Nutraceutical Strategy in Aging

Targeting Heat Shock Protein and Inflammatory Profile through Understanding Interleukin-6 Polymorphism

F. MAROTTA,[a] K. KOIKE,[b] A. LORENZETTI,[a] Y. NAITO,[c] F. FAYET,[a–d] H. SHIMIZU,[b] AND P. MARANDOLA[a]

[a] *G.A.I.A. Age-Management Foundation, Pavia, Italy*

[b] *BioScience Lab, Osato Research Institute, Gifu, Japan*

[c] *Immunity Research & Clinic, Nagoya, Japan*

[d] *School of Molecular & Microbial Biosciences, University of Sydney, Sydney, Australia*

ABSTRACT: The aging process is paralleled by two- to fourfold increases in plasma/serum levels of inflammatory mediators, such as cytokines and acute-phase proteins. In this study we assessed the inflammatory profile and polymorphism of healthy elderly subjects and the influence of a nutraceutical supplement. Forty elderly, generally healthy subjects were recruited, divided into two matched groups, and given either a fermented papaya preparation 9 g/day by mouth or the same amount of placebo. Treatments were carried out in a cross-over manner with a 3-month supplementation period followed by a 6-week washout period between treatments. Ten healthy young subjects served as controls. Interleukin-6 (IL-6) promoter −174 G/C polymorphism genotype was determined together with blood levels for redox status, proinflammatory cytokines, high sensitivity C-reactive protein, and serum 70 kDa heat shock protein (Hsp70) concentrations. Tumor necrosis factor-α and IL-6 were higher in elderly subjects ($P < 0.05$ versus young controls). The concentration of Hsp70 inversely correlated with markers of inflammation in −174 G/C-negative subjects ($r = 0.62$, $P < 0.05$). Nutraceutical intervention normalized the inflammatory parameters ($P < 0.05$) with a rise of Hsp70 ($P < 0.05$). This suggests that healthy elderly individuals may have a proinflammatory profile playing as a downregulating factor for inducible Hsp70, particularly if −174 G/C-negative. A nutraceutical intervention seems able to beneficially modulate such a phenomenon.

KEYWORDS: Hsp70, IL-polymorphism; fermented papaya preparation; pro-inflammatory cytokines

Address for correspondence: Prof. F. Marotta, MD, PhD, Via Pisanello, 4, 20146 Milano, Italy. Voice/fax: +39-024077243.
fmarchimede@libero.it

Ann. N.Y. Acad. Sci. 1119: 196–202 (2007).
doi: 10.1196/annals.1404.011

INTRODUCTION

Aging is accompanied by two- to four-fold increases in plasma/serum levels of inflammatory mediators, such as cytokines and acute-phase proteins. However, the physiological effects and efficacy of antioxidant supplements in human diets remain controversial. Experiments involving dietary intake of a single antioxidant or vitamin, often in high doses, may be seriously flawed given the interactive nature of antioxidants *in vivo*. The cellular response to environmental, pathological, or physiological stresses is followed by a rapid synthesis of molecular chaperones, such as the heat shock family of stress proteins (HSP). HSPs form a large family of proteins that are ubiquitously present in all organisms. In particular, the 70 kDa heat shock protein (Hsp70) is essential for cellular recovery, survival, and maintenance of cellular function.[1] In addition to the well-established role of HSPs in cell survival, widespread clinical interest exists in their chaperone function in a range of human diseases, including neurodegenerative conditions and a number of cardiovascular diseases.[2,3] However, the question as to whether this is an adaptation to a particular pathophysiologic status or the result of the suboptimal cellular environment associated with the disease remains unanswered. A negative correlation has recently been shown between the magnitude of Hsp70 production by monocytes and the serum concentration of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6). Moreover, it would appear that Hsp70 production decreases with increasing age and is negatively influenced in monocytes by proinflammatory cytokines.[4,5] Recent studies have definitively shown that antioxidant supplementation may beneficially modulate the synthesis of stress proteins in plasma, lymphocytes, erythrocytes, and skeletal muscle. This is of interest considering that muscles of young experimental animals have been shown to adapt rapidly following exercise by an increase in the production of HSP, while muscles of older animals show a severely diminished response. These data are in agreement with a recent double-blind controlled study[6] that antioxidant supplementation might improve muscular performance and decrease TNF-α in frail geriatric patients. Recently, the –174G>C gene polymorphism has been suggested as a risk factor for coronary heart disease, carotid atherosclerosis, and stroke, and this offers further insight into a tentative anticytokine intervention. In this study, we assess the inflammatory profile and polymorphism of healthy elderly subjects and the influence of a nutraceutical supplement that has been shown to possess effective antioxidant properties in *in vitro* and *in vivo* studies.[7–9]

