The effect of proteasome inhibitors on mammalian erythroid terminal differentiation

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Objective. Murine erythroblasts infected with the anemia-inducing strain of Friend virus (FVA cells) terminally differentiate to the reticulocyte stage after 48 hours of culture in vitro in response to erythropoietin (EPO). The objective of this study was to determine the possible role of proteasome-mediated proteolysis during the terminal differentiation of FVA cells.

Materials and Methods. The proteasome inhibitors MG132 and lactacystin were used to perturb the normal function of proteasomes during terminal differentiation. Effects of proteasome inhibitors on terminal differentiation were quantitated by evaluation of cellular morphology after benzidine staining and by Western blot analyses.

Results. Treatment of EPO-stimulated FVA cells with lactacystin or MG132 at later periods of culture increased accumulations of nuclear and cytosolic ubiquitinated proteins and decreased nuclear extrusion to less than 40% of controls.

Conclusion. Our results suggest that the proteasomal degradation of ubiquitinated proteins plays an important role in the enucleation of mammalian erythroblasts. © 2002 International Society for Experimental Hematology. Published by Elsevier Science Inc.

Among the most striking characteristics of mammalian erythroid differentiation are chromatin condensation, a decrease in cell volume, and the eventual extrusion of nuclei to produce reticulocytes. Murine erythroblasts infected with the anemia-inducing strain of Friend virus (FVA cells) complete terminal differentiation in vitro up to the stage of nuclear extrusion and reticulocyte formation in response to erythropoietin (EPO) [1–3]. FVA cells retain a maturation time course, an EPO dose responsiveness, and a sequence of differentiation events that appear similar to uninfected colony-forming units–erythroid (CFU-E) [2].

Many of the morphologic and physiologic alterations occurring during erythroid terminal differentiation involve the destruction and clearance of proteins that are no longer required for the function of the mature red cell. The ubiquitin-proteasome pathway is likely to play a role in this protein remodeling, as suggested originally by the high activity of this pathway in reticulocyte extracts [4]. Ubiquitin functions as a signal for substrate degradation in this pathway when covalently conjugated to the target molecule. We previously reported that the expression of two cytosolic ubiquitin-conjugating enzymes is strongly induced during the terminal differentiation of FVA cells [5], further suggesting a role for ubiquitin in erythroid terminal differentiation. Although it is likely that many ubiquitin conjugates are intermediates in the degradation of their respective target proteins, examples of stable ubiquitin-protein conjugates are also known. The best-characterized case is histone H2A. In higher eukaryotes about 10% of H2A exists in a monoubiquitinated form, even though H2A is a metabolically stable protein [6,7]. Other examples of stably ubiquitinated proteins include several receptors [8] and actin in insect flight muscle [9]. The existence of such stable conjugates suggests that other functions for ubiquitination, aside from targeting proteins for degradation, might exist.

The proteasome, a multicatalytic protease complex, is an essential component of the ATP-dependent proteolytic pathway that catalyzes the elimination of ubiquitinated proteins [10]. It is distributed in both the nucleus and cytosol, where it can comprise up to 0.5 to 1.0% of total cellular protein [11]. The mammalian 26S proteasome is composed of a 20S proteolytic core consisting of two outer α rings and two inner β rings, and two additional 19S regulatory complexes. The 19S regulatory complexes mediate ATP hydrolysis, substrate recognition, and presentation [11,12]. The 20S pro-
teolytic core contains proteolytic activities that are capable of cleaving proteins into small peptides [11,12]. The 26S proteasome catalyzes the rapid degradation of proteins that are covalently linked to polyubiquitin chains [12]. This pathway is highly regulated and selective, and it in turn regulates many important cellular processes such as transcriptional activation [13], cell-cycle progression [14], and antigen presentation [10]. Some studies have also shown that proteasomes may play an important role in regulation of cell proliferation [15–17], differentiation [16,18,19], and apoptosis [20,21].

