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Abnormally high expression of proteasomes in human leukemic cells

(multicatalytic proteinase/gene expression/leukemia/cell proliferation)

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ABSTRACT **Proteasomes are eukaryotic ring-shaped or cylindrical particles with multicatalytic protease activities. To clarify the involvement of proteasomes in tumorigenesis of human blood cells, we compared their expression in human hematopoietic malignant tumor cells with that in normal peripheral blood mononuclear cells. Immunohistochemical staining showed considerably increased concentrations of proteasomes in leukemic cells from the bone marrow of patients with various types of leukemia and the predominant localization of these proteasomes in the nuclei. Moreover, enzyme immunoassay and Northern blot analysis indicated that the concentrations of proteasomes and their mRNA levels were consistently much higher in a variety of malignant human hematopoietic cell lines than in resting peripheral lymphocytes and monocytes from healthy adults. Proteasome expression was also greatly increased in normal blood mononuclear cells during blastogenic transformation induced by phytohemagglutinin; their expression increased in parallel with induction of DNA synthesis and returned to the basal level with progress of the cell cycle. Thus, abnormally high expression of proteasomes may play an important role in transformation and proliferation of blood cells and in specific functions of hematopoietic tumor cells.**

Proteasomes are multicatalytic proteinase complexes that are thought to be major intracellular proteolytic enzyme complexes responsible for certain types of nonlysosomal pathways of protein breakdown that requires metabolic energy (1). They were found to catalyze the degradation of various proteins in an ATP-dependent fashion (2–4). Moreover, 20S proteasomes were demonstrated to assemble into 26S proteolytic complexes through association with certain subcellular factors and to catalyze ATP-dependent degradation of proteins conjugated with ubiquitin (5, 6). Thus, proteasomes appear to be essential for selective removal of unnecessary proteins, such as those with a rapid turnover and abnormal proteins generated in cells. Proteasomes may also be involved in RNA metabolism, because they are identical with the ubiquitous 20S ring-shaped or cylindrical particles in eukaryotes that have several possible functions, such as negative control of mRNA translation and pre-tRNA processing (7–9). Therefore, proteasomes are thought to be multifunctional enzyme complexes involved in the metabolism of both protein and RNA.

Since proteasomes are unusually large polysubunit complexes assumed to have several distinct enzyme activities in a single molecule (10, 11), it is of interest to determine the structure–function relationships of their individual components. For this, the primary structures of two subunits, named C2 (12) and C3 (13), from rat liver proteasomes and one component of 35 kDa of *Drosophila* proteasomes (14) have been determined by cDNA cloning. The overall amino acid sequence of rat C2 closely resembles that of the *Drosophila* 35-kDa protein, suggesting that these two components function similarly in most eukaryotes and that they evolved from the same ancestral gene. In contrast, no significant homologies of these components with any other known proteins were found by computer analysis, indicating that proteasomes are a novel type of enzyme complex.

Proteasomes are ubiquitous in cells of eukaryotes ranging from humans to yeast (1, 15, 16). Moreover, proteasomes or the related 20S particles have been found in both the cytoplasm and the nuclei of a variety of mammalian cells (8, 17, 18) and also various lower eukaryotes (14, 19–25), suggesting that their diverse roles depend on their differential localizations in the cells. However, the exact function of proteasomes is still unknown. One way to obtain information on their physiological role(s) is to study their expression in cells in abnormal states. In this work, we examined the expression of proteasomes in resting and growth-stimulated normal peripheral blood mononuclear cells and in a variety of human hematopoietic tumor cell lines.

MATERIALS AND METHODS

Materials. [*methyl*-^{3}H]Thymidine (2.0 TBq/mmol) and [α-^{32}P]dCTP (110 TBq/mmol) were from Amersham. ^{125}I-labeled protein A (1.5 TBq/mmol) was from DuPont/New England Nuclear. The fluorogenic substrate Suc-Leu-Leu-Val-Tyr-MCA (Suc, succinyl; MCA, 4-methylcoumaryl-7-amide) was from Peptide Institute, Minoh, Japan.