MATERIALS AND METHODS

Our study group consisted of 40 generally healthy, normofolemic male elderly patients. Major invalidating diseases and signs of dementia were regarded

as exclusion criteria. Seven subjects were taking angiotensin-converting enzyme inhibitors for mild hypertension and three of the seven were also on statins; all subjects had an erythrocyte sedimentation rate (ESR) below 15. Subjects were randomly divided into two groups matched for lifestyle, alcohol/tobacco use, body mass index, and physical activity. No vitamin/mineral supplements or medications were being taken by any of the subjects. The body composition of each subject was analyzed by bioimpedance. Subjects answered a dietary questionnaire, and information on the habitual intake of macronutrient and micronutrient content, using a seven-day diet history model, was collected. One group was given a good manufacturing practice-, ISO9001-certified fermented papaya preparation (FPP; Osato Research Institute, Gifu, Japan) of 9 g/day by mouth 1 h after breakfast and followed by fasting for a minimum of 30 min (FPP-supplemented group), while the other half (elderly control group) received the same amount of placebo (flavored powdered sugar). Treatments were carried out in a cross-over manner with a 3-month supplementation period followed by a 6-week washout period between treatments. Ten healthy young subjects were used as the young control group.

Blood Collection and Storage

Blood samples were drawn at the beginning of the study and on a monthly basis thereafter. Blood was refrigerated at 4 °C and allowed to coagulate for 4–6 h prior to processing via centrifugation. Sera were preserved at −70 °C.

IL-6 Promoter Polymorphism Analysis

For genetic analysis, blood samples were collected and immediately centrifuged at 3000 X *g* at 4 °C for 20 min; DNA was extracted and genotypes were determined in 2-ng genomic DNA with the Taqkman allelic discrimination assay. High-throughput analysis of the IL-6 −174 polymorphism was performed using the TaqkMan 5′ endonuclease assay on a 7900 HT Taqkman Prism 7900HT 384-wells format.

Assessment of Redox Status

The following parameters were measured: superoxide dismutase, glutathione (GSH), GSH-peroxidase (GSH-Px), and oxidized glutathione (GSSG) using hemoglobin-catalyzed oxidation of 10-N-methylcarmoyl-3,7-dimethylamino-10-H-phenothiazione after treatment with phospholipase.

Cytokine and C-reactive Protein Measurement

Serum concentrations of IL-6 and TNF-α were measured in duplicate. A standard curve was constructed and the cytokine concentrations were determined by using linear regression analysis. The plates were incubated and washed, and the amount of bound enzyme-labeled detection antibody was measured by adding a chromogenic substrate. The plates were then read at the appropriate wavelength with an enzyme-linked immunosorbent assay reader, which had previously been checked for the minimum detectable concentration of all cytokines. Dilutional experiments were also performed to accurately determine cytokine levels. Serum levels of C-reactive protein (CRP) were measured using purified protein and polyclonal anti-CRP antibodies.

Determination of Hsp70 in Serum

Serum Hsp70 concentration was determined using a Western dot blot. Briefly, 20 μL of serum at a 1:20 dilution was loaded by pipetting onto individual nitrocellulose membranes. These membrane pieces were rinsed with phosphate-buffered saline solution (PBS) and saturated with 100 μL of blocking buffer (PBS containing 5% skim-milk powder) for 1 h at 37 °C with gentle agitation. After washing the membrane pieces six times (10 min each) with 200 μL PBS-0.05% Tween 80, 100 μl of horseradish peroxidase-labeled goat antihuman immunoglobulin G (Sigma, St. Louis, MO) in blocking buffer (1:2500) was added and the incubation continued at 37 °C for 1 h. A rabbit antihuman Hsp70 antibody specific for the inducible Hsp70 and obtained through the expression of corresponding complementary DNA (cDNA) in NaCl-induced *Escherichia coli* GJ1168 cells using pET30 as the expression vector, was added at a dilution of 1:1000 in blocking buffer and the membranes incubated at 37 °C for 1 h with gentle agitation. The presence of anti-Hsp70 was then revealed with DAB (3,3-diaminobenzidine tetra hydrochloride) for 3–5 min.

Statistical Methods

Data are expressed as mean ± SD. The statistical analysis was performed with analysis of variance and post hoc analysis using Dunn's test, which was used because of the lack of a Gaussian distribution in the results. The frequency of results above the sensitivity limit of the system in all studied groups was compared with Fisher's exact test.

