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Research Article

Rapid reversion of aging phenotypes by nicotinamide through possible modulation of histone acetylation

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Abstract. Aging appears to be an irreversible process. Here we report that nicotinamide (NAA) can induce rapid and reversible reversion of aging phenotypes in human diploid fibroblasts in terms of cell morphology and senescence-associated β-galactosidase activity. Although NAA seems to enhance the replicative potential of the cells, it has little effect on their growth rate and life span, suggesting that NAA action is rather separated from the cellular replicative system. The effects are unique to NAA: none of the NAA-related compounds examined (an NAD precursor/niacin, NAD analogs, and poly(ADP-ribose) polymerase inhibitors) exerted similar effects. Thus, NAD-related metabolism and poly(ADP-ribosyl)ation are unlikely related to the NAA action. On the other hand, histone acetyltransferase (HAT) activity was elevated in NAA-exposed cells, while in aged cells, HAT activity and histone H4 acetylation were lowered. Taken together, the results suggest that NAA may cause rejuvenation by restoring, at least in part, altered gene expression in aged cells through its activation of HAT.

Key words. Cell aging; human diploid fibroblast; nicotinamide; histone acetylation; rejuvenation.

Aging is a physiological phenomenon common to dividing and nondividing cell systems and appears to be irreversible [1]. Although shortening of the telomere regions at the ends of chromosomes and accumulation of damage due to oxidative stress have been suggested as causes of aging at the cellular level [2], the mechanisms for cellular aging have yet to be established. Cultured human diploid fibroblasts, such as IMR-90 and BJ cells used in the present study, have been utilized as an in vitro model of aging cells, since they lose the ability to proliferate after a defined number of divisions, termed the Hayflick limit. When normal cells approach the end of their life span, they gradually exhibit size enlargement, shape change and senescence-associated β-galactosidase (SA-gal) activity [3]. These features have operationally been taken as aging phenotypes characteristic of aged cells. However, how these phenotypes or other aging-related functional defects are related to replicative senescence remains unclear.

Aging phenotypes emerge during diverse changes in gene expression [4, 5]. Thus, an analysis of regulators such as transcription factors and histone-modulating enzymes is of importance for understanding the aging phenomena and for controlling their progress. Modulation of histone acetylation by histone deacetylase (HDAC) inhibitors has been reported to induce senescence-related phenotypes in cultured human cells [6]. Furthermore, the protein Sir2, a gene silencer and putative longevity regulator in yeast, has been found to be an NAD-dependent HDAC (N-HDAC) [7]. Since aging is a universal phenomenon, the finding has prompted us to investigate a possible involvement of N-HDAC in the aging of mammalian cells.

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In the course of our attempt to examine N-HDAC involvement in mammalian cell aging, we have observed that nicotinamide (NAA), an NAD precursor, is capable of reversing aging phenotypes with little extension of the replicative life span. NAA has been found to elevate histone acetyltransferase (HAT) activity in vivo, while HAT activity and histone H4 acetylation are lowered in aged cells. Thus, these newly discovered effects of NAA on the phenotypic rejuvenation of aged cells might relate to its effects on histone modulation.

Materials and methods

Materials

N-*t*-butyl hydroxylamine was purchased from Tokyo Kasei (Tokyo, Japan). Other chemicals were from Sigma (USA), unless otherwise stated.

Cell culture

Human fibroblast strains IMR-90 and SV40-transformed IMR-90 were from the Coriell Institute for Medical Sciences (Camden, N. J.) and BJ was a generous gift from J.R. Smith. Unless otherwise specified, cells were cultured under standard conditions [8]. The in vitro life spans of IMR-90 and BJ cells were typically population doubling level (PDL) 48–55 and PDL 85–90, respectively. For 'quiescent' culture, confluent cells were fed with medium supplemented with 0.1% fetal bovine serum (FBS) and maintained for more than 48 h. These serum-starved cells were fed with 10% FBS and used 24 h later as 'stimulated' cultures. For H_2O_2 treatment, confluent cells were exposed to H_2O_2 at 300 µM for 2 h, rinsed with fresh medium and cultured in normal medium for 3–5 days before use. Treatment with chemicals was carried out by feeding cells with medium containing the chemicals on the next day of subculture and every 3–4 days. At intervals, cells were fixed with 2% formaldehyde/0.2% glutaraldehyde and stained with Giemsa solution or for SA-gal activity as reported elsewhere [3].

