Age-related decline in expression of calnexin

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Abbreviations: ER, endoplasmic reticulum; ESI Q-TOF MS, electrospray ionization quadrupole-time of flight mass spectrometry; HDF, human diploid fibroblast; HSP, heat shock protein; MALDI-TOF MS, matrix-assisted laser desorption/ionization-time of flight mass spectrometry

Abstract

Aging is accompanied by the changes in the cells that decrease their capacity to respond to various forms of stress. Cells are known to respond to stresses through expression of stress-response proteins, heat-shock proteins composed of molecular chaperones. Recent studies suggest that chaperone level and stress-induced chaperone expression could decrease with aging. The aim of the present study is to identify chaperones that show a significant change in protein expression with aging. We used an *in vitro* aging model system of human diploid fibroblasts (HDF). Proteome analysis of HDF showed that endoplasmic reticulum (ER) chaperone, calnexin, significantly decreased with aging. Oxidative stress-induced expression of calnexin also attenuated in old HDF compared to young cells. These findings suggest calnexin decreases with aging and might contribute to a cytoprotection in a variety of human age-related diseases.

Keywords: Calnexin; Chaperone; Heat shock protein; Proteomics; Senescence

Introduction

Aging is related to a decrease in the stress response *in vitro* as well as *in vivo* and could be seen via several cellular alterations, that can be responsible for

age-related diseases such as cancer, cardiovascular disorders, sclerosis and nephropathies (Salvioli *et al.*, 1994). In deed, cardiovascular diseases represent one of the fatal disease among an ageing population. Although blockade of the coronary vasculature is known as a common cause of damage to myocardium, other changes in cellular level that occur during the aging process result in decreasing function of myocardium and rendering it more susceptible to damages (Verbeke *et al.*, 2001; Taylor and Starnes, 2003). Several reports have already described a decreased capacity of the aging myocardium to different types of stresses (Gray *et al.*, 2000; Verbeke *et al.*, 2001; Boraldi *et al.*, 2003; Taylor and Starnes, 2003). Furthermore, age-related alterations in the expression and activation of proteins are known to be important for the cardioprotective process such as heat shock protein (HSP) 70 (Taylor and Starnes, 2003).

Heat shock response is one of primary intracellular defense mechanisms against stressful conditions. Number of heat shock proteins and molecular chaperones are known to be induced by variety of stimuli such as elevated temperature, ischemia, hypoxia, pressure overload and oxidative stress (Walsh *et al.*, 1997; Feder and Hofmann, 1999; Fehrenbach and Niess, 1999; Gray *et al.*, 2000; Jolly and Morimoto, 2000; Verbeke *et al.*, 2001). Interestingly, cells isolated from aged tissues and organisms, and cells undergoing replicative senescence *in vitro*, have a reduced heat shock response (Liu *et al.*, 1996; Verbeke *et al.*, 2001; Taylor and Starnes, 2003).

Proteomics analysis has provided a tool to examine minor changes in cellular protein expression as the technique develops in enhancing sensitivity and allowed to utilize the molecular mechanisms of cellular aging (Toda, 2000; Schoneich, 2003).

In this study, proteomics tool was used for the analysis of HSP in aging cells. The aim of the present study is to identify age-related HSP that shows a significant decline in protein expression with aging by employing an *in vitro* aging model system of human diploid fibroblasts (HDF). Proteins of the cells were separated by two-dimensional electrophoresis and protein identification was performed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS).

Materials and Methods

Cell culture

HDF was kept in 10-cm plates in Dulbecco's modified

Eagle's medium supplemented with 10% fetal bovine serum and antibiotics at a humid atmosphere (5% CO₂, 95% air) and subculture at a ratio of 1:4. We defined young cells as those resulting from less than 20 population doublings (PD), and old cells were from more than 40 PD. Cellular senescence of all old cells was confirmed by their delayed population doubling times and by a senescence-associated β -galactosidase activity assay, as described by Dimri *et al.* (1995).

Western blotting

Cells were harvested in the lysis buffer (20 mM Hepes pH 7.2, 1% TritonX-100, 150 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, and 1 μ g/ml aprotinin). After incubation for 30 min at 4°C, cellular debris was removed by centrifugation at 100,000 *g* for 30 min, and supernatants were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein concentration was determined by the Bio-Rad protein assay reagent (Bio-Rad Laboratories, LA). Samples (50 μ g) were prepared with the four volume of 0.5 M Tris-HCl buffer (pH 6.8) containing 4% SDS, 20% glycerol and 0.05% bromophenol blue at 95°C for 5 min. SDS-PAGE was performed in 10% slab gel. Proteins were transferred to nitrocellulose paper. The membrane was washed in blocking buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5% fat-free milk) for 60 min at room temperature with shaking and then washed with TBST (TBS, 0.01% Tween 20). Blots were incubated with the primary antibodies against calnexin (Santa Cruz, CA) followed by horseradish peroxidase-conjugated secondary antibody.