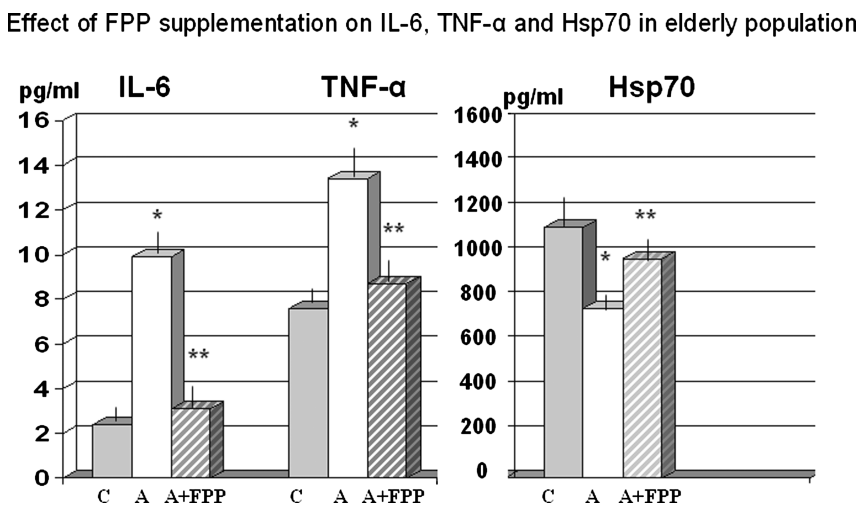

FIGURE 1. Effect of FPP supplementation on IL-6, TNF-α, and Hsp70 in an elderly population. C, young controls; A, elderly control subjects; A+FPP, elderly subjects supplemented with FPP. $^{*}P < 0.05$ vs. young controls; $^{**}P < 0.05$ vs. elderly control subjects.

RESULTS

IL-6 Promoter Polymorphism Analysis

The IL-6 −174 genotype frequencies of CC (20%), CG (45%), and GG (35%) were in accordance with the Hardy-Weinberg equilibrium as described in Caucasian controls.

Redox Status Assessment

Redox status parameters in elderly subjects were comparable to those of young controls. No significant difference was found as a result of FPP supplementation. However, five of the seven hypertensive patients, who were also on statins, showed a low GSH/GSSG ratio which was normalized by FPP supplements (statistic not applicable).

Cytokine and CRP Measurement

Significantly elevated levels of proinflammatory IL-6 (9.2 ± 2.7 pg/mL versus 2.4 ± 1.6 pg/mL, $P < 0.05$) and TNF-α (13.6 ± 3.3 pg/mL versus 7.6 ± 2.4 pg/mL, $P < 0.05$) were found in the elderly control group compared to young controls (FIG. 1). IL-6 level was not associated with body mass index in the elderly subjects. All cytokine parameters were significantly more

elevated in elderly subjects with the −174 GC genotype ($P < 0.05$). FPP caused a significant improvement in cytokine parameters of these elderly subjects ($P < 0.01$) with values comparable to young controls. High sensitivity (hs)-CRP did not show a significant increase in elderly subjects (0.8 ± 0.3 pg/mL versus 0.4 ± 0.2 pg/mL), but its log value using stepwise multiple linear regression analysis was independently associated with age, body mass index, and smoking ($P < 0.05$).

Determination of Hsp70 in Serum

The serum level of Hsp70 in the elderly control group was significantly lower than in young controls, irrespective of the −174 GC genotype ($P < 0.01$; FIG. 1). Only in the elderly subjects at baseline observation was there a significant inverse correlation between Hsp70 and IL-6 and hs-CRP ($r = 0.71$, $P < 0.01$) but not with TNF-α. FPP supplementation caused a significant rise in Hsp70 but only in elderly subjects with the −174GC genotype and with no detectable illness ($P < 0.05$).