Cells and Cell Culture. The human cell lines Daudi (Burkitt lymphoma cells), DG-75 (B cells), CCRF-CEM (acute lymphocytic leukemia cells), MOLT-10 (T cells), U-937 (histiocytic cells), HL-60 (promyelocytic cells), and K562 (erythroleukemia cells) were maintained as stationary suspension cultures in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2 in air. Lymphocytes and monocytes were separated from mononuclear cells in leukocyte concentrates collected from peripheral blood (200 ml) of healthy donors (26). More than 90% of these cells were collected in lymphocyte- and monocyte-rich fractions, as determined by nonspecific esterase staining, morphological examination, and staining with an anti-monocyte monoclonal antibody (mAb).

Biochemical Methods. DNA synthesis was determined by measuring the incorporation of [^{3}H]thymidine into the cellular DNA fraction (26). Protease activity was assayed by measuring the fluorescence liberated from a fluorogenic

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Abbreviations: Suc, succinyl; MCA, 4-methylcoumaryl-7-amide; mAb, monoclonal antibody; PHA, phytohemagglutinin.

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peptide (Suc-Leu-Leu-Val-Tyr-MCA; refs. 15 and 16). One unit of peptidase activity was defined as the amount degrading 1 nmol of substrate per hr. The protein concentration of the purified human proteasomes was calculated from their absorbance at 280 nm by assuming $A_{280}^{1\%,1cm} = 11.2$ (16). The protein concentration in cellular extracts was measured by the method of Bradford (27) with bovine serum albumin as a standard. Polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate (SDS/PAGE) was carried out by the method of Laemmli (28). Gel filtration was performed on a TSK-gel G4000PWXL column (Tosoh, Tokyo) in an HPLC apparatus.

Immunological Methods. Human liver proteasomes were purified and injected into rabbits to generate polyclonal antisera (16). mAbs against the purified human liver proteasomes were produced in mouse hybridoma cells by a standard method. For isolation of large amounts of mAbs, the cells producing antibodies were injected intraperitoneally into mice and the ascites containing antibodies were collected. The IgG fraction was separated by protein A-Sepharose chromatography. Serial sections of the tissue were stained immunohistochemically by the avidin–biotin–peroxidase complex (ABC) method (Vector Laboratories) (29). Tissues were treated with mixtures of culture media containing four mAbs against human proteasomes and then by the ABC method with normal goat serum, biotinylated anti-mouse IgG goat serum, and the avidin–horseradish peroxidase conjugate (Zymed Laboratories). For detection of proteasomes, the sections were washed extensively and treated by the peroxidase reaction with 3,3′-diaminobenzidine, which stained proteasomes brown. Sections were briefly counterstained with Mayer's hematoxylin solution, dehydrated, cleared, and mounted. The proteasome content of cultured cells was measured quantitatively by sandwich enzyme immunoassay with purified liver proteasomes as a standard (15). For immunoelectrophoretic blot analysis (30), proteins separated by SDS/12.5% PAGE were transferred electrophoretically to Durapore membranes (Millipore) with a semi-dry electroblotter (Sartorius). The membranes were pretreated with Block Ace (Dainippon Pharmacy, Tokyo) and then were treated with mAb 4-3 and anti-mouse IgG conjugated with alkaline phosphatase (ProtoBlot immunoblotting system, Promega) or ^{125}I-protein A (0.02 TBq/ml). They were then washed extensively with buffer containing 0.05% Tween 20 and stained by the alkaline phosphatase reaction, which stained proteasomes purple, or autoradiographed at −70°C.

RNA Blot Hybridization. Cultured or isolated cells were washed briefly with ice-cold phosphate-buffered saline, frozen in liquid nitrogen, and stored at −70°C until use. Northern blot analysis of total RNA (10 μg) extracted from various cells by the guanidinium thiocyanate/cesium chloride method (31) was performed as described (12, 13). The probe used with a *Hin*dIII–*Pvu* II fragment [≈1 kilobase (kb) long] of cDNA for subunit C2 of the rat proteasome (12). The RNA was transferred to a Hybond-N nylon membrane (Amersham) and hybridized with ^{32}P-labeled probe. The membrane was then washed (12) and exposed to Kodak XAR-5 film at −70°C with an intensifying screen.

RESULTS

mAbs Against Human Proteasomes. For determining the exact tissue distribution of proteasomes, we isolated four mAbs against human liver proteasomes, named 2-17, 2-21, 2-24, and 4-3. All were of the IgG1 subclass. Their specificities were examined by immunoelectrophoretic blot analysis (Fig. 1). mAb 4-3 reacted with only the largest component (31 kDa) of mammalian proteasomes; it reacted much more strongly with human proteasomes than with rat proteasomes.