Lactacystin, a specific proteasome inhibitor, was isolated from Actinomycetes based on its ability to promote neurite outgrowth from cultured neurons and to block cell division [22]. It acts by inhibiting proteasome function as a pseudo-substrate that becomes linked covalently to the hydroxyl groups on the active site threonine of the β subunits [23–25]. Lactacystin inhibits the three well-characterized, distinct peptidase activities of the proteasome: chymotrypsin-like, trypsin-like, and caspase-like, the first two irreversibly and all at different rates [23]. Lactacystin inhibits cell-cycle progression in both the G(0)/G(1) and G(2)/M phases in different cell types [26]. MG132, benzoyloxycarbonyl-leucyl-leucinal-leucinal, is a membrane-permeable peptide aldehyde that acts as a substrate analog that reversibly inhibits the proteasome with a K_i of 0.14 to 5.8 μM [10]. Unlike lactacystin, MG132 also inhibits certain lysosomal cysteine proteases and the calpains [10].

We employed proteasome inhibitors, morphological analyses, and Western blot analyses of total protein-ubiquitin conjugates in this study to investigate the possible role of the proteasome-mediated proteolysis of ubiquitinated proteins in erythroid terminal differentiation.

**Materials and methods**

**Chemicals and antibodies**

Lactacystin and z-leu-leu-leu-CHO (MG132) were purchased from BIOMOL Research Laboratories Inc. (Plymouth Meeting, PA, USA). All other chemicals, unless otherwise noted, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Mouse anti-ubiquitin antibody was a kind gift from Dr. Cecile M. Pickart (Johns Hopkins University, Baltimore, MD, USA). These antibodies recognize both free and conjugated ubiquitin on Western blots [27].

**FVA cell isolation and culture**

FVA cells were prepared as previously described [28]. Cells were cultured in Iscove’s modified Dulbecco’s medium (IMDM; GIBCO/BRL, Grand Island, NY, USA) containing 30% fetal calf serum (GIBCO/BRL), 0.1% deionized bovine serum albumin, 0.1 mM monothioglycerol, 100 U/mL penicillin, and 1 mg/mL streptomycin and maintained in 5% CO_2 in air at a density of 1 × 10^6 cells/mL.

**Treatment of FVA cells with proteasome inhibitors**

Lactacystin was dissolved in deionized water and MG-132 was dissolved in dimethyl sulfoxide (DMSO) to 0.25–10 mM, and the solution was added to culture medium to 1/1000 (v/v). The range of final concentrations used were 0, 0.25, 0.5, 1.0, and 5.0 μM for lactacystin, and 0, 0.25, 0.5, 1.0, 2.5, and 5.0 μM for MG132. The inhibitors were added to FVA cells after 29 hours of culture with EPO to investigate the role of the proteasome in the most terminal stages of FVA cell differentiation. Control cultures received either sterile deionized water or DMSO alone. Four slides of FVA cells were prepared using a CytoTek cytocentrifuge (Miles Scientific, Elkhart, IN, USA) just prior to adding the proteasome inhibitors to the cultures. The slides were stained, and the cells were counted as described below. The results of these four slides were used to determine baseline numbers of reticulocytes and extruded nuclei in cultures before FVA cells were treated with proteasome inhibitors (see Morphological Analyses below). At 41–43 hours of culture, cells treated with each concentration of the lactacystin or MG132 were collected, stained, and counted as described above, and nuclear and cytosolic protein fractions were collected for gel analysis and Western blotting as described below. Once the saturating dose of lactacystin was determined, the time required for lactacystin to have an effect on terminal differentiation was investigated. Lactacystin was added to FVA cells at a concentration of 1.0 μM at 29 hours of culture as described above. An equal volume of sterile deionized water was added to control cultures. Just prior to the addition of lactacystin (baseline) and at 1, 2, 4, 8, and 12 hours after addition, cytocentrifuge slides were prepared from 4 control and 4 lactacystin-treated cultures and subjected to morphological analysis as described below.