Histone proteins and histone-modulating enzyme activities

Cytosolic and nuclear extracts were prepared using NE-PER (Pierce, Rockford, Ill.) and used as enzyme sources for the assays described below. HAT activity was determined with [acetyl-^{3}H]acetyl-CoA (555 GBq/mmol, 15 Ci/mmol, ART-213; American Radiolabeled Chemicals, USA) according to Brownell and Allis [9]. For the assay of HDAC and N-HDAC activities, a radiolabeled histone fraction was prepared by labeling SV40-transformed cells metabolically with [methyl-^{3}H]acetic acid (370 GBq/mmol, 10 Ci/mmol, MT-634; Moravek Biochemicals, USA) as reported [10]. Using this histone preparation as substrate, HDAC activity was assayed according to Yoshida et al. [10] and N-HDAC activity was assayed in the presence of 1 mM NAD and 400 nM trichostatin A (Calbiochem, USA) [7]. Histone proteins were acid-extracted from isolated nuclei and separated by acid-urea-Triton (AUT) gel electrophoresis as reported elsewhere [10]. Coomassie blue-stained gels were analyzed densitometrically using the program NIH Image.

Reverse transcription-polymerase chain reaction

RNA preparation and reverse transcription-polymerase chain reaction (RT-PCR) were carried out as described elsewhere [8]. RT-PCR products were analyzed by agarose gel electrophoresis, ethidium bromide staining, and densitometry. The thermal program for β-actin and thymidine kinase (TK) was 94 °C, 30 s, 25 cycles of (94 °C, 30 s, 55 °C, 30 s, and 72 °C, 1 min), and 72 °C, 5 min, and the program for phosphatidylinositol 3-kinase (PI3K) IIα and IIβ was 94 °C, 30 s, 35 cycles of (94 °C, 30 s, 55 °C, 30 s, and 72 °C, 1 min) and 72 °C, 5 min. Primers were 5′-GGGCCGTCTTCCCCTCCATCGTGG-3′ and 5′-CCGTGGCCATCTCTTGCTCGAAGTC-3′ for β-actin; 5′-AGCACAGAGTTGATGAGACGC-3′ and 5′-GCTTCCTCTGGAAGGTCCCAT-3′ for TK, 5′-ACCATGGCTCAGATATTTAGCAACAGCG-3′ and 5′-ATTTTGGAATGCAGCCTGTTTAC-3′ for PI3K IIα and 5′-CTCACCATGTCTTCGACTCAGG-3′ and 5′-CTTCTAGGATCCGATGCTCTAGCAG-3′ for PI3K IIβ.

Results and discussion

Changes in N-HDAC activity due to aging and NAD-related chemicals

To examine the relationship of N-HDAC to mammalian cell aging, changes in N-HDAC due to aging of human fibroblasts were analyzed. As seen in figure 1, N-HDAC activity in the nuclear extracts prepared from old cells (or late-passage cells) was substantially lower than from younger cells. Young cells which had been converted to 'aged' cells by H_2O_2 treatment [11; Matuoka and Chen, unpublished data] also exhibited lowered N-HDAC activity. The observation implicated N-HDAC in aging and led us to hypothesize that modulation of N-HDAC activity might affect the aging process.

Since NAD is an essential cofactor for N-HDAC, we tested NAD-related compounds as potential N-HDAC modulators, including NAD, NADH, NADP, α-NAD, deamido-NAD, thio-NAD, NAA, and thionicotinamide. When we exposed middle-passage IMR-90 cells to these chemicals, they caused little change in cellular growth and morphology, although there was a slight decrease in cell growth with NADP (not shown). Upon careful examination, however, we observed some change in morph-

