H_2O_2 Accumulation by Catalase Reduction Changes MAP Kinase Signaling in Aged Human Skin *In Vivo*

Mi Hee Shin, Gi-eun Rhie, Yeon Kyung Kim, Chi-Hyun Park, Kwang Hyun Cho, Kyu Han Kim, Hee Chul Eun, and Jin Ho Chung

Department of Dermatology, Seoul National University College of Medicine, Laboratory of Cutaneous Aging Research, Clinical Research Institute, Seoul National University Hospital and Institute of Dermatological Science, Seoul National University, Seoul, Korea

To understand the molecular alterations occurring during the aging process, we compared mitogen-activated protein (MAP) kinase activities in the intrinsically aged and photoaged skins in the same individuals. Furthermore, we investigated the molecular events related to MAP kinase changes in intrinsically aged and photoaged skins. We found that extracellular-signal-regulated kinase (ERK) activity in photoaged skin was reduced, and that the activities of c-Jun N-terminal kinase (JNK) and p38 kinase were increased compared with intrinsically aged skin in the same individuals. Phospho-c-Jun levels and activator protein 1 activities in photoaged skin were also higher than in intrinsically aged skin. Moreover, catalase activity was found to be much reduced in primary dermal fibroblasts from photoaged skin, and as a result, H_2O_2 accumulated more in primary dermal fibroblasts in photoaged skin. In addition, treating primary dermal fibroblasts from photoaged skin with catalase reduced H_2O_2 levels, reversed aging-dependent MAP kinase changes, and inhibited matrix metalloproteinase (MMP)-1 expression. Our results indicate that the accumulation of reactive oxygen species due to catalase attenuation may be a critical aspect of the MAP kinase signaling changes that may lead to skin aging and photoaging in human skin *in vivo*. Thus, the induction and regulation of endogenous antioxidant enzymes including catalase may offer a strategy for preventing and treating skin aging.

Key words: skin/aging/MAP kinase/catalase/H_2O_2/MMP-1
J Invest Dermatol 125:221–229, 2005

Skin aging can be attributed to the intrinsic aging and photoaging processes. Damage to human skin due to repeated exposure to ultraviolet (UV) radiation from the sun (photoaging) and damage due to the passage of time (intrinsic aging) are considered distinct entities rather than similar skin aging processes. Naturally aged skin is smooth, pale, and finely wrinkled, whereas in contrast, photoaged skin is coarsely wrinkled, has poor elasticity, and is associated with dyspigmentation and telangiectasia.

The most dramatic histological changes in intrinsically and photoaged skin occur within the dermis (Lavker, 1979; Lavker and Kligman, 1988; Gilchrest, 1989), and alterations in collagen, the major structural component of the skin, have been suggested to be a cause of the skin wrinkling observed in photoaged and naturally aged skin (Fisher *et al*, 1997; Varani *et al*, 2000). UV irradiation induces the synthesis of matrix metalloproteinases (MMP) in human skin *in vivo* (Fisher *et al*, 1996, 1997), and MMP-mediated collagen destruction accounts for a large part of the connective tissue damage that occurs during photoaging (Fisher *et al*, 1996, 1997; Chung *et al*, 2001b). It was also recently suggested that collagen damage due to natural skin aging may be due to (as it is in photoaging) elevated MMP expression and a concomitant reduction in collagen synthesis (Varani *et al*, 2000; Chung *et al*, 2001b). These reports indicate that collagen deficiency in aged and photoaged skins is the result of increased MMP degradation, and that this is the cause of wrinkling in aged skin.

Studies indicate that mitogen-activated protein (MAP) kinase signal transduction pathways play an important role in regulating cell growth (Xia *et al*, 1995; Rosette and Karin, 1996), MMP expression (Gum *et al*, 1997), and type I procollagen synthesis (Davis *et al*, 1996; Chen and Davis, 1999). Three distinct, though related, families of MAP kinases exist: extracellular-signal-regulated kinase (ERK), c-Jun amino-terminal kinase (JNK), and p38 kinase. Previously, we demonstrated that ERK activity is reduced, whereas JNK activity is activated in intrinsically aged human skin *in vivo versus* young skin (Chung *et al*, 2000).

The downstream effectors of MAP kinases include several transcription factors such as c-Jun and c-Fos. Previously, we observed that the expressions of c-Jun mRNA and protein are upregulated in aged skin (Chung *et al*, 2000). In contrast, c-Fos mRNA and protein, which are constitutively expressed in young human skin *in vivo*, were unaltered in aged skin (Chung *et al*, 2000). c-Jun and c-Fos heterodimerize to form the activator protein 1 (AP-1) complex, and phosphorylation of c-Jun by JNK stimulates AP-1 transactivation activity (Karin and Hunter, 1995; Claret *et al*, 1996; Whitmarsh and Davis, 1996). The binding of activated AP-1 to its response element induces the expressions of

Abbreviations: AP-1, activator protein 1; ERK, extracellular-signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein kinase; MMP, matrix metalloproteinase; UV, ultraviolet

numerous genes, including certain members of the MMP family, such as collagenase, stromelysin, and 92 kDa gelatinase (Fisher *et al*, 1996). MMP specifically degrade connective tissue collagen, which forms the bulk of skin dermal connective tissue.

We previously found that the levels of MMP-1 and MMP-9 are higher in photoaged skin than in naturally aged skin (Chung *et al*, 2001b). Therefore, the degradation of collagen by elevated MMP levels in photodamaged skin may further attenuate collagen levels and induce more wrinkling. The molecular mechanisms underpinning enhanced MMP expression in photoaged skin, however, remain unknown.

In this study, we compared MAP kinase activities in the intrinsically aged and photoaged skins of the same individuals, and investigated the possibility of a signaling event triggered by MAP kinase changes. We found that ERK activity is attenuated in photoaged skin *in vivo*, whereas the activities of stress-activated protein kinases including JNK and p38 kinase were amplified *versus* the intrinsically aged skin of the same individuals. Furthermore, phospho-c-Jun expression and AP-1 activity in photoaged skin were higher than in intrinsically aged skin. In addition, we demonstrate that the accumulation of H_2O_2 due to reduced catalase activity during the aging and photoaging processes may cause changes in signaling pathways, and lead to skin aging and photoaging.

Results

The ERK activity was downregulated, whereas JNK and p38 kinase activities were upregulated in photoaged skin *versus* intrinsically aged skin on an individual basis We compared the activities of ERK, JNK, and p38 kinase in photoaged and intrinsically aged skin of the elderly on an individual basis. In order to investigate the effects of photoaging on changes in MAP kinase signaling pathways in human skin *in vivo*, the ratios of the MAP kinase activities in the sun-exposed (forearm) skin and in sun-protected (buttock) skin was compared in young and aged subjects. We found that the forearm to buttock ratio of ERK activity was significantly lower (82.6% $\pm$ 7.9%) in aged skin than in young skin (Fig 1*a*). The forearm to buttock ratios of total ERK protein, however, showed no significant difference with age (data not shown). Previously, it was reported that ERK activity is reduced with intrinsic aging (Chung *et al*, 2000), and we also observed that naturally aged skin has lower ERK activity than young skin (data not shown). Therefore, our results indicate that chronic UV exposure over a life time significantly accelerates aging-dependent reduced basal ERK activity in human skin *in vivo*.