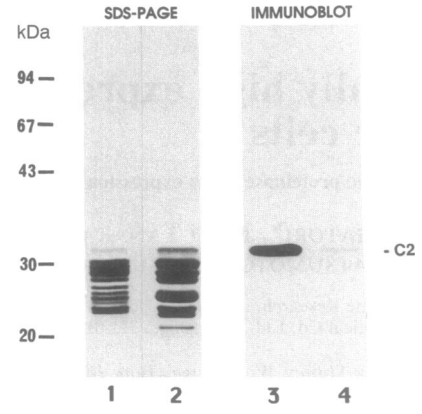

FIG. 1. Crossreactivity of mAb 4-3 with a single component of liver proteasomes. Samples (25 μg) of purified human (lanes 1 and 3) and rat (lanes 2 and 4) proteasomes were analyzed. Lanes 1 and 2, staining with Coomassie brilliant blue; lanes 3 and 4, immunostaining with mAb 4-3. Positions of marker proteins are shown at left.

This largest component was identified as that previously named C2 (11). The other three mAbs all reacted specifically with different epitopes of the C2 subunit, as judged by immunoblot analysis (data not shown). We examined whether mAb 4-3 could immunoprecipitate the whole proteasome complex (Fig. 2). mAb 4-3 or polyclonal antibody, together with protein A-Sepharose, precipitated the chymotrypsin-like (Suc-Leu-Leu-Val-Tyr-MCA-degrading) activity of proteasomes almost completely, although neither had any effect on the activity. Both antibodies also precipitated all the components of proteasomes, as judged by SDS/PAGE (Fig. 2 *Inset*). These findings indicate that C2 reflects the behavior of the whole proteasomal complex. This assumption is compatible with a previous finding that mAb 4-3 almost completely inhibited the ATP-dependent proteolytic activity of proteasomes in extracts of reticulocytes and muscle (4).

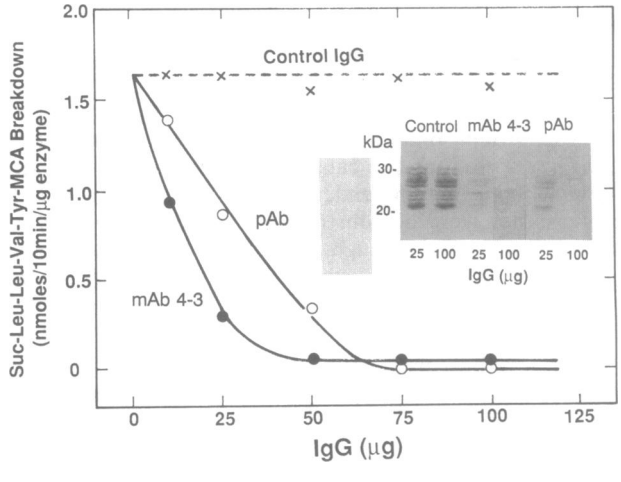

FIG. 2. Immunoprecipitation of proteasome complexes by monoclonal and polyclonal antibodies. Purified human liver proteasomes (50 μg) were treated for 60 min at room temperature with the indicated amounts of preimmunized control IgG (x), anti-proteasome mAb 4-3 IgG (●), or anti-proteasome polyclonal antibody (pAb) IgG (○). The mixture was then incubated at 4°C for 100 min with protein A-Sepharose 4B in an amount corresponding to 2-fold excess over the binding capacity of the added IgG. The precipitate formed was removed by centrifugation and the proteolytic activity of the supernatant fraction was measured. (*Inset*) SDS/PAGE profile of proteasomes included in the supernatant fraction. After immunoprecipitation, the enzymes in the supernatant fraction were precipitated by addition of 5 volumes of cold acetone and the precipitates were collected by centrifugation and subjected to electrophoresis.