CONCLUSION

Aging is associated with a wide range of impairments of regulatory systems, particularly those for surveillance and defence mechanisms. In this regard, it has been shown that aging is associated with decreased expression of HSP at the cellular level. The protective role of HSP is attributed to several properties, such as an active participation in the folding of proteins by minimizing incorrect interactions within and between molecules, a maintenance of proteins in their native folded states, and the repair or promotion of the degradation of misfolded proteins. An elevated serum level of Hsp70 has been associated with an improved outcome in patients suffering from a trauma[10] and in elderly people affected by heat-induced injury,[11] while lower levels were a feature of colon cancer patients in a recent study.[12] Both the production of cytokines *in vitro* and their plasma levels in elderly subjects follow different patterns compared to younger individuals. This is in agreement with the knowledge that a chronic inflammatory reaction may be particularly harmful to elderly individuals. In our study we found a significant increase in proinflammatory cytokines that were inversely correlated with Hsp70. However, these markers reverted to normal levels by FPP nutraceutical intervention, in agreement with results from our recent study in cirrhotic patients.[9] High serum levels of these markers are known to predict functional disability and an increased mortality rate in older individuals; these high serum levels, particularly in association with harmful IL-6-promoter polymorphism, may help identify high-risk individuals who might benefit from anti-inflammatory interventions.[13] Despite negative results and unanswered questions in studies on the effective role of a nutraceutical intervention,[14] the present preliminary data support the hypothesis

that an effective regimen of nutraceutical intervention may beneficially affect the proinflammatory cascade. Moreover, the nutraceutical intervention in this study caused a significant parallel increase of Hsp70 in the absence of any overlapping illness. This phenomenon occurred in those subjects with a genomic risk profile. Further investigations of these findings using a larger patient group over a longer duration are warranted.

REFERENCES

1. HARTL, F.U. 1996. Molecular chaperones in cellular protein folding. Nature **381:** 571–579.
2. JIN, X., C. XIAO, R.M. TANGUAY, *et al.* 2004. Correlation of lymphocyte heat shock protein 70 levels with neurologic deficits in elderly patients with cerebral infarction. Am. J. Med. **117:** 406–411.
3. GENTH-ZOTZ, S., A.P. BOLGER, P.R. KALRA, *et al.* 2004. Heat shock protein 70 in patients with chronic heart failure: relation to disease severity and survival. Int. J. Cardiol. **96:** 397–401.
4. REA, I.M., S. MCNERLAN & A.G. POCKLEY. 2001. Serum heat shock protein and anti-heat shock protein antibody levels in aging. Exp. Gerontol. **36:** 341–352.
5. NJEMINI, R., M.V. ABEELE, C. DEMANET, *et al.* 2002. Age-related decrease in the inducibility of heat-shock protein 70 in human peripheral blood mononuclear cells. J. Clin. Immunol. **22:** 195–205.
6. HAUER, K., W. HILDEBRANDT, Y. SEHL, *et al.* 2003. Improvement in muscular performance and decrease in tumor necrosis factor level in old age after antioxidant treatment. J. Mol. Med. **81:** 118–125.
7. RIMBACH, G., Q. GUO, T. AKIYAMA, *et al.* 2000. Ferric nitriloacetate induced DNA and protein damage: inhibitory effect of a fermented papaya preparation. Anticancer Res. **20:** 2907–2914.
8. ARUOMA, O.I., R. COLOGNATO, I. FONTANA, *et al.* 2006. Molecular effects of fermented papaya preparation on oxidative damage, MAP Kinase activation and modulation of the benzo[a]pyrene mediated genotoxicity. Biofactors **26:** 147–159.
9. MAROTTA, F., C. YOSHIDA, R. BARRETO, *et al.* 2007. Oxidative-inflammatory damage in cirrhosis: effect of vitamin E and a fermented papaya preparation. J. Gastroenterol. Hepatol. **22:** 697–703.
10. PITTET, J.F., H. LEE, D. MORABITO, *et al.* 2002. Serum levels of Hsp 72 measured early after trauma correlate with survival. Trauma **52:** 611–617.
11. WANG, Z.Z., C.L. WANG, T.C. WU, *et al.* 2001. Autoantibody response to heat shock protein 70 in patients with heatstroke. Am. J. Med. **111:** 654–657.
12. OZDEMIRLER ERATA, G., O. KANBAGLI, O. DURLANIK, *et al.* 2005. Induced oxidative stress and decreased expression of inducible heat shock protein 70 (ihsp 70) in patients with colorectal adenocarcinomas. Jpn. J. Clin. Oncol. **35:** 74–78.
13. REUBEN, D.B., A.I. CHEH & T.B. HARRIS. 2002. Peripheral blood markers of inflammation predict mortality and functional decline in high-functioning community-dwelling older persons. J. Am. Geriatr. Soc. **50:** 638–644.
14. KAUL, N., S. DEVARAJ, S.M. GRUNDY & I. JIALAL. 2001. Failure to demonstrate a major anti-inflammatory effect with alpha tocopherol supplementation (400 IU/day) in normal subjects. Am. J. Cardiol. **87:** 1320–1323.