Two-dimensional SDS-PAGE

After lysis buffer (8 M Urea, 4% CHAPS, 40 mM Tris base, 1% DTT, 0.5% immobilized pH gradient (IPG) buffer, protease inhibitor) was added to HDF, the sample suspension was sonicated in short bursts on ice. The lysate was centrifuged in a microcentrifuge at 30,000 *g* for 15 min at 18°C. The supernatant was saved at -80°C until used. The first dimension of 2-D electrophoresis was performed on an Amersham Pharmacia Biotech IPG phor Isoelectric Focusing System. Linear pH 3-10 Immobiline Dry Strips (24 cm) were rehydrated overnight at room temperature in rehydrating buffer (8 M Urea, 1% DTT, 2% CHAPS, 0.5% IPG buffer, BPB). Sample (500 μ g) was applied during rehydration. The first dimension was run for 53,500 V/h using the following conditions at 20°C: 500 V for 1 h, 1,000 V for 1 h; 8,000 V for 6 h 30 min. Next, gels were equilibrated for 30 min in equilibration buffer I (50 mM Tris-HCl (pH 8.8), 6 M Urea, 30% Glycerol, 2% SDS, 0.1% DTT) and equilibration buffer

II (50 mM Tris-HCl (pH 8.8), 6 M Urea, 30% Glycerol, 2% SDS, 0.25% IAA). The second dimension was run according to Ettan DALT II system operating and maintenance manual. A 12.5% SDS-polyacrylamide slab gel was used for the second dimension gel electrophoresis. The IPG strips were placed on the surface of the second dimension gel, and then the IPG strips were sealed with 0.5% agarose in SDS-electrophoresis buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS). The gels were placed into Ettan DALT II system chamber containing 1 $\times$ SDS electrophoresis buffer. The gels were run overnight at 110 V until the front dye reached the bottom of the gel.

Silver staining

Silver staining of gels was performed as modified silver staining protocol using Silver Stain Plus One kit. The use of glutaraldehyde in the sensitization step and formaldehyde in the silver impregnation step was omitted. After electrophoresis, the gels were fixed with 40% methanol/10% acetic acid for 30 min. The gels were sensitized by incubating in sensitizing solution (0.2% sodium thiosulphate, 30% methanol, sodium acetate 68 g/l), and it was then rinsed with three changes of distilled water for 5 min each. After rinsing, the gels were incubated in 0.25% silver nitrate for 20 min. After incubation, the silver nitrate was discarded, and the gels were rinsed twice with distilled water for 1 min and then developed in 0.15% formaldehyde in 2.5% sodium carbonate with intensive shaking. After the desired intensity of staining was achieved, the development was terminated with 1.46% EDTA.

Image analysis

We digitized the silver-stained gel image using a scanner (UMAX Power Look III). Image Master 2D (Amersham Pharmacia) was used to analyze the gel images.

Destaining

Silver-stained proteins were destained with chemical reducers to remove the silver as described previously with following critical modifications (Scheler *et al.*, 1998; Song and Lee, 2001). Potassium ferricyanide and sodium thiosulfate were prepared as two stock solutions of 30 mM and 100 mM, respectively and both dissolved in water. A working solution was prepared by mixing a 1:1 ratio prior to use. After interesting protein spots were excised from the gel, thirty to fifty microliters of working solution were added to cover the gels and occasionally vortexed. The stain intensity was monitored until the brownish color disappeared, then the gel was rinsed several

Figure 1. Typical electrophoretic patterns of proteins in young HDF cells (PD 21) and old HDF cells (PD 45) on a 2-D gel. Cellular proteins were separated by 2-D PAGE using IPG-Dalt, digested on membranes in the presence of 50% acetonitrile, then analyzed by MALDI-TOF MS. Arrows indicate proteins that were significantly and consistently different in young cells versus old cells on 2-D gel.

times with water to stop the reaction. Next, 200 mM ammonium bicarbonate was added to cover the gel for 20 min and was then discarded. Subsequently, the gel was cut into small pieces, washed with water, and was dehydrated repeatedly with changes of acetonitrile until the gel pieces turned opaque white. The gel pieces were dried in a vacuum centrifuge for 30 min.

Trypsin digestion of proteins in-gel

Enzymatic digestion was performed as previously described (Hellman *et al.*, 1985; Shevchenko *et al.*, 2001). Briefly, digestion was performed with 5–10 ng/ μ l of trypsin and 50 mM ammonium bicarbonate and incubated overnight at 37°C. Following enzymatic digestion, the resultant peptides were extracted three times with 10–20 μ l of 5% trifluoroacetic acid in 50% acetonitrile and dried using a vacuum centrifuge for 30 min.