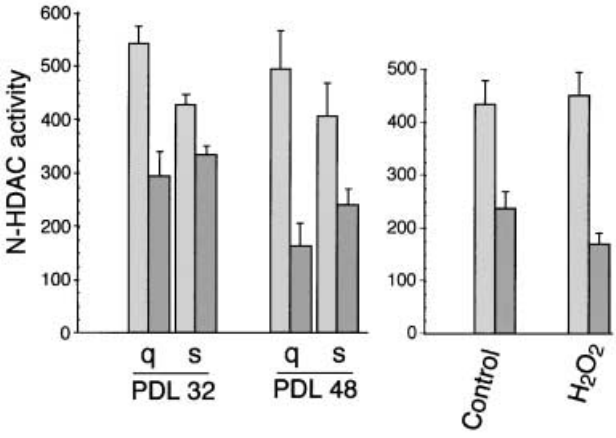

Figure 1. Changes in N-HDAC activity due to aging and chemical treatments. Nuclear (dark columns) and cytosolic (light columns) extracts of human fibroblasts were assayed for N-HDAC activity (arbitrary units calculated from dpm/assay normalized by mg protein and experimental factors; mean + SE, n = 3–6). q, quiescent IMR-90 cells; s, stimulated IMR-90 cells; Control, BJ cells (PDL 13); H_2O_2, BJ cells treated with H_2O_2 (see Materials and methods for details). Activity is lowered in the nuclear fractions from aged and H_2O_2-treated cells.

ology in NAA-exposed cells. The change was more apparent when NAA was applied to aged cells. Judging from the 'compactness of the cellular body and nucleus' typical of young cells, NAA caused morphological rejuvenation of aged IMR-90 cells in a dose-dependent manner (fig. 2A). Above 10 mM, NAA appeared to be growth suppressive or cytotoxic (not shown). The same change was observed on BJ cells, another human fibroblast strain (fig. 2B). Furthermore, NAA was also effective on H_2O_2-pretreated cells. An implication of this result is that cellular factors directly responsible for cell morphology might be common to natural aging and H_2O_2-induced aging. In this context, retinoblastoma protein (RB) has been reported to play a role in inducting of senescent morphology induced by H_2O_2 [12]. Given the failure in RB inactivation in aged cells [13], RB might be involved in the morphological changes during aging.

Effects of NAA on cell growth and life span

To further characterize the action of NAA, aged cells were examined after short- or long-term exposure to NAA. NAA also suppressed SA-gal expression, an aging marker (fig. 3A). The effects on cell morphology and SA-gal were apparent 2 days after exposure to NAA. Next, cells were exposed for 18 days through both growing and stationary phases with two subcultures and then split into two cultures either with or without NAA. Despite prior exposure for 18 days, the absence of exogenous NAA in the culture medium rapidly led to recovery of the aging phenotypes within 2 days of NAA removal (fig. 3A). Phenotypic rejuvenation was evident with either set of cells in the presence of NAA (fig. 3A). These observations show NAA action to be rapid, temporary, and reversible. NAA pretreatment for 54 days with six subcultures confirmed the lack of persistence of the effects (fig. 3B). Did the effects relate to modification of cell proliferation? NAA was found to greatly enhance colonial growth (fig. 4A). Aged cells did not form colonies of appreciable size, as previously reported [14]. However, NAA enhanced clonal cell growth and resulted in the formation of many large colonies. Again, the continuous presence of NAA was required for colony formation, since prior incubation with NAA had no effect on clonal growth (fig. 4A). Considering that colony size distribution may reflect the replicative age of a cell population [14], cells in the presence of NAA seem to be young and to have high replicative potential for their PDL. However, NAA did not

