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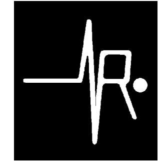
Free Radical Biology & Medicine 39 (2005) 1208 – 1215



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Original Contribution

Protein oxidation and degradation during postmitotic senescence

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Received 27 April 2005; revised 16 June 2005; accepted 20 June 2005

Abstract

Oxidized and cross-linked proteinacious materials (lipofuscin, age pigments, ceroid, etc.) have long been known to accumulate in aging and in age-related diseases, and some studies have suggested that age-dependent inhibition of the proteasome and/or lysosomal proteases may contribute to this phenomenon. Cell culture studies trying to model these aging effects have almost all been performed with proliferating (divisionally competent) cell lines. There is little information on nondividing (postmitotic) cells; yet age-related accumulation of oxidized and cross-linked protein aggregates is most marked in postmitotic tissues such as brain, heart, and skeletal muscles. The present investigation was undertaken to test whether oxidized and cross-linked proteins generally accumulate in nondividing, IMR-90 and MRC-5, human cell lines, and whether such accumulation is associated with diminished proteolytic capacities. Since both protein oxidation and declining proteolytic activities might play major roles in the age-associated accumulation of intracellular oxidized materials, we tested for protein carbonyl formation, proteasomal activities, and lysosomal cathepsin activities. For these studies, confluent, postmitotic IMR-90 and MRC-5 fibroblasts (at various population doubling levels) were cultured under hyperoxic conditions to facilitate age-related oxidative senescence. Our results reveal marked decreases in the activity of both the proteasomal system and the lysosomal proteases during senescence of nondividing fibroblasts, but the peptidyl–glutamyl-hydrolyzing activity of the proteasome was particularly inhibited. This decline in proteolytic capacity was accompanied by an increased accumulation of oxidized proteins.
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Keywords: Protein oxidation; Fibroblasts; Proteasome; Postmitotic senescence; Aging; Free radicals

Introduction

Various fluorescent age pigments accumulate in postmitotic cells, such as neurons and skeletal and cardiac myocytes [1–8], as well as in proliferating cells, such as hepatocytes [9,10]. The belief that accumulating age pigments are largely inert metabolic products is contradicted by studies from our group [11] and others [12], which have demonstrated an inhibitory effect of such accumulated proteinacious materials on normal protein turnover [11]. It is now import, therefore, to test whether the accumulation of oxidized/cross-linked intracellular proteins is causally related to decreased proteolytic activity of the proteasome and/or lysosomal proteases.

We have reported the selective degradation of oxidized proteins in both postmitotic cells [13–24] and in dividing cell lines [25–30]. These studies and a number of in vitro observations [31,32] demonstrate that proteins are inherently susceptible to oxidative damage, and that oxidative damage alters protein structure such that proteolytic susceptibility is modified. It now seems quite clear that mild oxidative damage increases proteolytic susceptibility, whereas extensive oxidative damage decreases proteolysis due to aggregation and cross-linking of the substrate proteins [33,35,36].

Abbreviations: PD, population doublings; ANEPPS, (4-(2-(6-dibutylamino)-2-naphthalenyl)ethenyl)-1-(3-sulfopropyl) hydroxide.

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doi:10.1016/j.freeradbiomed.2005.06.009

An age- or disease-related malfunction of the proteasomal system in degrading moderately oxidized proteins, resulting in further oxidation, aggregation, and cross-linking, is one suggested pathway for the formation of fluorescent age pigments, and it has been shown that oxidized proteins are located within such pigments [2,7]. To date, these relationships have mostly been studied in proliferative senescence, whereas proteolytic inhibition and the accumulation of oxidized and cross-linked protein aggregates have only been reported for a single postmitotic cell line [9].

We, therefore, decided to test the relationship(s) between changes in cellular proteolytic activities and the intracellular accumulation of oxidized/cross-linked proteins during non-proliferative senescence in two widely utilized human fibroblast cell culture lines: IMR90 and MRC-5 cells.