Identification of proteins

The dried samples were sent to Korea Basic Science Institute, Daejeon, South Korea for MALDI-TOF MS analysis and electrospray ionization quadrupole-time of flight mass spectrometry (ESI Q-TOF MS) analysis.

Results and Discussion

Protein profiles during replicative cellular senescence of HDF were analyzed by 2-D PAGE and stained with silver. We routinely observed between 650–800 spots from 400 mg of protein when stained with routine silver staining procedures. We found nine proteins that were significantly and consistently different in

young (PD 21) versus senescent cells (PD 45) on 2-D gels. Those proteins stained with silver were representatively shown in Figure 1. The spots were excised and destained. Then those were digested by trypsin overnight and were analyzed by MALDI-TOF MS for peptide finger printing as well as by ESI Q-TOF MS for peptide sequencing. The results of the MALDI-TOF MS and ESI Q-TOF MS indicated unequivocally that the identities of the spots were calnexin precursor (spot 2), DNA-repair protein XRCC2 (spot 4), and cytokine response protein CR6 (spot 7), respectively. We failed to identify the other proteins that were not enough amount to analyze by MALDI-TOF MS and ESI Q-TOF MS, although they were significantly and consistently different among the experimental groups. Among identified proteins, calnexin was significantly decreased in an age-dependent manner and the protein expression level of calnexin in senescent cells on 2-D gel was decreased to $26 \pm 10\%$ ($n = 6$) of young cells (Figure 2B). Subsequently, our next experiment was focused on age-related calnexin response to stress. To determine whether age affects the oxidative stress-induced calnexin expression, young cells and senescent cells were exposed to hydrogen peroxide (H_2O_2), respectively. The calnexin level was determined by Western blotting (Figure 3). H_2O_2 caused a significant dose-dependent increase of calnexin expression in young cells, but not in senescent cells.

Several studies have reported that the changes in ER chaperone expression may contribute to the extension of life spans. Caloric restriction (CR) delays most age-related physiologic changes and it is regarded as the only method known for extending life span in homeothermic vertebrates, and it also thought to be the most effective means known for

Figure 2. Calnexin spot significantly changed with aging. (A) Spot sizes indicate a clear decrease of calnexin in old cells (PD 45) compared to young cells (PD 21). (B) Histogram shows relative intensities of calnexin spots in both cells. Results are means $\pm$ SE, $n = 6$. Probability values were determined by ANOVA. (* $P < 0.001$).

reducing cancer incidence and increasing the mean age of onset of age-related diseases and tumors (Weindruch and Walford, 1982; Dhahbi *et al.*, 1997). It has been reported that CR-induced decrease of ER chaperone is responsible for extending the life span (Dhahbi *et al.*, 1997). In the present study, calnexin, a ER chaperone, exhibited a significant age-dependent decrease during replicative cellular senescence of HDF and furthermore oxidative stress-induced calnexin expression was attenuated in senescent cells compared to young HDF. These results strongly indicate that calnexin is closely related with aging process and it might contribute to establish a cytoprotective state in a variety of human age-related diseases.

There are a number of assay methods to measure the protein expression level through a variety of methods, including genomics, proteomics, Northern blotting, Western blotting, In-situ hybridization, and immunoassay. Most of those assay methods have each different principles and sensitivities, and then these methods are not fit to all experiments. Over the years, many theories of aging have been proposed in the field of aging research, but none of these theories are clearly testified yet. Therefore, it has been recently suggested that proteomics could offer great potential for molecular studies of biological aging and medicine

Figure 3. Age-related decline in oxidative stress-induced calnexin expression. Young HDF (PD 21) and old HDF (PD 45) were seeded on 6 well plates, respectively. After both cells were at the same time reached to confluency, these cells were treated with H₂O₂. These cells were incubated with various concentrations of H₂O₂ (0-100 μ M) at 37°C for 24 h. After incubation, the cells were lysed with the lysis buffer. Calnexin level was determined by Western blot analysis.

(Dierick *et al.*, 2002; Seong *et al.*, 2002; Schoneich, 2003; Eun *et al.*, 2004). As shown in Figure 3, no difference in calnexin level with aging was detected by Western blotting. Therefore, Western blotting could not provide us with information about the proteins that show slight changes during cellular senescence. However, proteomics makes it be possible for us to detect the changes of calnexin level with aging. Thus, this study indicates that proteomics could be applied potentially to gerontological research.

In conclusions, calnexin decreases with aging and oxidative stress-induced calnexin expression was attenuated in senescent cells. Thus, it might have a cytoprotective effect on a variety of human age-related diseases, which is recently known to be one of essential factors for extending of life span.

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