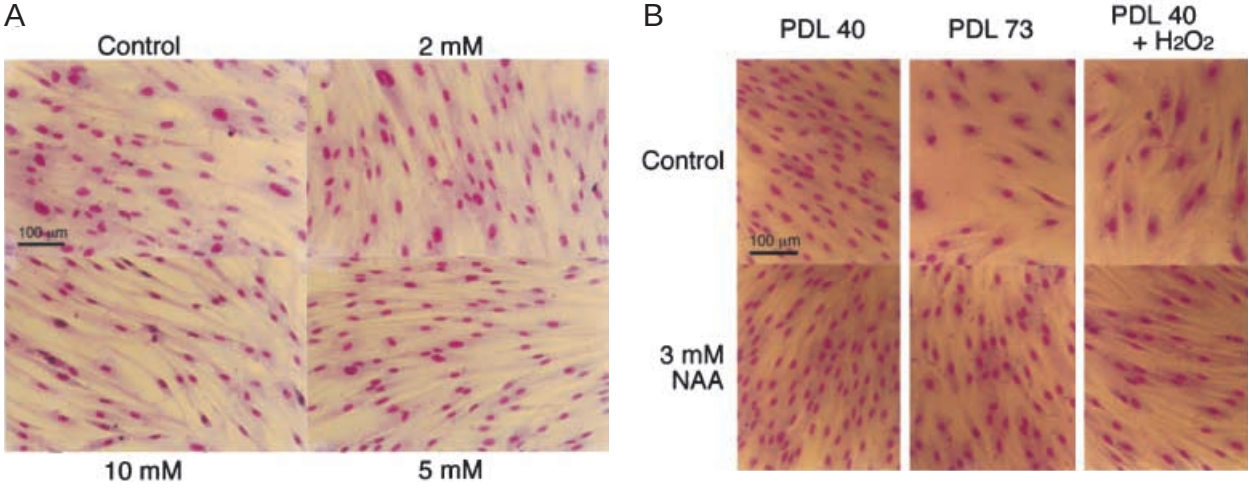

Figure 2. Changes in cell shape due to exposure to NAA. (*A*) Growing IMR-90 cells at PDL 44 were treated with NAA at indicated concentrations for 5 days. (*B*) Growing BJ cells were exposed to 3 mM NAA for 4 days. For H_2O_2-treated cells, see Materials and methods.

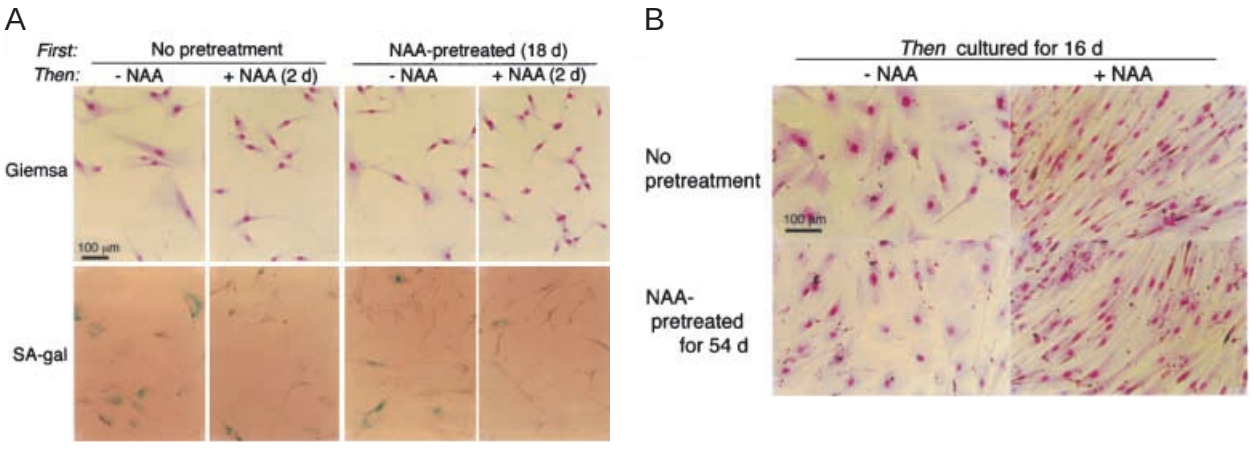

Figure 3. Rapid and reversible changes in cell shape and SA-gal by NAA treatment. (*A*) BJ cells were pretreated with 3 mM NAA for 18 days through two subcultures and, at PDL 79, inoculated cells were treated or untreated with 3 mM NAA for 2 days. At least one positive cell (stained blue/green) is deliberately included in every photograph to demonstrate the methodological adequacy of the staining. Changes in cell shape and SA-gal activity were apparent 2 days after addition or removal of NAA. (*B*) Starting at PDL 64, BJ cells underwent six subcultures in the presence or absence of 3 mM NAA for 54 days and were then inoculated at PDL 88 and cultivated for 16 days with or without NAA. The 'young-cell' phenotypes depended on the presence of NAA but not on pretreatment with NAA.