The forearm to buttock ratios of JNK (Fig 1*b*) and p38 kinase (Fig 1*c*) activities were significantly higher (189.6% $\pm$ 30.2%, $p<0.05$, and 191.4% $\pm$ 29.2%, $p<0.05$, respectively) in aged skin than in young skin. On the other hand, the forearm to buttock ratios of total JNK1/2 and p38 kinase protein did not show a significant difference with age (data not shown). Previously, it was reported that the basal activity of JNK increases with intrinsic aging (Chung *et al*, 2000), and we also observed higher basal JNK and p38 kinase activities in naturally aged skin than in young skin (data not shown). Therefore, our data suggest that chronic UV exposure during a lifetime augments the aging-dependent increase in basal JNK and p38 kinase activities, leading to constitutively higher activities of JNK and p38 kinase in photoaged skin than in intrinsically aged skin in the same individuals.

Phospho-c-Jun levels and AP-1 DNA-binding activities in photoaged skin were higher than in intrinsically aged skin The activation of JNK results in the induction and activation of c-Jun, which, together with c-Fos, forms the AP-1 transcription factor complex (Karin and Hunter, 1995;

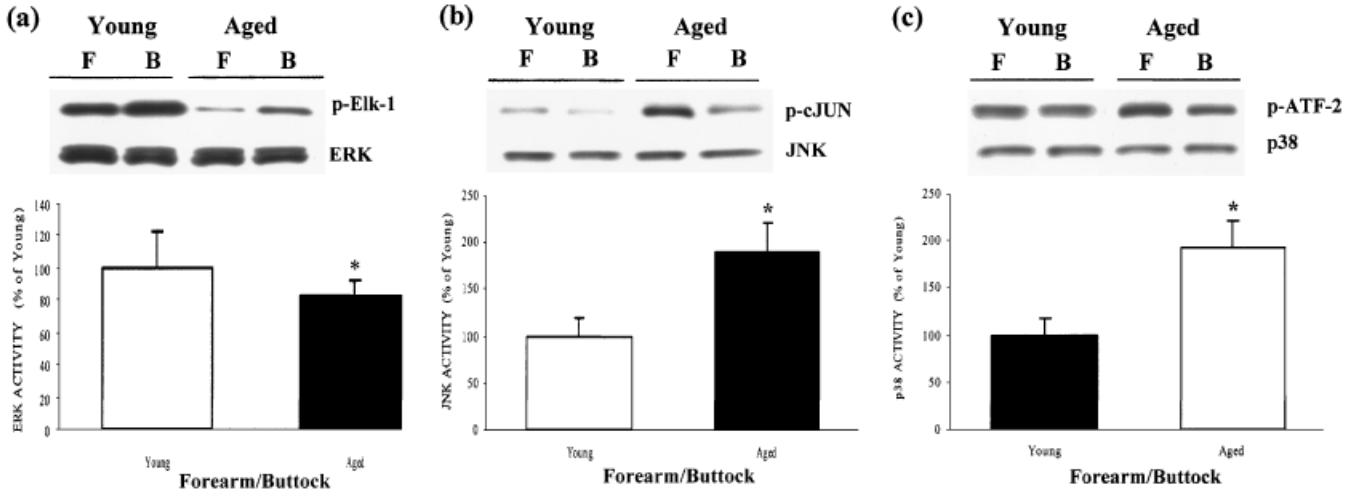

Figure 1
Decreased extracellular-signal-regulated kinase (ERK) and increased stress-activated mitogen-activated protein (MAP) kinase (c-Jun N-terminal kinase (JNK) and p38 kinase) activities in photoaged human skin *in vivo*, compared with intrinsically aged skin in the same individuals. The ratios of each MAP kinase in sun-exposed the forearm skin to sun-protected buttock skin were calculated. (*a*) ERK activity was measured in soluble protein extracts from forearm and buttock skins of young (mean age 22.2 y; N = 9) and aged (mean age 76.8 y; N = 12) subjects, using Elk-1 as a substrate. Total ERK protein was measured by western blot. (*b*) JNK activity of young (mean age 22.0 y; N = 7) and aged (mean age 77.5 y; N = 8) skin, using c-Jun as a substrate. Total JNK protein was measured by western blot. (*c*) p38 MAP kinase activity of young (mean age 21.6 y; N = 8) and aged (mean age 77.1 y; N = 11) skin, using ATF-2 as a substrate. Total p38 protein was measured by western blot. Ratios for young skin were taken to be 100%. Data are shown as means $\pm$ SEM. *$p<0.05$, aged skin *versus* young skin.

Claret *et al*, 1996; Whitmarsh and Davis, 1996). JNK and p38 kinase activate transcription of the factors c-Jun and activating transcription factor-2 (ATF-2), which bind to the c-Jun promoter and upregulate c-Jun gene expression. Since this study shows that photoaged skin has more JNK and p38 kinase activity than intrinsically aged skin, we hypothesized that photoaged skin may express more c-Jun protein and stimulate its phosphorylation. Immunohistochemical staining revealed that phospho-c-Jun levels in photodamaged facial skin were elevated with aging, and were always greater than those of buttock skin for the same individuals (Fig 2*a*). Phospho-c-Jun protein in sun-protected buttock skin increased with age, as was reported previously (Chung *et al*, 2000).

Because the higher expression of phospho-c-Jun in photoaged skin may have activated AP-1 transcription factor in photoaged skin, we performed DNA-binding assays for AP-1 transcription factor in total proteins extracted from sun-exposed (forearm) and sun-protected (inner arm) skins. We found that the forearm to inner arm ratio of AP-1 DNA activity was higher (138.5% $\pm$ 16%, $p<0.05$) in aged skin than young skin (Fig 2*b*). This result suggests that chronic sun exposure over a lifetime may increase the basal expression and phosphorylation of c-Jun, and thus activate AP-1 transcription factor constitutively in photoaged skin.

Catalase activity was markedly lower and H_2O_2 levels were higher in photoaged fibroblasts than in intrinsically aged fibroblasts We measured antioxidant enzyme activities in fibroblasts obtained from the sun-protected (inner arm) and sun-exposed (forearm) skins of young and elderly people. We found that catalase activity was significantly attenuated in aged fibroblasts, and that photoaged fibroblasts showed significantly less catalase activity than aged fibroblasts (Fig 3*a*), whereas the activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) remained unchanged (data not shown). 2,7-Dichlororofluorescein (DCF) staining showed that the levels of H_2O_2 in aged fibroblasts cultured from inner arm elderly skin were higher than in corresponding young fibroblasts (Fig 3*b*). These fibroblasts from aged skin showed higher fluorescence ($n=3$, 201.1% $\pm$ 36.1%, $p<0.05$) in the cytoplasm than young fibroblasts, and no nuclear staining. Furthermore, fibroblasts cultured from photoaged elderly skin showed much stronger fluorescence in the cytoplasm and nucleus ($n=3$, 214.6% $\pm$ 10.2%, $p<0.05$) than fibroblasts from intrinsically aged skin (Fig 3*b*). These results suggest that the level of H_2O_2 is elevated in aged fibroblasts, and that it accumulates more in photoaged skin fibroblasts, due to a lower level of catalase.

