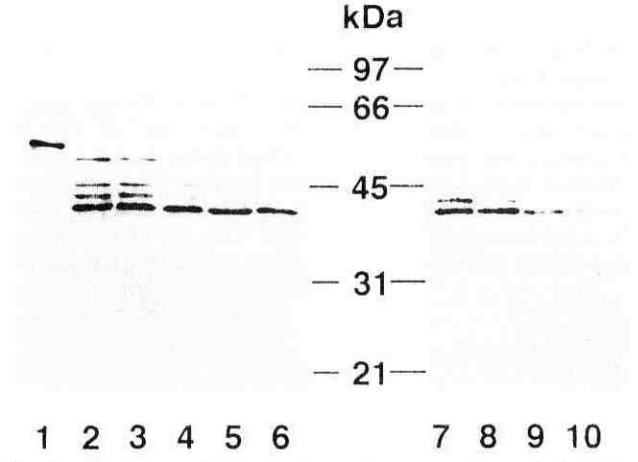

Fig. 4. Protease activity against endogenous vimentin in TPA-treated THP-1 cells. The cellular lysate (10 μg protein) from TPA-treated THP-1 cells was incubated for various periods in the absence (lanes 2–6) or presence (lanes 7–10) of 10 m*M* $CaCl_2$ and subjected to western blotting analysis with V9 antibody as described in "Materials and Methods." Lane 1, bovine eye-lens vimentin, 50 ng; lane 2, 0 time; lanes 3 and 7, 10 min; lanes 4 and 8, 30 min; lanes 5 and 9, 1 h; lanes 6 and 10, 2 h. The positions of molecular size markers are indicated in the center.

very rare component of the endogenous vimentin (Fig. 4). The molecular size of the smallest band in Fig. 5 is nearly the same as that of the small vimentin molecule

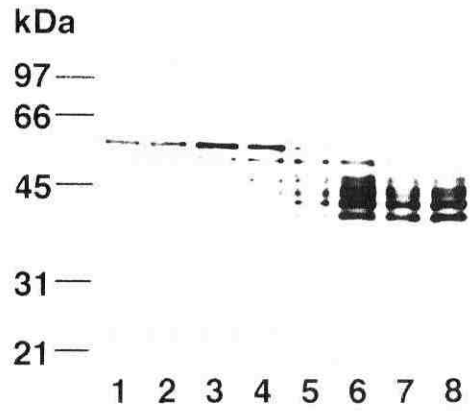

Fig. 5. Protease activity against exogenous bovine eye-lens vimentin in TPA-treated THP-1 cells. Bovine eye-lens vimentin (50 ng) was incubated with various amounts of the cellular lysate from TPA-treated THP-1 cells in the presence of 10 m*M* $CaCl_2$ for 10 min and subjected to western blotting analysis with V9 antibody. Lane 1, bovine eye-lens vimentin, 50 ng; lane 2, no enzyme; lane 3, 50 ng; lane 4, 0.1 μg; lane 5, 0.2 μg; lane 6, 0.3 μg; lane 7, 0.4 μg; lane 8, 0.5 μg. The positions of molecular size markers are shown on the left.

observed at 40 kDa in TPA-treated THP-1 cells (Fig. 2), despite the structural differences between human and bovine vimentins. These findings suggested that the small vimentin molecules in THP-1 cells had been generated by limited proteolysis.

To investigate whether the vimentin protease affects the expression of vimentin during differentiation of THP-1 cells, the effects of TPA on this protease activity were examined. As shown in Fig. 6, the proteolytic reaction was inhibited in TPA-treated cells. Although this observation suggests that the protease activity is reduced by the TPA treatment, we cannot exclude the possibility of a TPA-induced increase of endogenous inhibitor(s). This result would be consistent with the larger vimentin molecules having been observed when higher TPA doses were utilized (Fig. 2). These vimentin-degrading activities were enhanced by Ca^{2+} irrespective of TPA treatment (Fig. 6).