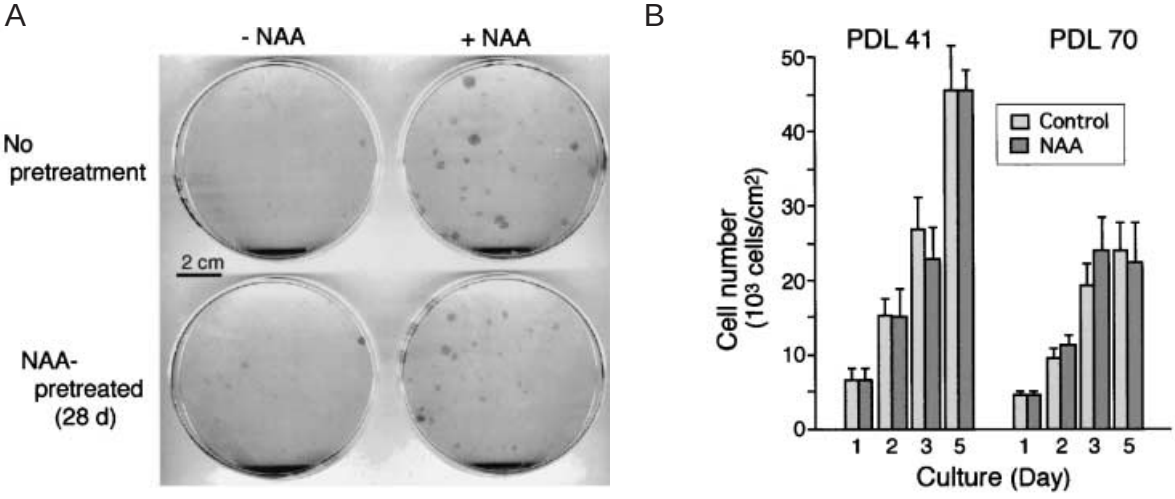

Figure 4. Changes in cell growth due to NAA. (*A*) BJ cells were pretreated or not with NAA for 28 days through three subcultures, inoculated at PDL 79 at a density of 200 cells/10-cm dish and cultured for 3 weeks in the presence or absence of 3 mM NAA. NAA enhanced colonial cell growth, which was unaffected by the pretreatment. (*B*) BJ cells were treated with 3 mM NAA from the next day of inoculation (day 1) on. No growth acceleration was apparent.

affect the rate of cell growth (fig. 4B). Given that NAA treatment brought about a noticeable increase in saturation cell density (as seen in figs. 2B, 3B), NAA is likely to act as a cell activator rather than a growth accelerator.

We next examined whether the continuous presence of NAA in the culture could extend the life span of normal human fibroblasts. Cells, either under a long-term NAA treatment or not, similarly senesced at PDL 88–91 and entered the terminal phase, where cells were gigantic and unable to reach confluency (fig. 5). It is also noteworthy that the NAA-treated cells maintained the rejuvenated state until they entered the terminal phase. The lack of a significant effect on the life span indicates that the action of NAA is distinct from other manipulations known to modulate the progress of aging, for example, those with telomerase, *N-t*-butyl hydroxylamine, and carnosine [15–17].

The aging of dividing cells, including fibroblasts, comprises both phenotypes related to cell replicative activity and those related to other cell functions. As to the experimental modulation of aging, in most cases, it could be regarded as retardation of the progress of aging rather than rejuvenation. One group has reported that kinetin (a plant growth hormone), garlic extract, and mild heat

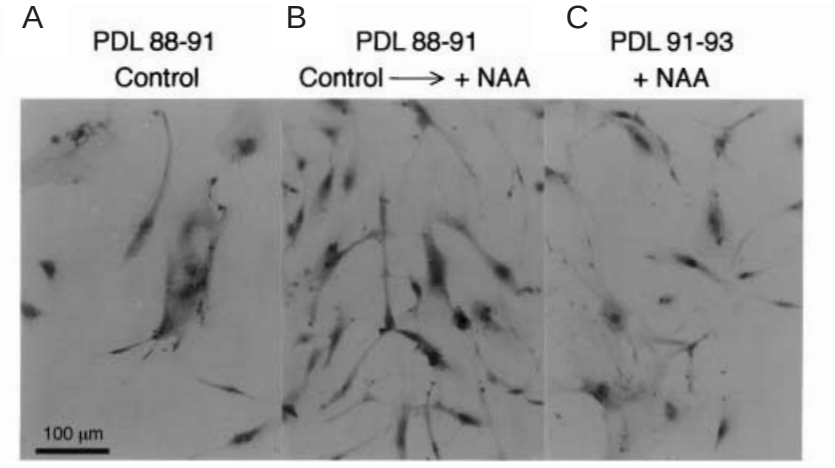

Figure 5. Effects of NAA on the cell replicative life span. Starting at PDL 64, BJ cells were cultured over 98 days either in the presence (*C*) or absence (*A*) of 3 mM NAA. One of the control cultures was treated with NAA after PDL 88 (*B*). Cells were fixed 16–22 days after final subculture.

shock all delay the onset of aging characteristics in human fibroblasts [18–20]. This might indicate that aging phenotypes are under the complex regulation of various factors.