**Morphological analyses**

FVA cells cultured with EPO alone or with EPO plus a series of concentrations of lactacystin or MG132 were stained with 3,3’-dimethoxybenzidine and hematoxylin [2]. Four slides were prepared for each treatment, and cells were evaluated in a double-blind manner using oil immersion optics. Three hundred nuclei were evaluated on each slide and classified as being 1) intracellular and normal, 2) intracellular and apoptotic, 3) intracellular, but being extruded, and 4) extruded. Reticulocytes were also counted in the same oil immersion microscope fields used to evaluate nuclei. Differences in the means between measurements at different doses were determined using Student’s t-test. The results were plotted as indicated in the figure legends. Cytoplasmic hemoglobin concentration was qualitatively evaluated by the intensity of benzidine staining.

**Isolation of nuclear and cytoplasmic proteins**

FVA cells were collected from culture dishes, counted with a hemocytometer, and washed twice in IMDM. Cells were lysed in 0.5 mL of cold lysis buffer (20 mM Tris-HCL, 1 mM EDTA, 5 mM iodoacetamide, 1 mM PMSF, 0.2 mM Na-p-tosyl-L-lysine chloromethyl ketone (TLCK), 10 μg/mL leupeptin, 20 μg/mL soybean trypsin inhibitor, and 0.1% Nonidet P-40) [5] and incubated for 10 minutes on ice. The lysate was centrifuged for 10 minutes at 14,000g. A 1.5 × 10^6-cell aliquot of the supernatant, containing primarily cytosolic components, was reserved for total-protein determination using the BioRad Protein Assay (BioRad, Hercules, CA, USA). The remaining supernatant was removed and mixed with an equal volume of 2X SDS-PAGE sample buffer,
boiled for 5 minutes, and stored at −70°C for further analysis. The pellet, consisting primarily of nuclei, was resuspended in SDS-PAGE sample buffer, sonicated to sheer DNA, boiled for 5 minutes, and stored at −70°C for further analysis.

**Gel and Western blot analysis**

Nuclear and cytoplasmic samples were analyzed by SDS/PAGE as previously described [29]. Gels were run in pairs. One gel was stained with Coomassie blue to quantify sample loading, while proteins in the second gel were transferred to polyvinylidene difluoride (PVDF) membranes as previously described [30]. Incubation of PVDF membranes with antibodies was also performed as previously described [31]. Blots were blocked overnight in Tris-buffered saline (TBS) with 5% powdered milk. Anti-ubiquitin antibodies were used at a concentration of 1:6000. Following incubation with the primary antibody, blots were washed and incubated with horseradish-peroxidase conjugated goat anti-rabbit secondary antibody diluted 1:1000. Blots were washed and incubated with PVDF membranes as previously described [30]. Incubation of PVDF membranes with antibodies was also performed as previously described [31]. Blots were blocked overnight in Tris-buffered saline (TBS) with 5% powdered milk. Anti-ubiquitin antibodies were used at a concentration of 1:6000. Following incubation with the primary antibody, blots were washed and incubated with horseradish-peroxidase conjugated goat anti-rabbit secondary antibody diluted 1:1000. Blots were carried through a chemiluminescent detection procedure as directed (Renaissance, Dupont/NEN, Boston, MA, USA) and exposed to x-ray film.

**Results**

**Effects of the proteasome inhibitors on enucleation of FVA cells**

The morphology of FVA cells during terminal differentiation has previously been described [1]. In order to begin our investigation of the role of the proteasome on FVA cell terminal differentiation in the current study, a series of concentrations of lactacystin and MG132 were added to FVA cells after 29 hours of culture with EPO and the resulting effects on the morphology of terminal differentiation of FVA cells were evaluated. Both lactacystin and MG132 had dose-dependent effects on the enucleation of FVA cells (Fig. 1). The saturating dose for both inhibitors was approximately 1.0 μM. At this dose there was a decrease in enucleation to less than 40% of controls in the cultures of FVA cells treated with inhibitors. At higher doses of inhibitors (>2.5 μM) FVA cells exhibited morphological abnormalities of nuclei, namely decondensed chromatin and apoptotic-like morphologies that were not evident in FVA cells treated with the 1-μM concentration (data not shown). The inhibition of enucleation did not appear to affect cellular hemoglobinization in that benzidine staining was identical in treated and untreated FVA cells. The inhibition of enucleation was statistically significant at all doses of lactacystin and at all but the lowest (0.25 μM) dose of MG132 (Fig. 1).

