Degradation of Oxidized Proteins in K562 Human Hematopoietic Cells by Proteasome*

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Tilman Grunet, Thomas Reinheckel‡, and Kelvin J. A. Davies‡§
From the ‡Department of Biochemistry and Molecular Biology, The Albany Medical College, Albany New York 12208 and §Andrus Gerontology Center, University of Southern California, Los Angeles, California 90089-0191

Exposure to various forms of oxidative stress (H₂O₂ and O₂⁻) significantly increased the intracellular degradation of both “short-lived” and “long-lived” cellular proteins in the human hematopoietic cell line K562. Oxidatively modified hemoglobin and superoxide dismutase used as purified proteolytic substrates were also selectively degraded by K562 cell lysates, but exposure of these protein substrates to very high hydrogen peroxide concentrations actually decreased their proteolytic susceptibility. Our studies found little or no change in the overall capacity of cells and cell lysates to degrade “foreign” oxidized proteins after treatment of K562 cells with hydrogen peroxide or paraquat, a finding supported by proteasome Western blots and unchanged capacity of cell lysates to degrade the fluorogenic peptide succinyl-leucine-leucine-valine-tyrosine-4-methylcoumarin-7-amide. Six days of daily treatment of K562 cells with an antisense oligodeoxynucleotide directed against the initiation codon region of the human proteasome C2 subunit gene dramatically depressed hydrogen peroxide-induced degradation of metabolically radiolabeled intracellular proteins. The actual amount of proteasome in antisense-treated K562 cells was also severely depressed, as revealed by Western blots and by measurements of the degradation of the fluorogenic peptide succinyl-leucine-leucine-valine-tyrosine-4-methylcoumarin-7-amide. The degradation of oxidatively modified foreign protein substrates was also markedly depressed in lysates prepared from K562 cells treated with the proteasome C2 antisense deoxyoligonucleotide. The inhibitor profile for the degradation of H₂O₂-modified hemoglobin by K562 cell lysates was consistent with a major role for the ATP-independent 20 S “core” proteasome complex. We conclude that proteasome, probably the 20 S core proteasome complex, is primarily responsible for the selective degradation of oxidatively damaged proteins in human hematopoietic cells. Since “oxidative marking” of cellular proteins by lipoxygenase has been proposed as an important step in red blood cell maturation, it is important to determine which protease or proteases could recognize and degrade such modified substrates. Our results provide evidence that proteasome can, indeed, conduct such selective degradation and appears to be the major cellular protease capable of fulfilling such a role in maturation.

Over the past several years a wide series of publications from our laboratory (1–5) and other groups (6–8) have reported a relationship between protein oxidation and the proteolytic susceptibility of oxidatively damaged proteins. In bacteria a number of proteolytic enzymes seem to be responsible for the degradation of oxidatively damaged proteins, whereas in rat liver cells and rabbit, human, and bovine erythrocytes and reticuloocytes the multicatalytic protease complex proteasome is integrally involved in the process. A number of studies have been conducted with cell lysates, cell extracts, and purified proteasome in an attempt to determine the role of proteasome in recognition and degradation of oxidized proteins (2, 3, 5). However, the role of proteasome in the degradation of mildly oxidized proteins in eucaryotic cells has been challenged in recent publications (9, 10).

Several reports have explored possible roles of ATP-dependent and ATP-independent proteases in the degradation of oxidized proteins during red blood cell maturation (11–15). The lack of specific inhibitors has long been a limiting factor in studying the role of proteasome in intracellular protein turnover. Progress has been made with certain synthetic inhibitors (16–19), and recently an apparently highly specific natural inhibitor has been found (20). One of the most specific ways to block the proteasome, however, is through the use of an antisense oligonucleotide, as we recently demonstrated in studies of clone 9 rat liver cells (5).

We undertook the present investigations to test whether human hematopoietic cells are able to degrade oxidized proteins preferentially and how the ATP-independent 20 S proteasome is involved in this process. For our studies we selected K562 cells, a well known human hematopoietic cell line that is able to differentiate in culture.

MATERIALS AND METHODS

Cells and Cell Culture—K562 cells (chronic myelogenous leukemia, human) were obtained from American Type Culture Collection (ATCC CCL243). The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. Cells were initially seeded at a density of 0.4 × 10⁶ cells/ml and grown for 3 days to a density of 5 × 10⁶ cells/ml before use. For the experiments the cells were seeded into 16 × 125-mm tissue culture tubes (2 ml of cell suspension per flask) without changing the media or cell density in order to perform the necessary washing procedures. Cell washing or media removal were performed by centrifuging the cells at 1500 × g for 10 min and resuspending in the necessary medium.