Comparison of the rejuvenating effects of NAA and related compounds

How does NAA exert the effects described above? These effects were unique to NAA: the NAD-related chemicals examined above (NADH, NADP, α-NAD, deamido-NAD, thio-NAD, and thionicotinamide) were all unable to reproduce the NAA action, except that NAD induced a similar, but weaker, morphological change around 0.1 mM (not shown). Thus, attributing the effects to an interaction or interference with an NAD-related cellular metabolism or function seems ruled out.

NAA is known as niacin (a vitamin B3 species) and as an inhibitor of poly(ADP-ribose) polymerases [21]. It has also been reported to prevent oxidative stress-induced DNA fragmentation [22]. We tested several compounds with known effects in these processes. Nicotinic acid (niacin/NAD precursor) and 3-aminobenzoamide and 3-acetylpyridine {both poly(ADP-ribose) polymerase inhibitors [21]} failed to exert the same effects as NAA (fig. 6). Also uneffective were *N-t*-butyl hydroxylamine, L-ascorbic acid, and (+)-α-tocopherol, which are antioxidants, suggested to retard the progress of aging or to activate functions in aged cells [16, 23, 24]. The results imply that the NAA action is distinct from such biological functions as vitamin B3, NAD metabolisms, poly(ADP-ribosyl)ation, or redox modulation.

Changes in histone modulation due to NAA and aging

Since N-HDAC is dependent on NAD, and NAA is an NAD precursor, NAA might possibly influence N-HDAC activity. We exposed aged cells to NAA for 2 days and assayed the extracts for the activities of histone-modulating enzymes. A 2-day exposure was used in this experiment because NAA affected cell morphology in as little as 2 days (fig. 3 A). If NAA affected morphology through modulation of these enzymes, they should have been affected within this time frame. In addition, a one-time, transient change is unlikely to trigger the biological event, since NAA is required continuously to sustain its effects (figs. 3, 4).

The results showed that NAA did not affect HDAC, including N-HDAC (table 1). Unexpectedly, however, it elevated HAT activity *in vivo*. Direct addition of NAA to the HAT assay reaction *in vitro* had no effect (not shown), suggesting an indirect action of NAA on HAT. Speculatively, on the assumption that a decrease in HAT activity leads to cell aging, NAA might rejuvenate aged cells through its activation of HAT.

To test this possibility, we analyzed age-related changes in HAT activity (fig. 7). HAT activity seemed to be lowered in aged cells and, in addition, in the cells which had undergone H_2O_2-induced senescence. Furthermore

Table 1. Changes in histone-modifying enzyme activities in NAA-treated cells.

Enzyme	Control	NAA treated
Histone acetyl-transferases (HAT)	815 ± 13	1022 ± 26 (125)
Histone deacetylases (HDAC)	1786 ± 55	1745 ± 71 (98)
NAD-dependent histone deacetylases (N-HDAC)	321 ± 30	333 ± 20 (104)

Growing BJ cells at PDL 66 were exposed to 3 mM NAA for 2 days. Nuclear extracts from the cells were assayed for the enzyme activities (arbitrary unit calculated from dpm/assay normalized by mg protein and experimental factors; mean ± SE; n = 3–6; percent of control in parentheses).