The observation that a complete inhibition of enucleation was not achieved with any of the doses of either lactacystin or MG132 used in the experiments above led us to perform another series of experiments to determine the time course of the effect of lactacystin on FVA cells. We found that inhibition of nuclear extrusion did not occur simultaneously with the addition of lactacystin to the FVA cell cultures.

Figure 1. Effects of MG132 and lactacystin on nuclear extrusion and reticulocyte formation in terminallydifferentiating FVA cells. (A,B): Number of extruded nuclei (A) or reticulocytes (B) observed per 300 total nuclei. BL, baseline number of extruded nuclei or reticulocytes observed per 300 nuclei just prior to addition of proteasome inhibitors. 0.00, number of extruded nuclei or reticulocytes observed per 300 nuclei after 14 hours of culture in the absence of proteasome inhibitors. The concentrations of inhibitors used are indicated at the bottom of each figure. Values represent the mean ± one standard deviation of quadruplicate measurements. *, significantly different from value in untreated cultures (*p < 0.05). (C,D): Effects of MG132 and lactacystin on nuclear extrusion (C) and reticulocyte formation (D) normalized to the amount of each observed in control (untreated) cultures. The baseline number of extruded nuclei or reticulocytes was subtracted from each measurement prior to normalization.

Figure 2 illustrates that there was a lag of approximately 4 to 8 hours between the time lactacystin was added to cultures of FVA cells, and statistically significant differences were observed in the number of reticulocytes and extruded nuclei in treated vs untreated cultures.

**Effects of the proteasome inhibitors on the accumulation of ubiquitinated proteins during the terminal differentiation of FVA cells**

Previous studies have shown that inhibiting the activities of the proteasome prevent the rapid degradation of ubiquitin-conjugated proteins and cause an accumulation of these proteins in cells [10,32,33]. Western blot analysis was performed to confirm that proteasome inhibitors were blocking the breakdown of ubiquitinated proteins in the current study. Figure 3 illustrates the results of a Western blot analysis of nuclear and cytoplasmic fractions collected from FVA cells cultured for 14 hours with or without 1.0 μM lactacystin. Nuclear and cytosolic protein blots showed an in-
tense reaction over a broad range of molecular weights, with an increase specifically in high-molecular-weight ubiquitin protein conjugates, which correspond to the preferred substrates for the 26S proteasome [11]. There were clearly observable increases in the relative amounts of protein ubiquitin conjugates in the nuclei (compare lane 7 to lane 6, Fig. 3) and cytoplasm (compare lane 9 to lane 8, Fig. 3) of lactacystin-treated vs untreated FVA cells, though the increase was most dramatic in the nuclear fraction.

**Discussion**

The results of this study demonstrate that inhibition of the functions of the proteasome greatly decreased nuclear extrusion and reticulocyte formation in terminally differentiating FVA cells, suggesting that the proteasome may play an important role in nuclear extrusion. The decreased nuclear extrusion and reticulocyte formation observed after lactacystin treatment was accompanied by an increase in nuclear and cytosolic ubiquitin conjugated proteins in the lactacystin-treated FVA cells as compared to control FVA cells not treated with lactacystin, confirming that the concentration of lactacystin used in the experiment did inhibit generalized proteasomal degradation of both cytosolic and nuclear proteins.