Treatment with catalase decreased H_2O_2 levels and reversed ERK and JNK activity changes in photoaged fibroblasts To determine whether H_2O_2 levels in photoaged fibroblasts can be reduced by supplementing catalase, purified catalase was administered for 24 h to culture media of photoaged fibroblasts from elderly forearm skins. Western blotting showed that the levels of catalase in cultured photoaged fibroblasts were increased in a concentration-dependent manner by this exogenous catalase treatment. The mean catalase protein level in cell extracts increased significantly to 129.7% $\pm$ 6.16% at 50 U per mL, 190.6% $\pm$ 34.0% at 100 U per mL, and 209.7% $\pm$ 26.0% at 200 U per mL compared with control (Fig 4*a*). Moreover, catalase treatment significantly reduced H_2O_2 levels within photoaged fibroblasts in a concentration-dependent manner (Fig 4*b*). DCF fluorescence intensities were significantly decreased to 53.4% $\pm$ 1.5% ($p<0.05$) at 50 U per mL, 42.4% $\pm$ 2.9% ($p<0.05$) at 100 U per mL, and 40.0% $\pm$ 3.4% ($p<0.05$) at 200 U per mL. These results suggest that exogenous catalase can scavenge intracellular H_2O_2 in the fibroblasts of photoaged skin.

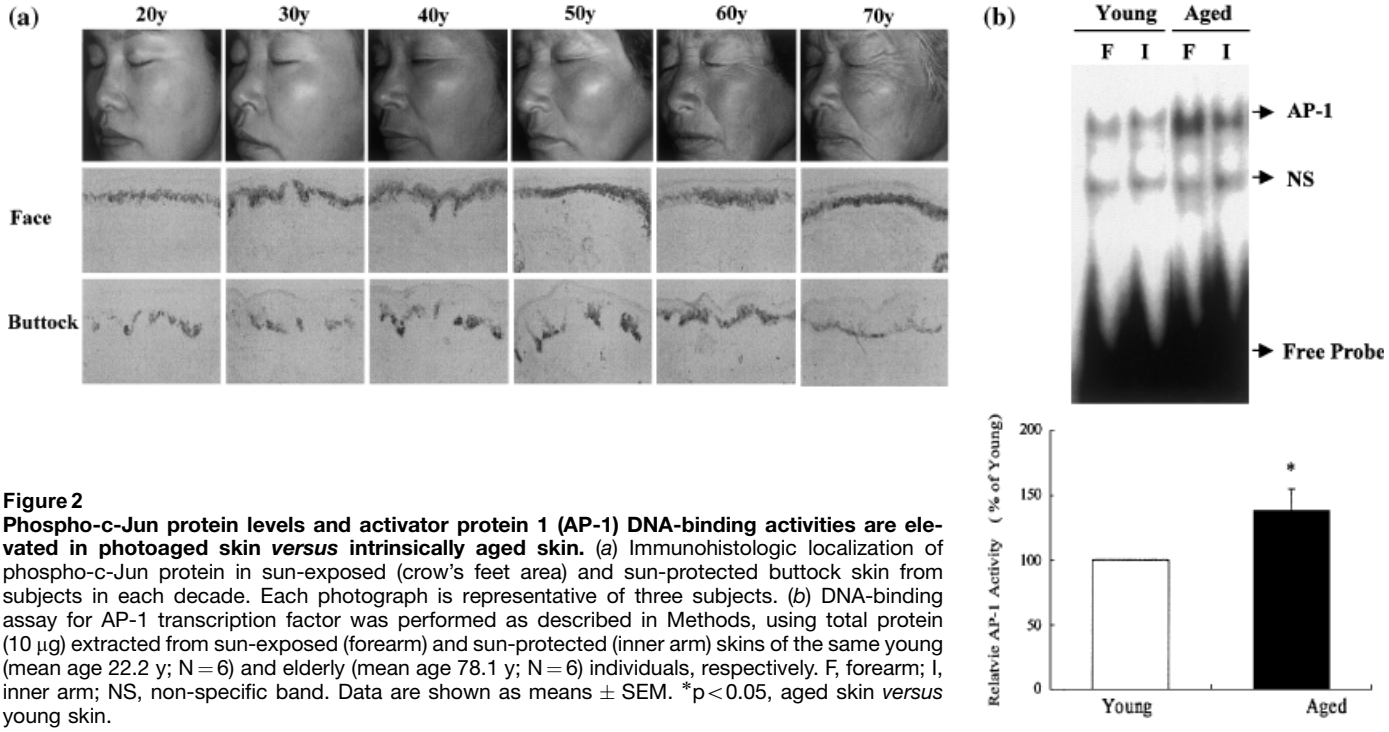

Figure 2
Phospho-c-Jun protein levels and activator protein 1 (AP-1) DNA-binding activities are elevated in photoaged skin *versus* intrinsically aged skin. (*a*) Immunohistologic localization of phospho-c-Jun protein in sun-exposed (crow's feet area) and sun-protected buttock skin from subjects in each decade. Each photograph is representative of three subjects. (*b*) DNA-binding assay for AP-1 transcription factor was performed as described in Methods, using total protein (10 μg) extracted from sun-exposed (forearm) and sun-protected (inner arm) skins of the same young (mean age 22.2 y; $N=6$) and elderly (mean age 78.1 y; $N=6$) individuals, respectively. F, forearm; I, inner arm; NS, non-specific band. Data are shown as means $\pm$ SEM. *$p<0.05$, aged skin *versus* young skin.