Although the degrading activity was enhanced by Ca^{2+} (Figs. 4 and 6), Ca^{2+} was not essential, because proteolysis proceeded in the presence of EDTA (Fig. 7). Among the protease inhibitors tested, PMSF inhibited this activity (Fig. 7), suggesting that the enzyme involved is a serine protease. Sulfhydryl reagents tested in this study did not affect the protease activity (Fig. 7).

DISCUSSION

We investigated the expression of an intermediate filament protein, vimentin, during the TPA-induced differ-

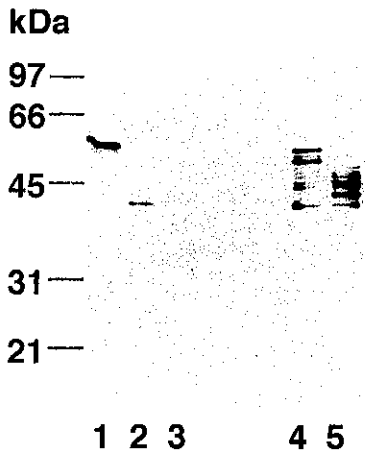

Fig. 6. Effects of TPA treatment on the vimentin protease activity in THP-1 cells. Bovine eye-lens vimentin (50 ng) was incubated with cellular lysates (0.5 μg protein) from untreated (lanes 2 and 3) or TPA-treated (lanes 4 and 5) THP-1 cells in the absence (lanes 2 and 4) or presence (lanes 3 and 5) of 10 mM $CaCl_2$ for 10 min and then subjected to western blotting analysis. Lane 1, bovine eye-lens vimentin, 50 ng. The positions of molecular size markers are shown on the left.

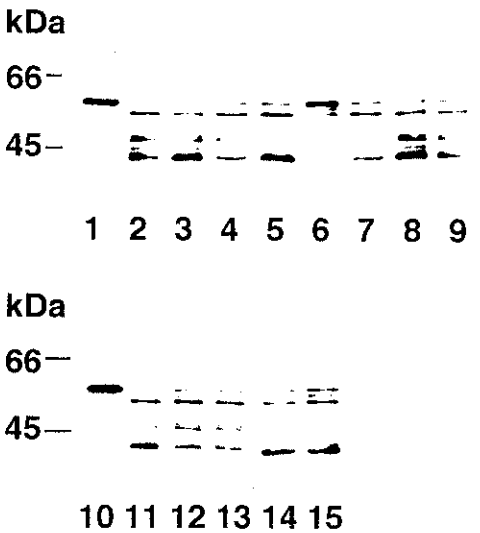

Fig. 7. Effects of protease inhibitors on the protease activity against vimentin. Bovine eye-lens vimentin (50 ng) was incubated for 30 min with cellular lysates (0.5 μg protein) from TPA-treated THP-1 cells in the presence of various protease inhibitors, and subjected to western blotting analysis. The protease sources were preincubated with the inhibitors for 30 min before the protease reaction. Lanes 1 and 10, bovine eye-lens vimentin, 50 ng. Inhibitors: lane 2, none; lane 3, 5 mM EDTA; lane 4, 6 mM β-mercaptoethanol; lane 5, 10 mM E-64; lane 6, 0.5 mM phenylmethylsulfonyl fluoride; lane 7, 0.1 mM leupeptin; lane 8, 0.1 mM antipain; lane 9, 0.1 mM chymostatin; lane 11, 1 μM pepstatin; lane 12, 0.5 mM tosyl phenylalanylchloromethyl ketone; lane 13, 0.5 mM tosyl lysyl chloromethyl ketone; lane 14, 10 mM iodoacetate; lane 15, 10 mM iodoacetamide. The positions of molecular size markers are shown on the left.