The mechanism by which nuclei are extruded during terminal erythroid differentiation, and thus what role the proteasome might play in the process, are both unknown. One possible mechanism for the function of the proteasome in the extrusion of nuclei from differentiating erythroblasts is for the proteasome to have an effect on a regulatory protein or proteins responsible for triggering enucleation. In this model of erythroblast enucleation, proteasome inhibitors would block the proteolytic activation of a key regulatory molecule, or prevent the destruction of molecules whose presence serves to inhibit enucleation. Proteasomal degradation of ubiquitinated cyclins has been shown to be important for cell-cycle progression in eukaryotic cells [14], lend-
ing theoretical support for such a model. We observed a
generalized increase in the amount of high-molecular-
weight ubiquitinated proteins in both the nucleus and cyto-
sol of lactacystin-treated FVA cells as compared to controls. The majority of the ubiquitinated proteins likely represent those that would normally be degraded during terminal dif-
ferraturation as part of the cellular remodeling process in-
volved in converting a nucleated precursor cell into an anu-
cleate mature red blood cell, making it impossible to
directly visualize which of the ubiquitinated proteins are candidates for being involved in nuclear extrusion in such a
model.

Many studies have postulated that cytokinesis and enu-
cleation are similar processes and that actin filaments are
essential for enucleation [34–38]. A previous study visual-
ized F-actin in the region between the incipient reticulocyte
and extruding nucleus in enucleating FVA cells, and dem-
onstrated that treatment of FVA cells with cytochalasin D
blocks nuclear extrusion [39]. Proteasomes are observed to
associate with a number of cytoskeletal elements, including
both intermediate filaments and actin [39,40]. One study
demonstrated that in vitro polymerization of actin in the
presence of stoichiometric amounts of proteasomes yielded
ladder-like F-actin filament networks, with the proteasomes
forming the “rungs” between the adjacent actin filaments at
regular intervals [39]. These authors postulated that protea-
somes might be involved directly in the regulation of actin
filaments [39]. Thus, another mechanism by which protea-
some inhibitors could inhibit nuclear extrusion in FVA cells
is that proteasomes might regulate F-actin functions impor-
tant to FVA cell enucleation. In this model, when functions
of the proteasome are blocked by MG132 or lactacystin,
F-actin function related to enucleation would be downregu-
lated and cause decreases in free nuclei and enucleating cells.
Further studies are required to determine whether F-actin and
proteasomes can be colocalized in the constriction ring seen
between the incipient reticulocyte and extruding nucleus dur-
ing FVA cell enucleation.

Neither lactacystin nor MG132 completely blocked nu-
clear extrusion in FVA cells. We hypothesized at least two
possible explanations for this finding. The first was that
there is a lag period after the addition of the proteasome in-
hibitors before they had an effect. The small increase in ex-
truded nuclei and reticulocytes that occurred even in the
proteasome inhibitor–treated FVA cells as compared to un-
treated FVA cells could thus be explained simply by the
amount of nuclear extrusion that occurs during the lag pe-
riod. Our findings indicated that there was a lag period of
4–8 hours for lactacystin to inhibit FVA cell enucleation as
compared to controls, suggesting that at least part of the in-
complete inhibition of enucleation by the proteasome inhib-
itors can be explained by this mechanism. Lactacystin needs
to be converted to a β-lactone derivative while in the culture
medium before it can pass through the plasma membrane
and gain access to the cell interior [22]. One explanation for
the observed lag period may be that this represents the time
required for the derivitization of lactacystin to take place
and for a critical intracellular concentration of the β-lactone
derivative capable of inhibiting proteasome function to be
reached. MG132, however, has been reported to be mem-
brane permeable without further modification in other cell
types [10], and yet its effects on nuclear extrusion are iden-
tical to those caused by lactacystin. If FVA cell membranes
are as equally permeable to MG132 as are the cell mem-
branes of other cell types, then the lag period might be bet-
ter explained by a model in which a proteolytic regulatory
event precedes actual nuclear extrusion by a period of sev-
eral hours.

A second explanation for the incomplete block of enu-
cleation may be that the loss of proteasome function can be
partially compensated by other proteolytic systems in FVA
cells. Two studies reported that a giant protease [41] and a
proteolytic system [42] can compensate for the loss of pro-
teasome functions in other cell models. In the former study,
however, lactacystin was reported to inhibit specific func-
tions of the giant protease [41]. Further studies will be re-
quired to determine the mechanisms by which proteasome
inhibition blocks enucleation and whether an alternative
protease system can at least partially compensate for the
loss of proteasome function in lactacystin- or MG132-
treated FVA cells.

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