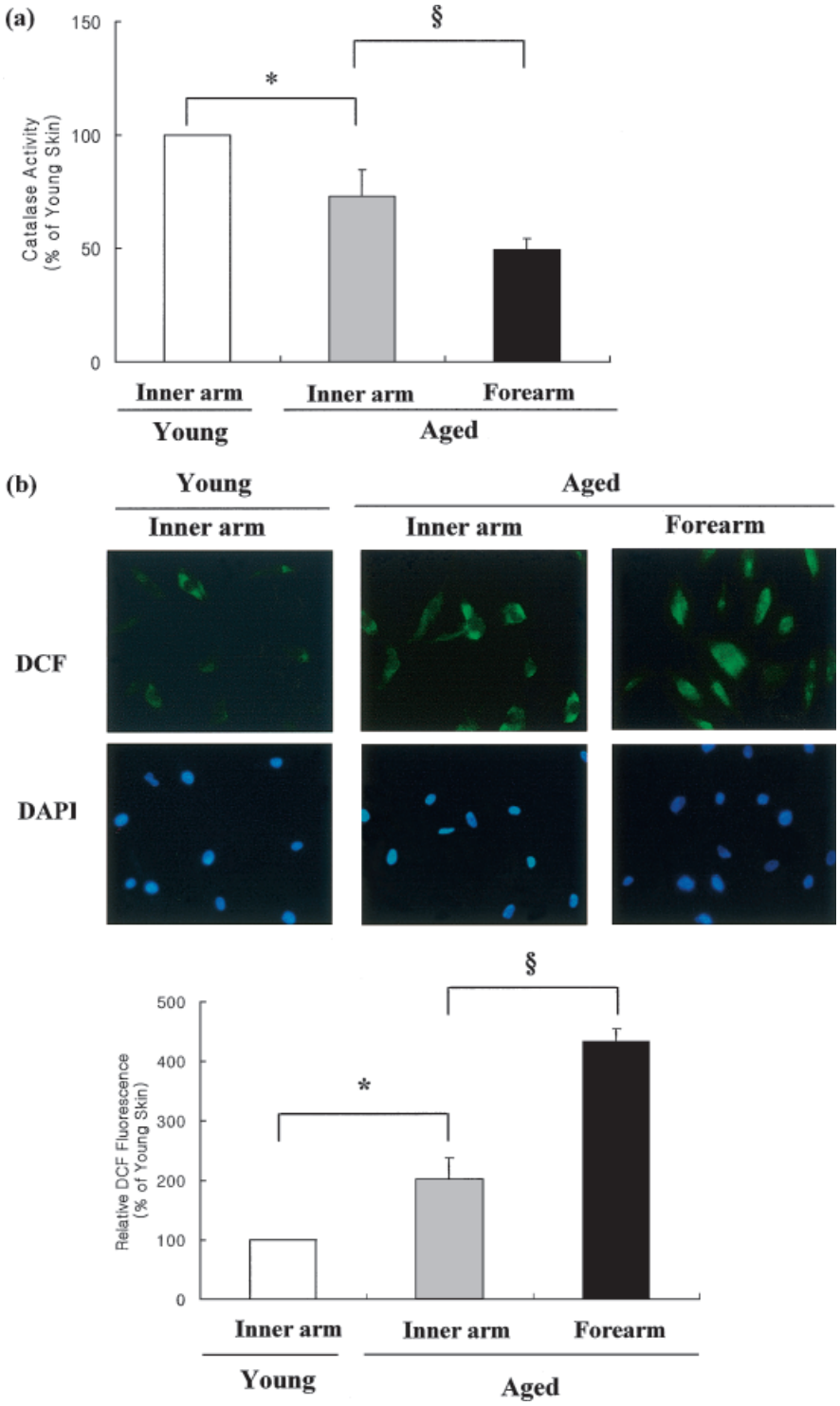

Figure 3
Catalase activity was reduced and H_2O_2 elevated in fibroblasts from aged and photoaged skin *versus* young photoaged skin. (*a*) Catalase activities were measured in young (mean age 22.5 y; N = 5), aged, and photoaged fibroblasts of the same elderly individuals (mean age 78.3 y; N = 5). Results are expressed as percentages of young fibroblast values. Means ± SEM. $^*p < 0.05$, young inner arm *versus* aged inner arm fibroblasts. §$p < 0.05$, aged *versus* photoaged fibroblasts. (*b*) Intracellular H_2O_2 levels in fibroblasts from the inner arm skin of young subjects (mean age 22.5 y; N = 3), the inner arm, and forearm skin of elderly subjects (mean age 78.3 y; N = 3) were visualized after 2,7-dichlororofluorescein staining, as described in Methods. Figures show representative fluorescence images from three independent experiments. The amounts of H_2O_2 in aged and photoaged fibroblasts are expressed as percentages of those in young human skin fibroblasts. Results are expressed as means ± SEM. $^*p < 0.05$, young *versus* aged fibroblasts. §$p < 0.05$, aged *versus* photoaged fibroblasts.

We then investigated whether this accumulated H_2O_2 causes the observed changes in MAP kinase activities in aged human skin. We found that, as in human skin *in vivo*, ERK activity was reduced (52.5% ± 6.7%, $p < 0.05$, Fig 5*a*), and that JNK activity (192.0% ± 53.0%, $p < 0.05$, Fig 5*b*) and p38 kinase activity (150.9% ± 27.2%, $p < 0.05$, Fig 5*c*) were increased in cultured aged fibroblasts. Aged fibroblasts were then treated with catalase (0–200 U per mL) for 24 h, and cells were harvested to observe the effects of catalase on MAP kinase activities. ERK phosphorylation, which was found to be constitutively lower in aged fibroblasts, was increased by catalase treatment in a concentration-dependent manner to 169.1% ± 24.1% at 50 U per mL, 263.4% ± 57.2% at 100 U per mL, and 302.9% ± 49.7% at 200 U per mL, compared with control fibroblasts (Fig 5*d*). JNK activity, which was constitutively higher in aged cells, was decreased to 50.8% ± 7.0% at 50 U per mL, 51.6% ± 12.5% at 100 U per mL, and 40.9% ± 11.1% at 200 U per mL, compared with control (Fig 5*e*). But, p38 kinase activity was not reduced by catalase treatment (Fig 5*f*).