Immunohistochemical Location of Proteasomes in Human Leukemia Cells. To compare the proteasome levels of normal blood cells with those of malignant tumor cells, we used a mixture of the four mAbs to stain proteasomes in specimens of bone marrow from patients with various leukemias. Abnormally high expression of proteasomes was observed in typical, irregular-shaped leukemic cells from patients with acute lymphocytic leukemia, adult T-cell leukemia, and acute myelocytic leukemia (Fig. 3). Similar results were obtained with bone marrow cells from patients with chronic lymphocytic leukemia and chronic myelocytic leukemia (data not shown). Moreover, the nuclear content of proteasomes in leukemic cells (Fig. 3, thick arrows) was much higher than those in normal cells, indicating that the proteasomes in leukemic cells were mainly localized in the nuclei. The proteasome levels differed in cells from different patients, possibly due to differences in the proliferation rates or states of differentiation and/or maturation of the cells. Proteasomes were also detected by staining in some, but not all, nuclei of apparently immature normal megakaryocytes (Fig. 3, thin arrows).

Proteasome Levels in Malignant Hematopoietic Cells. We next examined the expression of proteasomes in various types of malignant hematopoietic cell lines by quantitative enzyme immunoassay (Table 1). For some unknown reason, the proteasome contents of normal mononuclear cells were much lower than those of cells in normal tissues of rats (15) and humans (unpublished data). In contrast, the proteasome contents of four malignant hematopoietic cell lines derived from lymphocytes, Daudi (Burkitt lymphoma cells), DG75 (B cells), CCRF-CEM (acute lymphocytic leukemia cells), and MOLT-10 (T cells), were about 10 times those in normal lymphocytes. Likewise, the proteasome contents of three hematopoietic tumor cell lines derived from monocytes, U-937 (histiocytic cells), HL-60 (promyelocytic cells), and K562 (erythroleukemia cells), were much higher than those in normal monocytes, although the content varied greatly in different cell types. These findings indicate that proteasomes are expressed at abnormally high levels in malignant human hematopoietic cells.

For determination of whether the increased content of material that reacted with anti-proteasome antibody in these malignant cells was present as a proteasome complex or in subunit form, crude extracts of Daudi cells were fractionated by gel filtration on a TSK-gel G4000PWXL column. A proteasome complex, detected by its ability to degrade Suc-Leu-Leu-Val-Tyr-MCA (Fig. 4 *Lower*), and subunit C2, detected by reaction with mAb 4-3 (Fig. 4 *Upper*), were eluted in the same fraction with an apparent molecular mass of 600 kDa. No immunopositive bands were detected in fractions 14–16 corresponding to the elution position (≈30 kDa) of free subunit C2. Thus, the C2 protein was found in proteasomes, but not in the subunit form in cell extracts.

Table 1. Proteasomes in malignant human hematopoietic cells and normal blood mononuclear cells

Cells	Proteasome content, ng/mg of cell protein
Lymphocytes	135
Daudi (Burkitt lymphoma cells)	904
DG75 (B cells)	1483
CCRF-CEM (ALL* cells)	1598
MOLT-10 (T cells)	1358
Monocytes	687
U-937 (histiocytic cells)	3264
HL-60 (promyelocytic cells)	3939
K562 (erythroleukemia cells)	2065

Cells were isolated and cultured as described in *Materials and Methods*. The cells were harvested, sonicated, and centrifuged at 30,000 × *g* for 1 hr. The resulting cell extract was used for quantitative enzyme immunoassay of proteasomes with mAb 4-3 and polyclonal antibodies and for determination of protein concentration. Values are means for two independent experiments.
*Acute lymphocytic leukemia.

Next, we examined whether the increase in proteasomes in these malignant hematopoietic cells was due to an increase in their mRNAs. To measure the level of mRNA encoding a proteasomal protein, we used a full-length cDNA for the largest subunit, C2, of the rat liver proteasome complex (12). This cDNA identifies a single mRNA of 1.3–1.4 kb in both rat and human tissues (12). The levels of mRNA hybridized to the cDNA were greatly increased in all seven malignant hematopoietic cell lines examined: Daudi, DG75, CCRF-CEM, MOLT-10, U-937, HL-60, and K562 (Fig. 5). In contrast, the levels of mRNA for the C2 subunit were quite low in normal peripheral lymphocytes and monocytes isolated from healthy donors. We obtained essentially similar results for the mRNA levels of C3, another subunit of rat liver proteasomes (13), suggesting that the proteasome complex, presumably all the components, is expressed at very high levels in these malignant tumor cells.