Proteolysis Measurements—The measurement of degradation of metabolically radiolabeled proteins (5, 21) was performed after either a 2-h labeling procedure or a 16-h labeling procedure (followed by a 2-h “cold chase”) in different experiments. During the labeling procedure cells were incubated with a (³⁵S)methionine-cysteine mixture in methionine-free minimal essential Eagle’s medium. After 2 or 16 h of incubation at...
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**RESULTS**

Oxidative Stress Affects the Degradation of Metabolically Labeled Proteins—The effect of H$_2$O$_2$ and paraquat on the degradation of metabolically labeled short-lived proteins (i.e. proteins synthesized during 2 h of radiolabeling) is reported in Fig. 1. After treatment of the K562 cells with 0.4 mM H$_2$O$_2$, we found an increase in release of acid-soluble counts in comparison to the control (Fig. 1A). After 24 h the acid-soluble counts were twice the level of the control. However, an increase of the H$_2$O$_2$ concentration to 1.0 mM was not accompanied by any further increase in acid-soluble counts but instead caused a slight decrease.

We next tested the effects of the superoxide-generating agent paraquat (Fig. 1B). Cells were treated with paraquat at two different concentrations, 20 and 300 μM. Both concentrations increased the release of acid-soluble counts by about 30% at the 24-h time point (Fig. 1B).

The effect of these two oxidative stresses on the degradation of a longer-lived fraction of cell proteins was tested after labeling the cells for 16 h (followed by a 2-h cold chase) as shown in Fig. 2. Both concentrations of H$_2$O$_2$ used in the experiments as detailed above were twofold more potent than paraquat in the release of acid-soluble counts (Fig. 2).

**Degradation of Short-Lived Proteins in K562 Cells**

![Fig. 1. Degradation of short-lived proteins in oxidatively stressed human K562 hematopoietic cells.](image)

**Degradation of Long-Lived Proteins in K562 Cells**

![Fig. 2. Degradation of long-lived proteins in oxidatively stressed human K562 cells.](image)

**Degradation of H$_2$O$_2$ Modified Hb by K562 Cell Lysates**

![Fig. 3. Degradation of H$_2$O$_2$ modified foreign hemoglobin by human K562 cell lysates.](image)
showed similar effects on the degradation of long-lived proteins (Fig. 2A) as were seen in the experiments with short-lived proteins (Fig. 1), except that no biphasic proteolytic responses were seen with the long-lived protein pool. The effect of the superoxide-generating agent paraquat on long-lived proteins (Fig. 2B) was also similar to its effects on short-lived proteins (Fig. 1). Proteolysis after cell treatment with 300 μM paraquat was decreased in comparison to treatment with 20 μM paraquat but was still higher than proteolysis in the control cells (Fig. 2B).

Degradation of Foreign Purified Protein Substrates—To further explain the results shown in Figs. 1 and 2 we added oxidatively damaged tritiated hemoglobin as an exogenous substrate to lysates of K562 cells. The tritiated hemoglobin was oxidized with different concentrations of H2O2 prior to incubation with K562 cell lysates to test whether there is an optimum H2O2 treatment for proteolytic susceptibility. Whereas untreated hemoglobin was a poor substrate for proteolysis in K562 cell lysates, increasing H2O2 treatments up to 20 mM H2O2 caused a progressive increase in proteolytic susceptibility

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Degradation of Short-Lived Proteins in K562 Cells Treated with Proteasome C2 Oligodeoxynucleotides

FIG. 6. Degradation of short-lived proteins in human K562 cells treated with antisense oligodeoxynucleotides. Cells were either untreated (none) or treated for 6 days with daily 1 μmol/liter sense or antisense oligodeoxynucleotides to the initiation codon of the human proteasome C2 subunit mRNA (37). The sense oligodeoxynucleotide used was 5'-CACCATGGTTGAAA-3' and the antisense oligodeoxynucleotide was 5'-TTCCACATGTTG-3'. Both oligodeoxynucleotides were synthesized on an ab130 DNA synthesizer (Applied Biosystems, Foster City, CA) and extensively purified prior to use according to the manufacturer's protocol. Short-lived cell proteins were then labeled with [35S]methionine/cysteine for 2 h as described in Fig. 1. The cells were next either exposed to 0.4 mM H2O2 or used as controls. Trichloroacetic acid-soluble counts as a measure of proteolysis were assayed 24 h after treatment (main panel). The values represent the mean ± standard errors of nine independent experiments. The inset shows a Western blot of the 20 S proteasome, demonstrating the depletion of several subunits (at least three are obvious) after treatment with antisense oligodeoxynucleotide. The Western blot was performed according to Towbin et al. (39). Thirty μg of protein of centrifuged cell lysates were used per lane. The rabbit anti-rat 20 S proteasome antisemur was the kind gift of Dr. Keiji Tanaka (University of Tokushima, Tokushima, Japan). The lowest of the three obvious bands in the Western blot is the C2 subunit (molecular mass 30,000–32,000).