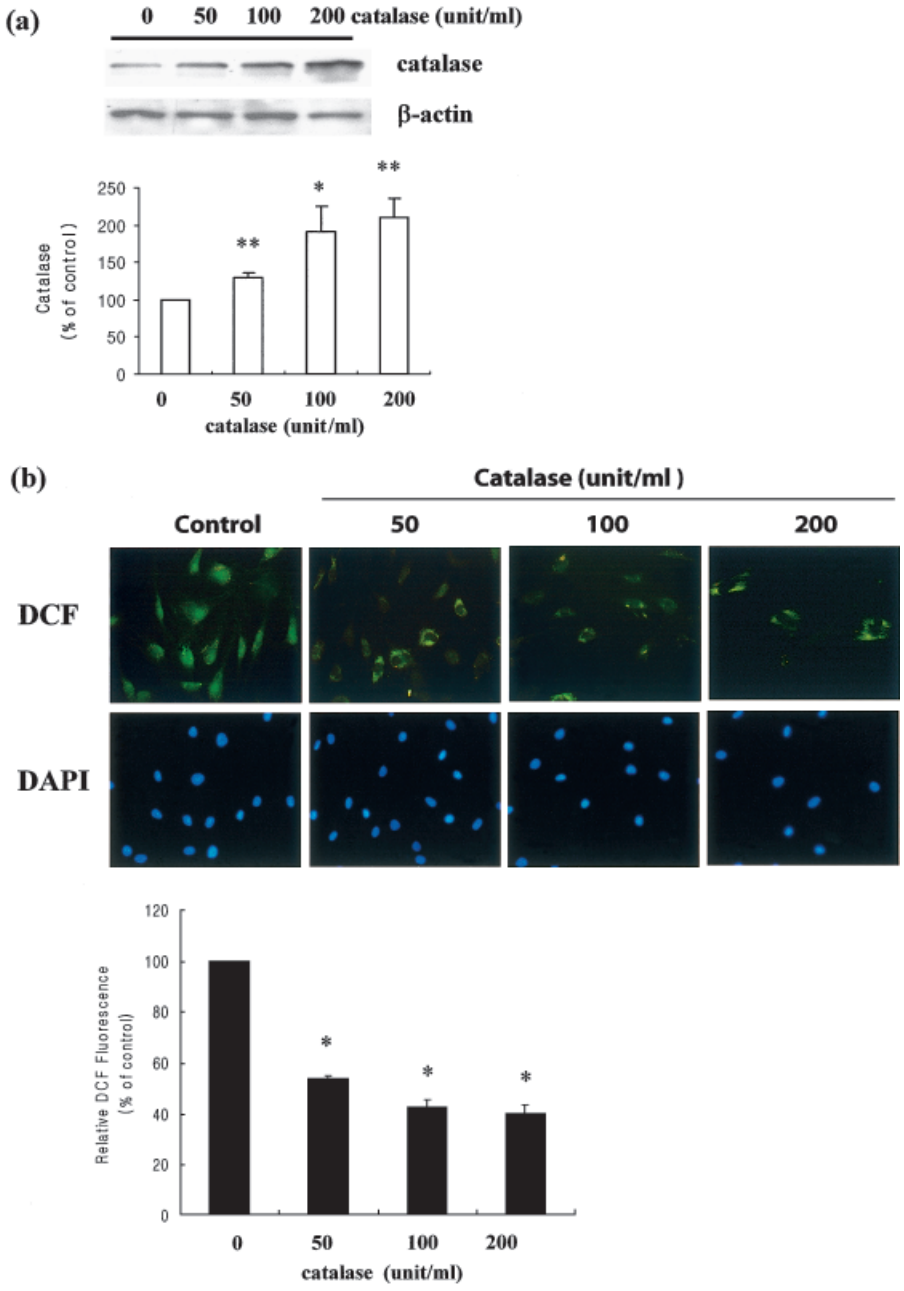

Figure 4
Exogenous catalase treatment increased intracellular catalase protein levels and reduced H_2O_2 levels in photoaged fibroblasts. (*a*) Human dermal fibroblasts cultured from the forearm skins of elderly subjects (mean age 78.6 y; N = 3) were treated with catalase (0–200 U per mL) for 24 h, and then harvested. Intracellular catalase levels were measured in soluble cell extracts by western blotting. (*b*) H_2O_2 levels in photoaged fibroblasts after exogenous catalase treatment (0–200 U per mL) were measured by 2,7-dichlororofluorescein staining. The figure shows a fluorescence image representative of three independent experiments. Fluorescence intensities are expressed as percentages of the control. Means ± SEM. *$p < 0.05$, *versus* control cells.

Catalase treatment reduced basal MMP-1 expression in aged fibroblasts In order to investigate the effects of catalase on MMP-1 expression, aged human dermal fibroblasts were treated with catalase for 24 h. This treatment reduced MMP-1 protein expression significantly to 59.0% ± 10.4% ($p < 0.05$) at 50 U per mL, 68.1% ± 4.2% at 100 U per mL, and 67.4% ± 10.9% at 200 U per mL (Fig 6). These results suggest that H_2O_2 elevation due to catalase attenuation may contribute to increased MMP expression in aged fibroblasts.

Discussion

The ERK pathway is associated with cell proliferation, differentiation, and survival (Xia *et al*, 1995). Moreover, stress-activated MAP kinase (JNK, p38) pathways are associated with growth arrest, apoptosis, and stress responses (Ham *et al*, 1995; Xia *et al*, 1995; Verheij *et al*, 1996). It has been reported that ERK activity is lower in aged (80 + year old) human skin *in vivo*, and that JNK activity is higher than in young skin (Chung *et al*, 2000). Therefore, the balance between ERK activity and JNK and p38 kinase activities shifts in favor of reduced cell growth and increased stress response in aged human skin (Chung *et al*, 2000). In this study, we observed that the sun-exposed skins (forearm) of elderly subjects showed less ERK activity and more JNK and p38 kinase activities than sun-protected (buttock) skin in the same individuals. The sun-exposed forearm skin of elderly subjects had been exposed to sunlight for more than 70 y and showed severe photodamage. Therefore, our results suggest that chronic UV exposure shifts the balance of the MAP kinase pathways to the stress-activated MAP kinase (JNK and p38) pathways.

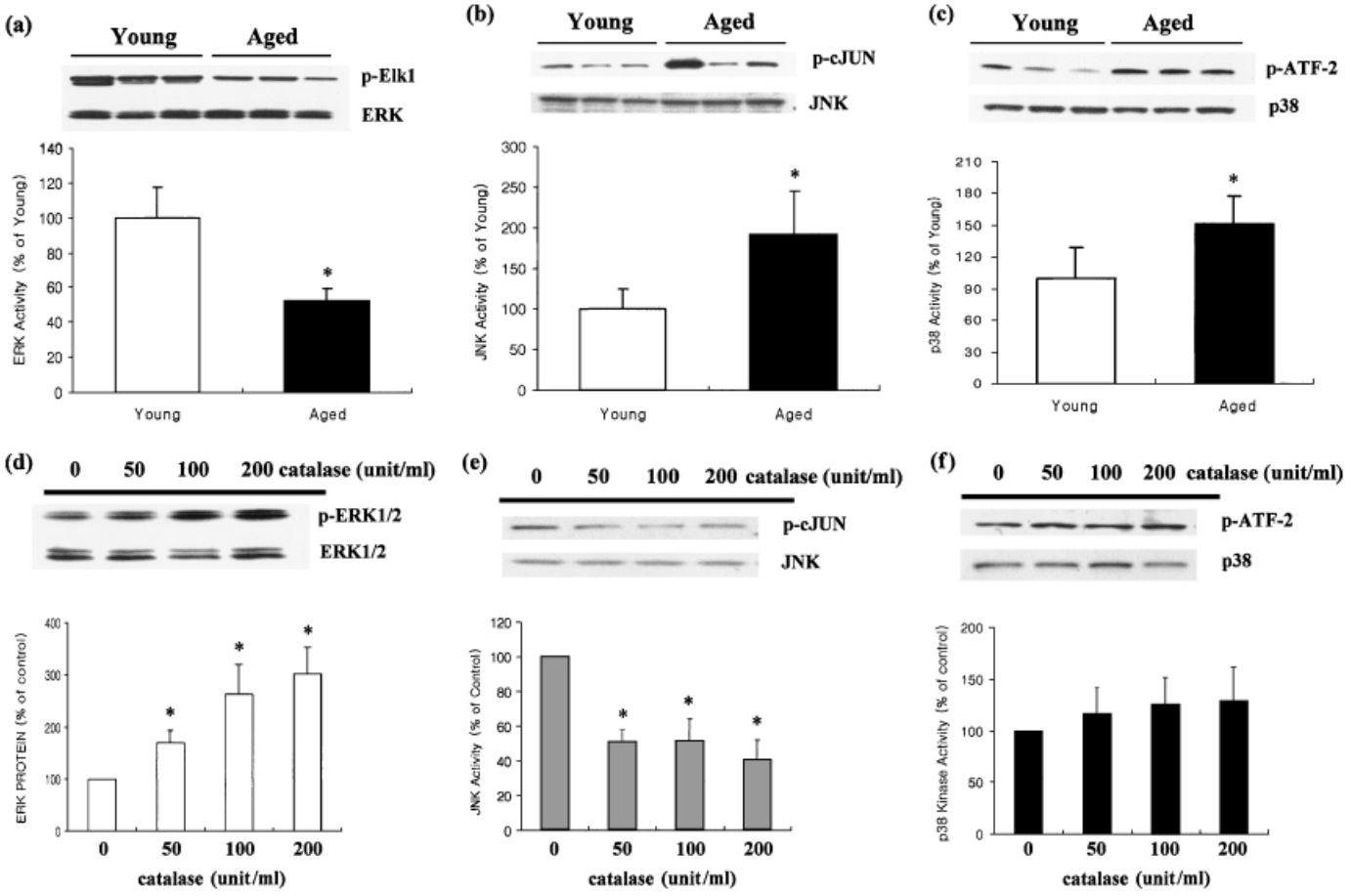

Figure 5
Catalase treatment reversed aging-dependent extracellular-signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) activity changes. (*a*) ERK, (*b*) JNK, and (*c*) p38 MAP kinase activities were measured in soluble cell extracts of young (mean age 22.2 y; N = 6) and aged (mean age 77.5 y; N = 6) fibroblasts, as described in Methods. Total ERK, JNK, and p38 proteins were measured by western blot. The activities of aged fibroblasts are expressed as percentages of those of young fibroblasts. Means ± SEM. *p < 0.05, aged skin *versus* young skin. Human dermal fibroblasts from aged (mean age 78.6 y; N = 3) skin were treated with catalase (0–200 U per mL) for 24 h, and then harvested for western blotting and kinase assays. (*d*) The levels of phospho- and total- ERK were measured by western blotting, and the activities of (*e*) JNK and (*f*) p38 kinase were measured. Total JNK and p38 proteins were measured by western blot. Means ± SEM. *p < 0.05, *versus* control cells.