Materials and methods

Cell culture

IMR-90 and MRC-5-fibroblasts were obtained from the European Collection of Cell Cultures (Salisbury, UK). Since the supplier could not give the exact number of population doublings (PDs), the initial PD was arbitrarily set at zero. Both cell lines were cultivated in Dulbecco's minimal essential medium (Seromed, Berlin, Germany) supplemented with 10% fetal calf serum (US origin; Seromed) under normal atmospheric conditions (5% CO_2, 20% O_2). The experiments using nonproliferating cells were performed under hyperoxic conditions (5% CO_2, 40% O_2) for 4, 8, or 19 weeks in order to facilitate age-related oxidative changes. The medium was changed once a week. After various periods of hyperoxia, parameters of protease activity and protein oxidation were investigated. Cells were counted at each experimental time point to determine the loss of living cells under hyperoxic conditions.

Determination of proteolytic activities

The maximal activities of the lysosomal cathepsins and the proteasomal system were analyzed according to Inubushi et al. [37] and Grune et al. [26]. Between 0.1 and 2×10^6 cells were washed twice with PBS and then lysed in 150 μl of 1 mM dithiothreitol during vigorous shaking for 1 h at 4°C. The lysates were immediately used for determination of proteolytic activities.

Proteasome activity

Nonlysed cells, membranes, and nuclei were removed by centrifugation at 14,000*g* for 30 min. The supernatants were incubated in a buffer consisting of 50 mM Tris-HCl (pH 7.8), 20 mM KCl, 0.5 mM Mg-acetate, and 1 mM dithiothreitol. After a 1-h incubation with 200 μM of one of the fluorogenic peptides (vide infra), hydrolysis was stopped by the addition of an equal volume of ice-cold ethanol and by further dilution with 0.125 M sodium borate (pH 9.0). Three fluorogenic peptide substrates were used to measure the three distinct proteasome activities: suc-LLVY-MCA for the chymotrypsin-like activity, z-PFR-MCA for the trypsin-like activity, and z-LLE-βNA for the peptidyl–glutamyl-hydrolyzing activity. The fluorescence of the reaction products was monitored with 380 nm excitation and 440 nm emission for MCA, and 335 nm excitation and 410 nm emission for βNA, using free MCA or βNA, respectively, as standards.

Activity of lysosomal cathepsins

Lysates were sonicated for 2 min on ice in a Sonoplus GM70. The proteolytic activity assay was performed by incubation of lysates at 37°C for 30 min in a buffer containing 50 mM sodium acetate (pH 5.5), 8 mM cysteine hydrochloride, and 1 mM EDTA, in the presence of 200 μM z-FR-MCA as a fluorogenic peptide substrate. The reaction was terminated by the addition of an equal volume of ice-cold ethanol, and measurements of MCA release were performed as described for the determination of proteasome activity above.

Protein carbonyl measurement

The protein carbonyl content of cell lysates (4 mg/ml) was determined by the ELISA of Buss et al. [38], with modifications described by Sitte et al. [39]. The detection system consisted of an anti-dinitrophenyl-rabbit-IgG-antiserum (Sigma, Deisenhofen, Germany) as a primary antibody, and a monoclonal anti-rabbit-IgG-antibody peroxidase conjugated (Sigma, Deisenhofen, Germany) as secondary antibody. Development was performed with *o*-phenylenediamine.

Oxidized/cross-linked materials

Oxidized/cross-linked materials (lipofuscin-like or ceroid-like materials) of about 3×10^5 cells were determined by measuring the cellular autofluorescence in the red, green, or orange range of the spectrum (563–607 nm) by flow cytometry using a Becton-Dickinson FACScan as described previously [40].

Results

Proliferation of IMR-90 fibroblasts

Since the aim of the present study was to investigate protein oxidation and changes in protease activity in postmitotic cells, we first tested how long the IMR-90 cells were still able to divide under in vitro conditions. As shown in Fig. 1A, a number of approximately 12 population doublings was reached after 40 days under our in vitro

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