(HFig. 3). Hydrogen peroxide concentrations over 20 mM caused more extensive hemoglobin damage, as described previously (22), but actually decreased proteolytic susceptibility. Our explanation of this finding is that mildly oxidatively damaged proteins are susceptible to degradation, whereas extremely damaged proteins form cross-links and aggregates that are poor substrates for proteolysis (1–5, 21, 22).

To test whether protein degradation depends on increased proteolytic susceptibility of substrates or on protease induction or activation, we harvested cells after various time intervals of oxidative stress (H2O2 or paraquat), lysed the cells, and measured the ability of these cell lysates to degrade foreign hemoglobin. The results of these measurements are shown in Fig. 4. Oxidized hemoglobin was degraded up to four times faster by cell lysates than was unmodified hemoglobin. This was true for lysates from control cells, hydrogen peroxide-treated cells, and paraquat-treated cells. There were no time-dependent changes in the ability of the K562 cell lysates to degrade unmodified or control hemoglobin after oxidant treatment of the cells. Thus, protease induction/activation does not appear to be a significant contributor to overall proteolysis, which rather appears to depend on substrate modification.

Importance of Proteasome for Intracellular Degradation of Oxidatively Modified Proteins—Previous work in red blood cells demonstrated that the 20 S proteasome is responsible for about 70% of the increased proteolysis observed with H2O2 exposure, and our recent studies indicated a similarly important role for proteasome in cultured rat liver cells (5). The fluoropeptide s-LLVY-MCA is widely used as a preferential substrate for the 20 S proteasome. To test the possible importance of proteasome in degrading oxidatively modified proteins in K562 cells, we examined the ability of K562 cell lysates to degrade this fluoropeptide after oxidative treatment of the cells (Fig. 5). While we did observe in all cells a 10–15% decrease in proteolysis during the first 5 h, followed by a 30–35% increase in comparison with initial degradation rates, there were no observable H2O2 (Fig. 5A) or paraquat (Fig. 5B) effects. While we cannot explain these small changes in activity, it is important to note that they were limited to the s-LLVY-MCA peptidase activity of the lysates and not the activity against intact proteins.

We decided to further test whether there is a real induction of the proteasome using an immunoblot as indicator. The antibody used to recognize the 20 S proteasome was a purified IgG fraction of an anti-20 S proteasome antiserum. In none of the well known proteasome bands was there significant change in intensity, again indicating that proteasome is not induced in our system (data not shown).

To clarify the involvement of the proteasome in the increased degradation of oxidatively damaged proteins we decided to decrease proteasome activity in K562 cells using an antisense oligodeoxynucleotide against the initiation codon region of the H2C subunit of the proteasome. We used six daily treatments of K562 cell suspensions with 1 μmol/liter of either sense or antisense oligodeoxynucleotide. Cell proteins were then metabolically radiolabeled for 2 h with [35S]methionine/cysteine, treated with H2O2, and the release of trichloroacetic acid-soluble counts was measured after 24 h (Fig. 6). With both control cells and "sense" oligodeoxynucleotide-treated cells we measured an increase in proteolysis after treatment with 0.4 mM H2O2 of about 100%. This increase was almost totally abolished in the case of K562 cells that had been pretreated with the antisense oligodeoxynucleotide. To test whether there was a real decrease in the actual cellular proteasome content we performed an immunoblot (Fig. 6, inset) and found a decrease in several subunits of the proteasome.

To quantify the effect of the antisense oligodeoxynucleotide...
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TABLE I

Inhibitor profile for the degradation of H$_2$O$_2$-modified hemoglobin

| Inhibitor | Human K562 cell lysates | Clone 9 liver cell lysates | RBC lysates | RBC proteasome |
|----------|------------------------|---------------------------|------------|---------------|
| PMSF     | 78                     | 82                        | 84         | 84            |
| DFP      | 72                     | 78                        | 81         | 86            |
| NEM      | 66                     | 69                        | 78         | 79            |
| EDTA     | 51                     | 49                        | 49         | 45            |
| 8-Hydroxyquinoline | 54            | 50                        | 50         | 56            |
| Leupeptin | 7                      | 12                        | 10         | 9             |
| ATP/Mg$^2+$ | 22                   | 25                        | 22         | 19            |