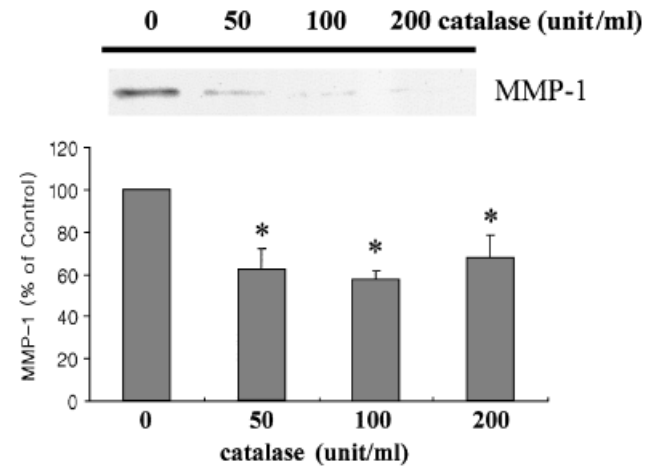

Figure 6
Catalase treatment reduced the basal expression of matrix metalloproteinase (MMP)-1 in aged fibroblasts. Human dermal fibroblasts from the skins of aged subjects (mean age 78.6 y; N = 3) were treated with catalase (0–200 U per mL) for 24 h. MMP-1 protein levels in culture media was measured by western blotting. Means ± SEM. *p < 0.05, *versus* control cells.

MAP kinases are involved in the activation and expression of c-Jun and c-Fos, the main components of AP-1 transcription factor (Karin and Hunter, 1995; Claret *et al*, 1996; Whitmarsh and Davis, 1996). JNK and p38 kinase activate transcription factors c-Jun and ATF-2, which bind to the c-Jun promoter and upregulate c-Jun gene expression (Angel *et al*, 1988; Fisher *et al*, 1998). Recently, it was demonstrated that c-Jun kinase activity is elevated in intrinsically aged skin; thus, the expressions of c-Jun mRNA and protein are higher in intrinsically aged skin than in young skin (Chung *et al*, 2000). The observed increase in the forearm to upper inner arm ratios of JNK and p38 kinase activity would cause the activation of c-Jun and the upregulation of c-Jun gene expression more in photoaged skin than in intrinsically aged skin. Consistent with this expectation, we found that the expression of phospho-c-Jun protein is higher in photoaged (face) skin than in intrinsically aged (buttock) skin on an individual basis. Sun-exposed facial skin expresses high levels of phospho-c-Jun protein from the twenties, compared with sun-protected buttock skin in the same individuals. This may be because facial skin is always exposed to sunlight, and 20 y of exposure to sunlight may

upregulate the basal expression of c-Jun protein in the facial skins of young subjects. We also observed that the expression of phospho-c-Jun protein increased with age in sun-protected (buttock) skin, which is consistent with a previous report, which showed elevated c-Jun mRNA and protein expressions in aged skin (Chung *et al*, 2000).

Collagen bundles form the bulk of skin connective tissue in the dermis, and are gradually lost with age (Shuster *et al*, 1975; Burke *et al*, 1994; Varani *et al*, 2000). Fisher *et al* (1996, 1997) showed that UV irradiation induces MMP synthesis in human skin *in vivo*. And, recently, we demonstrated that MMP-1 protein levels are higher in the dermis of photoaged skin than in naturally aged skin (Chung *et al*, 2001b). It has also been suggested that collagen damage may occur during natural skin aging, as it does during photoaging, due to elevated MMP expression, and that total MMP-1 and MMP-9 levels are elevated, in human sun-protected aged skin *in vivo versus* comparable young skin (Varani *et al*, 2000; Chung *et al*, 2001b). With regard to MMP expression, it has been demonstrated that the transcriptions of MMP-1 and MMP-9 require AP-1 *in vivo* (Fisher *et al*, 1996, 1997). Since c-Jun protein expression was higher in photoaged skin, we hypothesized that photoaged skin may have more activated AP-1 than intrinsically aged skin. As was expected, we found that the DNA binding activity of AP-1 is higher in photoaged skin than in intrinsically aged skin on an individual basis. Therefore, it is possible that this greater AP-1 activity in chronically photodamaged skin produces more MMP, like MMP-1 and MMP-9, which degrade more collagen in the dermis and cause skin wrinkling.

Previously, we reported that catalase activity, and not SOD, GPx, or glutathione reductase (GR), is reduced in the dermis of aged and photoaged human skin *in vivo*, whereas the activity of catalase increased significantly in photoaged and aged epidermis (Rhie *et al*, 2001). In this study, we found that catalase activity was reduced more so in photoaged fibroblasts than in intrinsically aged fibroblasts, and that as a result, H_2O_2 accumulated more so in photoaged fibroblasts. Moreover, treating aged fibroblasts with catalase reduced H_2O_2 levels, and reversed the aging-dependent changes in ERK and JNK activities and reduced the basal expression of MMP-1. Exogenous catalase could enter the cells by endocytosis and remove H_2O_2 both outside and inside the cultured fibroblasts. Therefore, it is possible that the accumulation of H_2O_2 due to catalase attenuation in aged and photoaged fibroblasts may increase oxidative stress and affect MAP kinase signaling pathways to promote skin aging and photoaging. The reason for different regulation of catalase activity in the epidermis and dermis during photoaging and intrinsic aging is not clear (Rhie *et al*, 2001). It is possible that decreased catalase activity in the aged and photoaged dermis might be due to the aging process. On the other hand, the induction of catalase in the epidermis may be a defense response to environmental oxidative stress (Rhie *et al*, 2001). Chronic oxidative stress to our skin from the external environment over a lifetime might stimulate the epidermal keratinocytes to upregulate catalase expression in order to scavenge ROS, which is generated and accumulated much more in the epidermis. Therefore, it may be possible that ROS would be accumulated in both aged epidermis and dermis, even

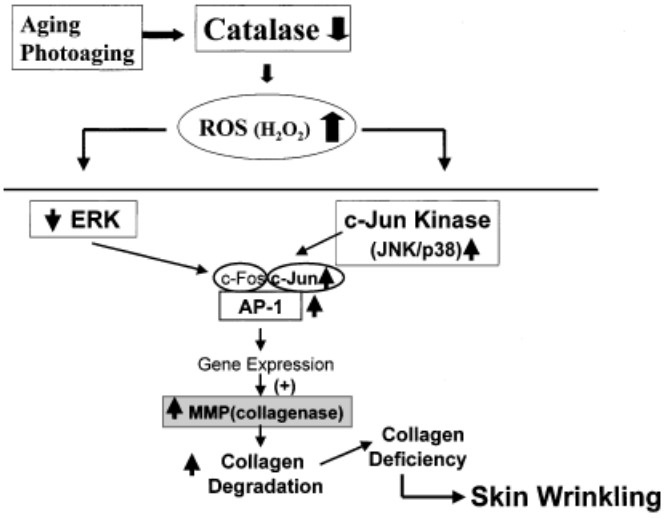

Figure 7
Mitogen-activated protein kinase signaling pathway alterations led to skin aging and photoaging in human skin *in vivo*.

though catalase is regulated differently. Many of the molecular alterations resulting from chronological aging and exposure to UV irradiation are believed to arise from oxidative stress (Davis *et al*, 1996; Chen and Davis, 1999). This central role of oxidative stress may explain, at least in part, the observed similarities between the signaling changes involved in UV-induced skin aging (photoaging) and chronological skin aging.