Proteasome Expression During Blastogenesis of Lymphocytes. To examine whether lymphocyte proliferation is associated with increased expression of proteasomes, we studied the effect of phytohemagglutinin (PHA) treatment of resting human peripheral blood mononuclear cells, consisting of both lymphocytes and monocytes, on their expression of proteasomes, because PHA is known to be a potent T-cell

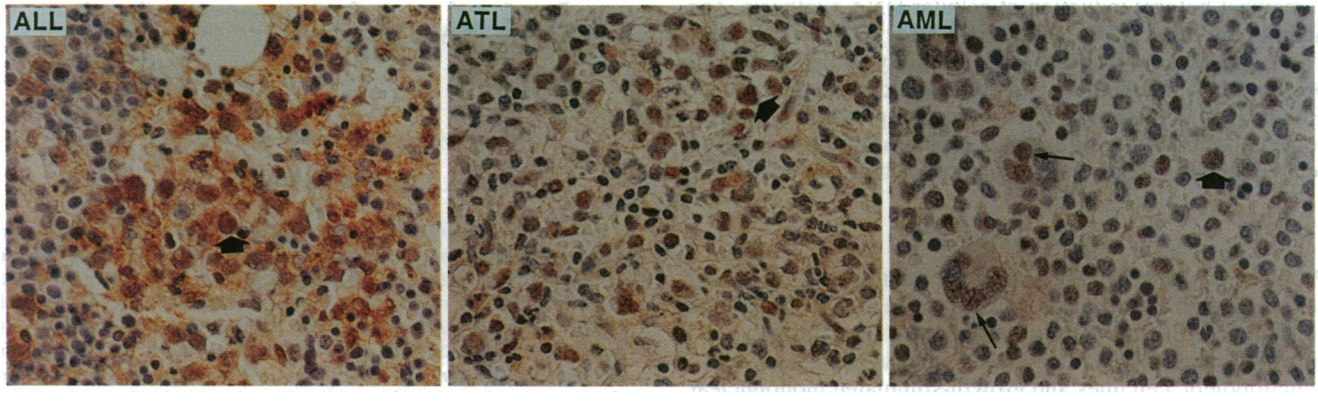

FIG. 3. Immunohistochemical staining of proteasomes (brown) in bone marrow from patients with various leukemias. Specimens of human bone marrow were obtained at autopsy from patients with acute lymphocytic leukemia (ALL), adult T-cell leukemia (ATL), or acute myelocytic leukemia (AML). (×340.)