entiation of the human monocytic leukemia cell line THP-1. The current study focused on vimentin expression at both the mRNA and the protein level. On northern blotting analysis, vimentin mRNA was found to be increased by TPA. A similar TPA-induced up-regulation of vimentin mRNA has been demonstrated in HL-60 and U937 human myeloid leukemic cells.[13, 14] Transcription of the vimentin gene has been suggested to be enhanced via a double AP-1 binding site in the 5′-upstream region of the gene.[24, 25]

Vimentin also increased at the protein level during the course of TPA-induced differentiation. However, the vimentin protein was recovered from the soluble fraction and was smaller than that of other cells.[22, 23] Such small vimentin molecules were also observed in HL-60 cells (data not shown). All vimentin mRNA in these cells was 2.1 kb, corresponding to the normal length found in other cells,[13, 14, 21] making truncation at the transcriptional level such as alternative splicing and so on, highly unlikely. When treated with a high dose of TPA, larger vimentin molecules including the intact 57 kDa molecule were also detected. These findings suggested that post-translational processing, probably limited proteolysis, leading to the appearance of small vimentin molecules had occurred intracellularly and prompted us to explore the possibility of protease activity against vimentin in THP-1 cells. As expected, we were able to demonstrate protease activity against endogenous vimentin and exogenous bovine eye-lens vimentin in THP-1 cells. Interestingly, this proteolysis was suppressed by TPA treatment.

Intermediate filament protein monomers are highly elongated fibrous molecules possessing a central rod domain flanked by globular amino- and carboxyl-terminal domains.[7, 8] The α-helical rod domains of the two monomers are coiled around one another, after which the dimers assemble into tetramers. Soluble tetramers are the fundamental subunit from which intermediate filaments assemble. The globular terminal domains are assumed to be involved in assembly of the tetramers into the final intermediate filaments.[7, 8] As a breakdown product of vimentin (lacking the amino-terminal 3 kDa peptide) cleaved by an intermediate filament-specific protease cannot form the filamentous structure in Ehrlich ascites tumor cells,[26] the amino-terminal domain appears to be essential for filament assembly. Presumably, the small soluble vimentin molecules in THP-1 cells cannot assemble into intermediate filaments, although the actual cleav-

age sites remain to be determined. Only the residual intact vimentin or nascent vimentin may participate in the filament formation confirmed by immunocytochemical analysis. Thus, the reduction in protease activity, which leads to an increase in the level of intact vimentin, may promote the assembly of vimentin intermediate filaments in differentiated THP-1 cells.

The protease activity against vimentin was not inhibited by sulfhydryl reagents or pepstatin, indicating that it is different from the Ca^{2+}-activated vimentin-specific protease in Ehrlich ascites tumor cells[27] and human immunodeficiency virus type 1 protease.[28] As the protease activity against vimentin demonstrated in this study was inhibited by PMSF, the enzyme involved appears to be a serine protease. TPA causes transcriptional down-regulation of three related serine proteases, myeloblastin, neutrophil elastase and cathepsin G, in azurophilic granules in HL-60 and U937 cells during the course of differentiation into the monocyte/macrophage lineage.[29-31] Gene expression of proteasome subunits has also been shown to be down-regulated during the TPA-induced monocytic differentiation of THP-1, U937, K562, and HL-60 cells.[32] Interestingly, cathepsin G has been characterized as a vimentin-specific protease in murine myeloid leukemia cells.[33] However, cathepsin G is different from the present THP-1 protease in that cathepsin G is independent of Ca^{2+} and is completely inhibited by chymostatin.[33] Further study is needed to characterize the enzyme and to elucidate the biological significance of its down-regulation in the differentiation of leukemia cells.

In conclusion, the regulation of vimentin expression and vimentin-degrading activity may work in coordination to maintain the assembly of vimentin intermediate filaments in differentiated THP-1 cells. The results of this study should lead to a better understanding of the cellular function of the intermediate filament protein and the intracellular proteases in human leukemia cells.

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