In conclusion, this study demonstrates that ERK activity is reduced and JNK and p38 kinase activities are increased, and that c-Jun expression and AP-1 activity are increased during the aging and photoaging of human skin *in vivo* (Fig 7). High AP-1 activity levels may stimulate the transcription of MMP such as MMP-1 and MMP-9, which leads to collagen destruction and skin wrinkling in aged and photoaged skin. We found that these changes are more prominent in photoaged skin than in naturally aged skin in the same individuals, and that this difference is caused by the much-reduced activity of catalase and the accumulation of H_2O_2 in photoaged fibroblasts. Thus, the induction and regulation of endogenous antioxidant enzymes, including catalase, offer a strategy for treating and preventing skin aging.

Methods

Human skin biopsies A total of 12 young Koreans (12 men, mean age 21.6. age range 19–29 y) and 12 elderly Koreans (11 men and 1 woman: mean age 76.8, age range 70–83 y), without current or prior skin diseases, provided skin samples. All elderly subjects had severely photodamaged skin, of $>$grade 5 according to our photographic wrinkle grading scale (Chung *et al*, 2001a). Skin specimens were obtained from all subjects by punch biopsy from photodamaged extensor (forearm) skin and from sun-protected (buttock and inner arm) skin. Another group of volunteers (ten men and eight women, three subjects in each decade of age from their twenties to seventies) provided both buttock and facial (crow's feet area) skin samples. 2-mm punch biopsy specimens were obtained from the face. Specimens required for western blotting and kinase activity assays were immediately snap-frozen in liquid nitrogen, and specimens for immunohistochemical stain were oriented immediately

into a cryomatrix (Shandon, Pittsburgh, Pennsylvania) and stored at −70°C. This study was conducted according to the Declaration of Helsinki Principles. All procedures involving human subjects received prior approval from the Seoul National University Institutional Review Board, and all subjects provided written informed consent.

Cell culture and catalase treatments Human dermal fibroblast cultures were established from sun-protected (buttock and inner arm) and sun-exposed (forearm) skin of both young (n = 6, mean age 22.2, age range 19–26 y) and elderly (n = 6, mean age 77.5, age range 75–84 y) subjects. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM:GIBCO-BRL, Eggenstein, Germany) supplemented with 10% heat-inactivated fetal bovine serum and penicillin–streptomycin at 37°C in a humidified 5% CO_2 atmosphere. Cultures were treated with various doses (0–200 U per mL) of catalase (Sigma, St Louis, Missouri) and harvested at indicated time points.

Sample preparation and extraction Punch biopsied skin samples or cultured fibroblasts were homogenized in WCE buffer [25 mM HEPES (pH 7.7), 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM ethylenediamine tetraacetic acid (EDTA), 0.1% Triton X-100, 0.5 mM dithiothreitol (DTT), 20 mM β-glycerolphosphate, 0.1 mM Na_3VO_4, 2 μg per mL leupeptin, 2 μg per mL aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF), and a protease inhibitor cocktail tablet from Boehringer Mannheim (Indianapolis, Indiana)]. The tissue suspension was rotated at 4°C for 10 min, supernatants were collected, kept at −70°C, and used for western blotting, kinase assays, or electrophoretic mobility shift assays, as described below. Protein concentrations were determined using the Bio-Rad DC protein assay (Bio-Rad, Hercules, California) using bovine serum albumin as a reference protein.

Western blot analysis Western blot analysis was performed, as described previously (Chung *et al*, 2000). Antibodies against total and phospho-ERK, JNK, p38 were purchased from New England Biolabs (Beverly, Massachusetts), antibody against MMP-1 from Oncogen (Boston, Massachusetts), and antibody against catalase from Calbiochem-Novabiochem (La Jolla, California). Protein–antibody complexes were detected using an enhanced chemifluorescence western blotting system (Amersham Bioscience, Buckinghamshire, UK), and were exposed to a Kodak X-ray film. Band intensities were measured using TINA2.0 software (TINA; Raytest Isotopenmeβgerate, Straubenhardt, Germany).

MAP kinase assays The activities of MAP kinases, ERK, JNK, and p38 kinase, were assayed using the New England Biolabs protocol. In brief, protein samples were incubated with phospho-p44/42 MAP kinase (Thr202/Tyr204) monoclonal antibody, c-Jun(1–89) fusion protein beads, or phopho-p38 kinase (Thr180/Tyr182) monoclonal antibody overnight at 4°C. Pelleted beads were washed twice with WCE buffer containing 1mM phenylmethylsulfonyl fluoride and twice with kinase buffer (25 mM Tris, pH 7.5, 5 mM β-glycerolphosphoate, 2 mM DTT, 1 mM Na_3VO_4, 10 mM $MgCl_2$). Kinase reactions were carried out in the presence of ATP at 30°C for 30 min, and subjected to SDS polyacrylamide gel electrophoresis. Elk-1 phosphorylation was used to measure ERK activity, c-Jun phosphorylation for JNK activity, and ATF-1 phosphorylation for p38 kinase activity. Gels were exposed to Kodak X-ray film, and band intensities were measured using TINA2.0 software (TINA; Raytest Isotopenmeβgerate).

Electrophoretic mobility shift assay Electrophoretic mobility shift assays (EMSA) were performed using a commercial kit by following the manufacture's instructions (Promega, Madison, Wisconsin). Briefly, AP-1 (5′-CGC TTG ATG CAG CCG GAA-3′) consensus oligonucleotides were ^{32}P end labeled by incubating them for 10 min at 37°C with 10 U of T4 polynucleotide kinase in a reaction containing 10 μCi (γ-^{32}P) of ATP (3000 Ci per mmol, Amersham-Pharmacia Biotech., Piscataway, New Jersey). Ten micrograms of total proteins extracted from punch-biopsied skin samples were equilibrated for 10 min in a binding buffer (4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 50 μg per mL poly(dI–dC)). For competition assays, a cold probe was added to this buffer before adding the labeled probe to the reaction and incubating for 20 min at room temperature. DNA–protein complexes were loaded on a 6% DNA retardation gel (Invitrogen, Carlsbad, California) and run in 0.5 × tris-borate EDTA (TBE) buffer at 100 V for 120 min. Gels were dried and detected by autoradiography.