on proteolytic activity we next measured the degradation of a foreign protein substrate, hemoglobin, in K562 cell lysates. As one can see in Fig. 7A, the antisense oligodeoxynucleotide decreased the degradation of oxidatively damaged hemoglobin by >60%, whereas the sense oligodeoxynucleotide had no effect. There were no significant differences between any of the samples in the degradation of undamaged hemoglobin. The same results were also obtained using either peroxide-treated or untreated superoxide dismutase as a proteolytic substrate (data not shown). In Fig. 7B degradation of the fluoropeptide s-LLVK-MCA is shown. Whereas the antisense oligodeoxynucleotide decreased the proteolytic degradation of the peptide by >60%, the sense oligodeoxynucleotide had no significant effect. One can conclude from these data that proteasome plays a major role in the selective degradation of oxidatively damaged proteins in human K562 cells.

Inhibition profiles for the degradation of oxidatively modified hemoglobin by human K562 cell extracts reveal a striking similarity with similar experiments in clone 9 liver cell lysates, erythrocyte lysates, and purified erythrocyte 20 S core proteasome complex (Table I). These results indicate that, as previously reported for erythrocytes, reticulocytes, and rat liver cells (1–5, 21, 22), the 20 S (670 kDa) core proteasome complex is primarily responsible for the degradation of oxidatively modified proteins in human erythroid progenitor cells.

DISCUSSION

Our studies indicate that mild forms of oxidative stress are able to increase the intracellular degradation of both short-lived and long-lived proteins in human K562 cells. Similar findings were previously reported from studies with bacteria, chloroplasts, isolated rat mitochondria, rabbit and human erythrocytes and reticulocytes, rat muscles, and rat liver cells (1–5, 21, 22). It seems clear that oxidative stress-induced intracellular proteolysis is a general biological phenomenon.

Our antisense studies using an oligodeoxynucleotide directed against the human proteasome C2 subunit indicate that proteasome is the main protease responsible for degradation of oxidized proteins in K562 hematopoietic cells. This is in good agreement with results from red blood cells (1–4, 21, 22) and rat liver cells (5, 22).

At least two forms of the multicyclase protease complex proteasome are known to co-exist in eucaryotic cells, the ATP-independent 20 S (670–700 kDa) form and the ATP-stimulated 26 S (1,500 kDa) form (23–37). Previous work with red blood cells (1–4, 21, 22) and rat liver cells (5, 22) provided experimental evidence that the ATP-independent 20 S (670–700 kDa) core proteasome complex is the form that selectively recognizes and degrades oxidatively damaged proteins. The degradation of oxidized proteins in red cell and rat liver cell lysates and by the purified erythrocyte or reticulocyte 20 S proteasome complex are actually inhibited 15–20% by ATP as shown in both the present work and in previous studies (2–5, 21, 22). This observation, plus the overall inhibitory profile reported in Table I, suggest that the ATP-independent 20 S (670–700 kDa) core proteasome is also largely responsible for the degradation of oxidized proteins in human hematopoietic cells.

We also looked for a possible net increase in total proteasome activity or cellular content, since oxidative stress might either activate proteasome or induce proteasome synthesis. Our present studies involving comparisons of control and oxidatively stressed cells, however, revealed no evidence to suggest either of these possibilities. By Western blot analyses with polyclonal antiproteasome antibodies and actual proteinase assays measuring cellular capacity to degrade control and oxidatively modified protein substrates such as hemoglobin and superoxide dismutase, no evidence for net increased amounts of proteasome protein or increased total activities were found. Small changes in proteolytic activity toward the fluoropeptide s-LLVY-MCA were not related to oxidation but may be the result of induction or activation of a single 20 S proteasome subunit, which is responsible for degradation of this peptide, as was described for individual yeast proteasome subunits (40, 41), or may result from increased activity of another cellular protease. We cannot yet discount the possibility that proteasome activation or induction may have occurred but might have been masked by an equal and opposite decline in activity or synthesis or by an increase in proteasome degradation during oxidative stress. Nevertheless, in the absence of any net increase in proteasome activity or content, it seems highly probable that oxidative modification of substrate proteins is the major cause of increased cellular protein degradation following oxidative stress.

The role of various proteases in the maturation of red blood cells has been widely studied (11–15). An important role for lipoxigenase as an initiator of oxidative damage in maturing red cells has been suggested (15). Furthermore, proteasome has been suggested as a candidate for the protease that may recognize lipoxigenase-damaged cellular proteins and initiate their selective degradation. Our current finding that proteasome appears largely responsible for the selective degradation of proteins damaged by oxidation in human erythroid progenitor cells adds credence to the concept that proteasome may perform a similar but more extensive function in the maturation process. Thus our initial studies also appear to validate the use of oligodeoxynucleotides to test this hypothesis.

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