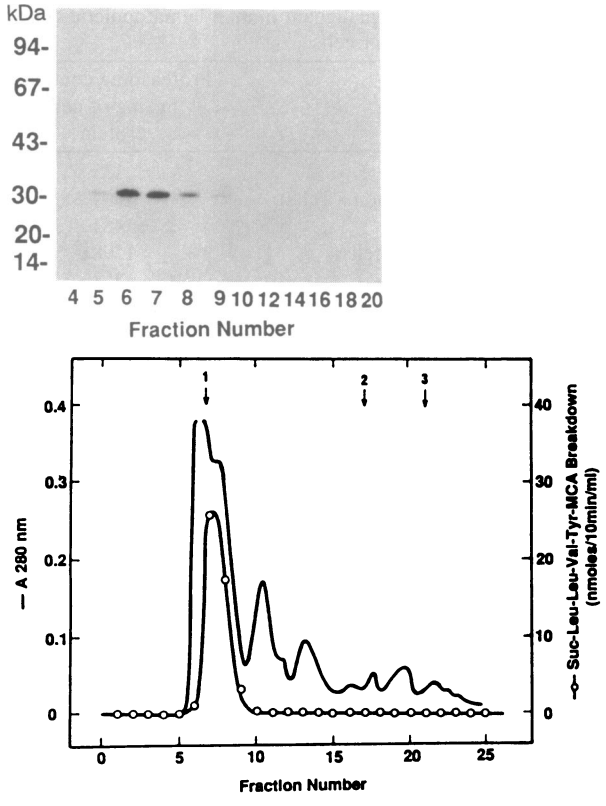

FIG. 4. Gel filtration of proteasomes and component C2 in Daudi cell extracts. Daudi cells (2×10^7) were lysed by sonication and centrifugation for 60 min at 20,000 × *g*. The resulting supernatant (0.5 mg of protein) was fractionated on a TSK-gel G4000PWXL column (7.8 × 300 mm) in 50 mM Tris·HCl, pH 7.5/20% (vol/vol) glycerol/0.5 mM dithiothreitol at a flow rate of 0.5 ml/min. Fractions (0.5 ml) were collected and 50 μl of each fraction was used for protease assay (*Lower*); arrows indicate elution positions of the molecular size markers: 1, thyroglobulin (660 kDa); 2, ribonuclease A (13.7 kDa); 3, acetone (total column volume). For immunoblotting analysis with mAb 4-3 (*Upper*), proteins included in 0.25 ml of each fraction were precipitated with acetone, subjected to SDS/PAGE, transferred to nitrocellulose membrane, and immunostained.

mitogen inducing blastogenic transformation. The level of proteasome C2 mRNA was low in untreated mononuclear cells from healthy donors, but when the cells were stimulated by addition of PHA, the C2 mRNA increased markedly (Fig. 6). During culture with PHA, the level of this mRNA was increased on day 2, was maximal on day 4, and returned to almost the unstimulated level on day 6. These changes in proteasome mRNA expression occurred in parallel with (or somewhat before) induction of cellular DNA synthesis. The number of cells increased ≈2-fold after PHA stimulation. Thus, expression of proteasomes is closely related to cellular proliferation, presumably in a cell-cycle-dependent manner. However, it is unknown whether high expression of proteasomes is simply a result of cell proliferation or is causally related to blastogenic transformation of these cells.

DISCUSSION

Proteasomes presumably play an essential role in crucial cellular activities, because they are ubiquitous in eukaryotic cells (1, 15, 16). In the present study, we found that expression of proteasomes was abnormally high in malignant human hematopoietic cell lines and growth-stimulated mononuclear cells (Table 1 and Fig. 6). In addition to these findings with cells in culture, we observed high expression of proteasomes in leukemic cells in bone marrow from patients with various

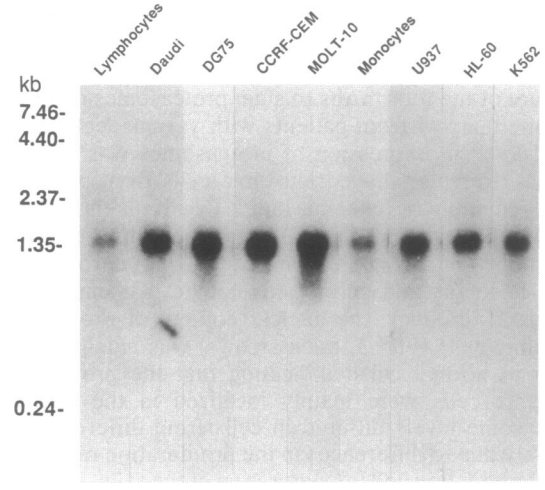

FIG. 5. Expression of mRNA encoding proteasome subunit C2 in various malignant hematopoietic cells and normal peripheral mononuclear cells. The cells used are described in Table 1. The lymphocyte- and monocyte-rich fractions from peripheral blood of healthy donors were prepared as described in *Materials and Methods*. Northern blot was probed with a *Hin*dIII–*Pvu* II fragment (≈1 kb) of cDNA for subunit C2.

types of leukemia (Fig. 3), suggesting possible involvement of proteasomes in the onset of human leukemia. Recently, we found that the mRNAs for proteasomes were also greatly increased in primary human hepatomas and in the human hepatoblastoma cell line Hep G2 (A.K., K.T., and A.I., unpublished data) and in various human renal cancer cells (H.

