Phage-Displayed Antibodies for the Detection of Glycated Proteasome in Aging Cells

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ABSTRACT: Accumulation of posttranslationally damaged proteins during aging could explain the decline of cell performance with age. N^{ε}-carboxymethyllysine (CML) is the major glycation product on damaged proteins, causing dysfunction and cross-linking. The proteasome, a multicatalytic degradation complex, is one of the pathways for eliminating damaged proteins, and thus regulating their accumulation within the cell. However, the proteinase activities of the proteasome decline during aging. This may be due to posttranslational modifications of the subunits forming the proteasome complex. Using phage display technology, we have selected 16 single-chain variable fragments (scFv) recognizing the CML-modified α7 subunit of the proteasome. Using one of them, Ab3, we have observed a five-fold increase of CML-α7 in old human skin fibroblasts in comparison with young fibroblasts and telomerase-immortalized bone marrow cells (hTERT-BMCs).

KEYWORDS: proteasome; fibroblasts; bone marrow; protein modification; phage display

Advanced glycated end-products (AGEs) are irreversible posttranslational protein modifications that result from an intricate network of complex and spontaneous nonenzymatic reactions. These modifications can result in loss of functionality, misfolding, and cross-linking of proteins.[1] AGE accumulation has been reported to be associated with several pathological conditions, such as diabetes, uremia, atherosclerosis, and Alzheimer's disease, as well as with the normal aging process. AGE levels are considered to be good biochemical markers of the progression of these pathologies, and are thought to be the cause of some complications. Among AGEs are the glycoxidative products, Nα-(carboxymethyl)lysine (CML) and pentosidine, which require oxidation

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for their formation.[2,3] CML is the major AGE product on account of the numerous pathways leading to the formation of this glycoxidative product.[4,5] However, the reaction of glyoxal with the ε-NH2 group of a lysine is the main pathway leading to the generation of CML adducts.

One of the main defenses against accumulation of AGEs and other damaged proteins is the protein degradation machinery, the proteasome, which is a nonlysosomal multicatalytic proteinase ubiquitous in living forms.[6,7]

The activity of the proteasome has been observed to decrease with aging,[8] leading to the accumulation of damaged proteins. Several explanations have been proposed in order to explain this reduction of activity: downregulation of the expression of the proteasome subunits,[9] inhibition of the proteasome by damaged proteins,[10] and loss of activity by posttranslational modifications.[11] Posttranslational modification leading to the formation of AGEs occurs randomly, so it is difficult to determine which modifications cause the loss of function of certain macromolecule, such as the proteasome. The generation of antibodies by use of the phage display technology allows the production of a diverse range of different monoclonal antibodies specific for a given antigen. When antibodies are generated using damaged protein as antigen, molecular tools become available, making it possible to study the influence of single modifications on proteins.

The basic principle in phage display relies on the introduction of a foreign DNA into the bateriophage genome, creating fusion proteins between coat proteins of the filamentous bacteriophage and the foreign protein, which subsequently will be expressed on the surface of the phage particle.[12] When the foreign DNA is introduced in the phage genome code for fragments of human antibodies, large libraries of human antibody fragments will be displayed on the surface of the phage, thus allowing us to perform selections in which specific antibodies are isolated on the basis of their affinity for the antigen. In this study we used the phage display technology to select specific antibodies against the CML-modified α7 subunit of the proteasome.

SELECTION OF SPECIFIC scFv ANTIBODIES

Four micrograms of CML-modified α7 subunit were used to coat an immunotube and were subsequently incubated with the Tomlinson J scFv repertoire.[13] After washing off the nonbinding phages, specific binders were eluted and were able to infect a TG-1 bacterial culture. More than 2,000 colonies were obtained and 768 were monoclonally reproduced. Phages from one plate were tested for specificity in enzyme-linked immunosorbent assay (ELISA) against α7 and CML-α7, using GST and CML-GST as negative control. Fifty positive signals were obtained in the ELISA recognizing either α7 or CML-α7, of which 16 were specific against the CML-α7. One of the 16 specific clones against CML-α7, Ab3, was recloned in the pKBJ3 expression vector,[14]

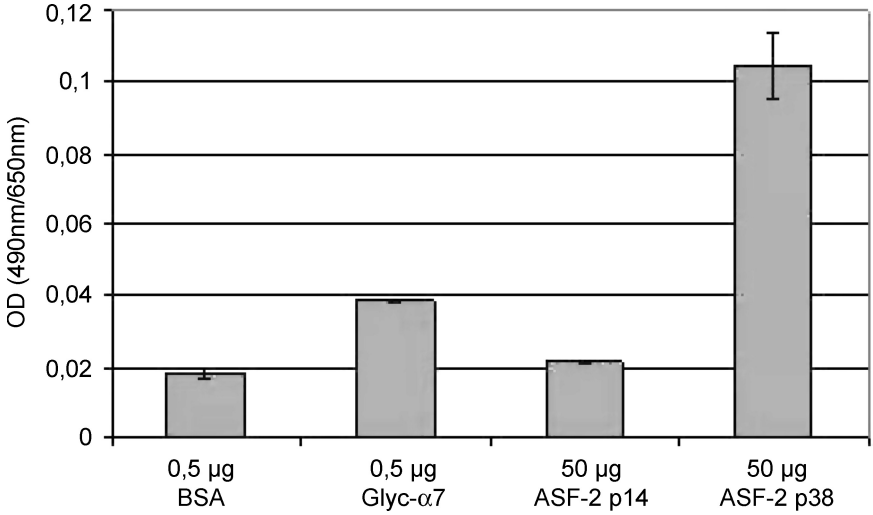

FIGURE 1. Analysis of the presence of CMLα7 in different cellular extracts in ELISA using Ab3. ELISA of old (p36) and young (p14) fibroblasts extract, with BSA as negative control, and glyc-α7 as positive control.

allowing an efficient expression of soluble antibody in bacteria, which were then purified using a his-tag. The activity of the purified Ab3 was tested and used in the analysis of the importance of CML modifications on a specific site of the proteasome.

CML-α7 ACCUMULATES IN FIBROBLAST DURING AGING

Cell extracts of serially passaged old (>90% of the life span completed) and young (<25% life span completed) human skin fibroblast ASF-2 were coated in an ELISA plate and the ELISA was performed using Ab3. In young human skin fibroblast and in fast-dividing hTERT-MSC cells (human telomerase immortalized mesenchymal stem cells) minute amounts of CML-α7 could be detected. Following serial passing of fibroblast we observed an increase in the accumulation of CML-α7, resulting in a five-fold accumulation of CML-α7 in fibroblasts, which have reached more than 90% of their life span (FIG. 1).

In conclusion, we have observed an age-related increase in glycated α7 subunit of the proteasome, or in other words damaged proteasome, in human cells undergoing aging *in vitro*. The absence of accumulation of damaged proteasome in the hTERT-MSCs and young fibroblasts in comparison with the old fibroblasts could be due to a faster dispersion of the possible damaged proteins into the daughter cells. On the contrary, old cells with a lower replication rate accumulate damaged proteins. Finally, we have developed a tool for analyzing

a marker of proteasomal malfunction, cellular viability, and a potential marker of aging. Additionally, the proteasome condition can be determined by using this antibody after a detrimental or beneficial treatment of cells, such as the addition of glyoxal, cytokinins, and heat shock. Moreover, Ab3 will allow us to determine whether decreasing cellular proliferation will result in the accumulation of damaged proteasome and inhibition of *de novo* synthesis of proteasome.

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