Immunohistochemical staining Serial sections of 6-μm thickness were mounted onto silane-coated slides (Dako, Glostrup, Denmark). Acetone-fixed frozen sections were stained with antibody for phospho-c-Jun (Cell Signaling, Beverly, Massachusetts) for 1 h at room temperature. After rinsing in phosphate-buffered saline, the sections were visualized using an LSAB kit (Dako, Glostrup, Denmark), which uses a biotinylated secondary antibody and horseradish–streptavidin conjugate; 3-amino-9-ethylcarbazole was used as the chromogenic substrate. Sections were counterstained briefly in Mayer's hematoxylin.

Antioxidant enzyme assays The cultured fibroblasts were sonicated in buffer A (NaCl 130 mM, glucose 5 mM, EDTA 1 mM, and Na_2HPO_4 10 mM, pH 7.0). Lysates were rotated at 4°C for 10 min, and supernatants collected, kept at −70°C, and used for antioxidant enzyme assays. Protein concentrations were determined using the Bio-Rad DC protein assay (Bio-Rad, Hercules, California) and bovine serum albumin as a reference protein. The activities of SOD, catalase, and GPx were assayed spectrophotometrically on a Beckman DU650 spectrophotometer (Beckman Instruments, Fullerton, California), as described previously (Rhie *et al*, 2001).

Measurement of intracellular H_2O_2 Intracellular H_2O_2 levels were determined by measuring 2,7-dichlororofluorescein diacetate (DCFDA, Molecular Probes, Eugene, Oregon) fluorescence. Briefly, cells were stabilized in serum free DMEM without phenol red, for at least 30 min before staining. To measure intracellular H_2O_2, cells were incubated for 5 min with 20 μM DCFDA, an H_2O_2-sensitive fluoroprobe, which fluorescently labels intracellular H_2O_2. Cells were then immediately observed under a fluorescent microscope (Venox AHBT3/Q imaging system, Olympus, Tokyo, Japan) at an excitation wavelength of 488 nm and an emission wavelength of more than 515 nm. DCF fluorescence was measured in 50 randomly selected cells per experiment, and fluorescence intensity was measured using image analysis software (IMT (VT)-morphology program, Seoul, Korea), and values are expressed with respect to the control. To normalize cell number, 4, 6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI: 1 μg per mL, Molecular Probes) was used as a fluorescent marker for the nucleus at an excitation wavelength of 364 nm and an emission wavelength of 480 nm under a fluorescent microscope. The experiments were repeated at least three times per treatment.

This work was supported by grant Molecular and Cellular Biodiscovery Research Program (M1-0311-00-0152) from the Ministry of Science and Technology.

DOI: 10.1111/j.0022-202X.2005.23823.x

Manuscript received November 11, 2004; revised March 10, 2005; accepted for publication April 6, 2005

Address correspondence to: Jin Ho Chung, MD, PhD, Department of Dermatology, Seoul National University Hospital 28 Yongon-dong, Chongno-Gu, Seoul 110-744, Korea. Email: jhchung@snu.ac.kr

References

Angel P, Hattori K, Smeal T, Karin M: The jun proto-oncogene is positively autoregulated by its product, Jun/AP-1. Cell 55:875–885, 1988

Burke EM, Horton WE, Pearson JD, Crow MT, Martin GR: Altered transcriptional regulation of human interstitial collagenase in cultured skin fibroblasts from older donors. Exp Gerontol 29:37–53, 1994

Chen A, Davis BH: UV irradiation activates JNK and increases alphal(I) collagen gene expression in rat hepatic stellate cells. J Biol Chem 274:158–164, 1999

Chung JH, Kang S, Varani J, Lin J, Fisher GJ, Voorhees JJ: Decreased extracellular-signal-regulated kinase and increased stress-activated MAP kinase activities in aged human skin *in vivo*. J Invest Dermatol 115:177–182, 2000

Chung JH, Lee SH, Youn CS, *et al*: Cutaneous photodamage in Koreans: Influence of sex, sun exposure, smoking, and skin color. Arch Dermatol 137:1043–1051, 2001a

Chung JH, Seo JY, Choi HR, *et al*: Modulation of skin collagen metabolism in aged and photoaged human skin *in vivo*. J Invest Dermatol 117:1218–1224, 2001b

Claret FX, Hibi M, Dhut S, Toda T, Karin M: A new group of conserved coactivators that increase the specificity of AP-1 transcription factors. Nature 383:453–457, 1996

Davis BH, Chen A, Beno DW: Raf and mitogen-activated protein kinase regulate stellate cell collagen gene expression. J Biol Chem 271:11039–11042, 1996

Fisher GJ, Datta SC, Talwar HS, Wang ZQ, Varani J, Kang S, Voorhees JJ: Molecular basis of sun-induced premature skin ageing and retinoid antagonism. Nature 379:335–339, 1996

Fisher GJ, Talwar HS, Lin J, *et al*: Retinoic acid inhibits induction of c-Jun protein by ultraviolet radiation that occurs subsequent to activation of mitogen-activated protein kinase pathways in human skin *in vivo*. J Clin Invest 101:1432–1440, 1998

Fisher GJ, Wang ZQ, Datta SC, Varani J, Kang S, Voorhees JJ: Pathophysiology of premature skin aging induced by ultraviolet light. N Engl J Med 337:1419–1428, 1997

Gilchrest BA: Skin aging and photoaging: An overview. J Am Acad Dermatol 21:610–613, 1989

Gum R, Wang H, Lengyel E, Juarez J, Boyd D: Regulation of 92 kDa type IV collagenase expression by the jun aminoterminal kinase- and the extracellular signal-regulated kinase-dependent signaling cascades. Oncogene 14:1481–1493, 1997

Ham J, Babij C, Whitfield J, Pfarr CM, Lallemand D, Yaniv M, Rubin LL: A c-Jun dominant negative mutant protects sympathetic neurons against programmed cell death. Neuron 14:927–939, 1995

Karin M, Hunter T: Transcriptional control by protein phosphorylation: Signal transmission from the cell surface to the nucleus. Curr Biol 5:747–757, 1995

Lavker RM: Structural alterations in exposed and unexposed aged skin. J Invest Dermatol 73:59–66, 1979

Lavker RM, Kligman AM: Chronic heliodermatitis: A morphologic evaluation of chronic actinic dermal damage with emphasis on the role of mast cells. J Invest Dermatol 90:325–330, 1988

Rhie G, Shin MH, Seo JY, *et al*: Aging- and photoaging-dependent changes of enzymic and nonenzymic antioxidants in the epidermis and dermis of human skin *in vivo*. J Invest Dermatol 117:1212–1217, 2001

Rosette C, Karin M: Ultraviolet light and osmotic stress: Activation of the JNK cascade through multiple growth factor and cytokine receptors. Science 274:1194–1197, 1996

Shuster S, Black MM, McVitie E: The influence of age and sex on skin thickness, skin collagen and density. Br J Dermatol 93:639–643, 1975

Varani J, Warner RL, Gharaee-Kermani M, *et al*: Vitamin A antagonizes decreased cell growth and elevated collagen-degrading matrix metalloproteinases and stimulates collagen accumulation in naturally aged human skin. J Invest Dermatol 114:480–486, 2000

Verheij M, Bose R, Lin XH, *et al*: Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis. Nature 380:75–79, 1996

Whitmarsh AJ, Davis RJ: Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. J Mol Med 74:589–607, 1996

Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME: Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 270:1326–1331, 1995