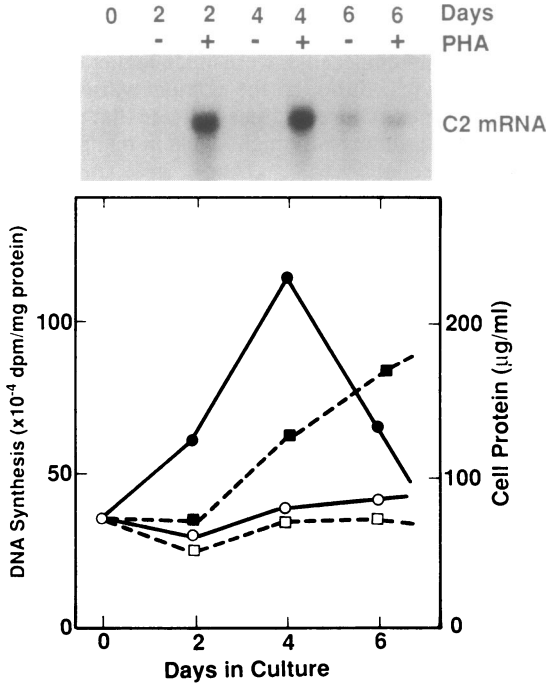

FIG. 6. Effect of PHA on the mRNA level for proteasome subunit C2 and proliferation of human mononuclear cells. Cells were cultured at 10^6 per ml as described in *Materials and Methods*, except that PHA (DIFCO) was added at a final concentration of 0.1% (vol/vol). (*Upper*) For preparation of total RNA fractions, samples of $\approx 10^8$ cells were taken at the indicated times and Northern blot analysis was carried out. (*Lower*) DNA synthesis was determined by measuring the incorporation of [^{3}H]thymidine into the cellular DNA fraction of 10^5 cells in 15 hr with (●) or without (○) PHA. As the cells aggregated during PHA treatment, cell growth was assayed by measuring total cellular protein in cultures with (■) or without (□) PHA.

Kanayama, K.T., and A.I., unpublished data). Thus, proteasomes may function similarly in three different types of cancer cells. Increased expression of the proteasome genes in a variety of malignant human hematopoietic cells in culture (Fig. 5) and *in vivo* (Fig. 3) may be essentially the same phenomenon as that in proliferating mononuclear blood cells during blastogenesis induced by PHA (Fig. 6), because these malignant cells are probably formed by transformation of immature hematopoietic cells. One possible reason why proteasome expression is activated in such cells is that proteasomes somehow function in the G_1 stage of the cell cycle, as judged by the parallel found between proteasome expression and alteration of DNA synthesis in PHA-stimulated mononuclear cells (Fig. 6). As malignant hematopoietic cells grown continuously, this may also be why expression of the proteasome genes in these cells is increased continuously. Thus, abnormally high expression of proteasomes may be important in proliferation or transformation of these cells. Very recently, we found that inactivation of the chromosomal genes of two major subunits of yeast proteasomes by a one-step gene-disruption method created independent recessive lethal mutations, indicating that proteasomes are also essential for proliferation of these cells, (T. Fujiwara, K.T., and A.I., unpublished data). This finding seems to be compatible with the present findings that the proteasome content increases during growth of normal human mononuclear cells and is abnormally high in tumor cells.

The molecular mechanism(s) of enhanced expression of proteasomes is unknown, but the finding that proteasomes are located predominantly in the nucleus of various leukemic cells (Fig. 3) seems to be significant in considering the physiological role of their elevated expression. For example, proteolysis catalyzed by proteasomes may be essential in division of the nucleus during mitosis, such as in dissociation and rearrangement of the chromatin. Proteasomes may also be responsible for selective removal of unnecessary proteins generated in the nucleus of proliferating cells. The c-*myc* and c-*myb* oncogene products have been shown to be degraded very rapidly through an energy-dependent pathway (32). The nuclear localization of proteasomal 19–20S particles has been observed in transcriptionally active hepatocytes (17), actively dividing mammalian cells (8, 18), and avian erythroblastosis virus-transformed erythroleukemic cells (23) and in cells of the sea urchin (19, 20), newt (24), axolotl (25), and *Drosophila* (14) in certain stages of oogenesis, embryogenesis, and development, but the relation of these particles to proliferation or transformation has not been determined. Another possible explanation for enhanced expression of proteasomes in cancer cells is that these complexes may be responsible for functions related to RNA metabolism, as has been proposed for the 20S ribonucleoprotein particles mentioned in the Introduction.

Although these findings suggest essential functions of proteasomes in carcinogenesis, the exact nature of these functions is unknown. Some proteases on cell membranes are involved in tumor metastasis by catalyzing proteolysis of the extracellular matrix (33). Wong *et al.* (34) reported that a cytoplasmic factor, ADR (activator of DNA replication), is an intracellular protease in growing lymphoid cells. The present study provides strong evidence that proteasomes are involved in carcinogenesis. The exact function in this process requires further study.

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