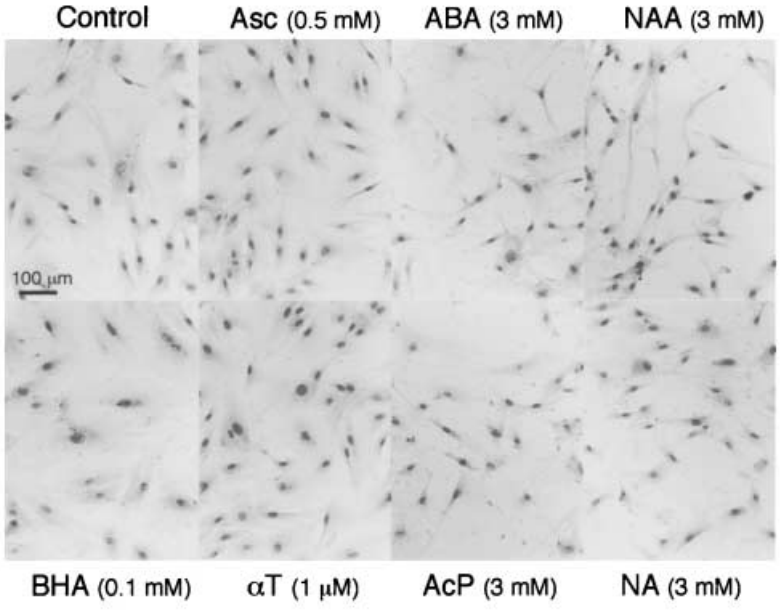

Figure 6. Changes in cell shape due to exposure to poly(ADP-ribose) polymerase inhibitors and antioxidants. Growing BJ cells at PDL 67 were treated for 5 days with the chemicals indicated. Representative results are shown from the experiments using five or more different doses. Asc, L-ascorbic acid, sodium salt; ABA, 3-aminobenzamide; BHA, *N-t*-butyl hydroxylamine; αT, (+)-α-tocopherol; AcP, 3-acetylpyridine; NA, nicotinic acid, sodium salt. Upon chemical treatment, only NAA-treated cells became slimmer, with most of them bearing small nuclei.

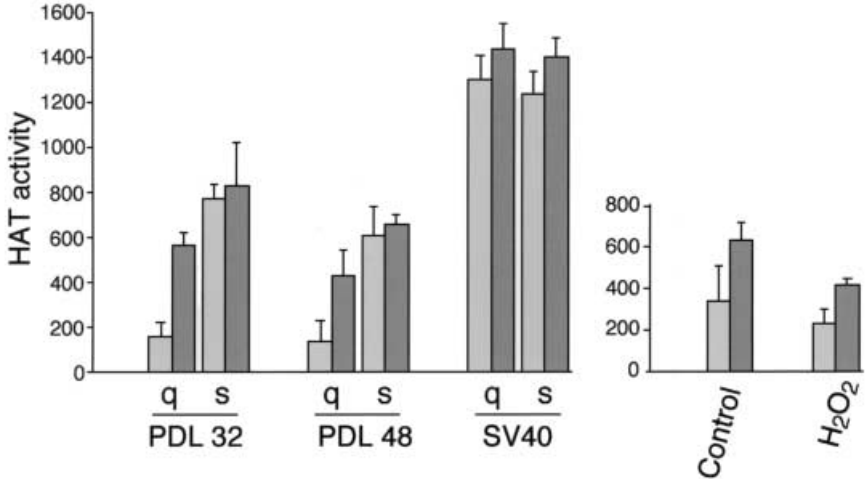

Figure 7. Changes in HAT activity due to aging and H_2O_2 treatment. Extracts of human fibroblasts were assayed for HAT activity (see legend to fig. 1). SV40, SV40-transformed IMR-90 cells.

SV40-transformed cells exhibited enhanced HAT activity. To assess the reliability of the assay, we compared the results of two assays, which were performed independently. Although there were substantial fluctuations in the net dpm between the assays (as large as 30%), the relationship among the samples in each assay was sustained: for HAT activity in the nuclear extracts, the ratios of 'aged' to 'young' cells were 0.67 in one assay and 0.61 in the other assay for 'quiescent culture' and 0.66 and 0.76 for the 'stimulated culture' (not shown). The results showed the reproducibility of the assay.

Next, to confirm that these changes in HAT activity reflect the state of histone acetylation in the cell in vivo, nuclear proteins were acid-extracted and analyzed by AUT gel electrophoresis (fig. 8). There was no particular difference in the protein banding pattern between quiescent and stimulated cells in each set of the cell samples. In terms of cellular age, however, several changes were noted, among which those indicated by arrows in the figure were most apparent. The band marked 'b' in the region of histone H1 might reflect a change in H1 phosphorylation. The band marked 'c' was confirmed to

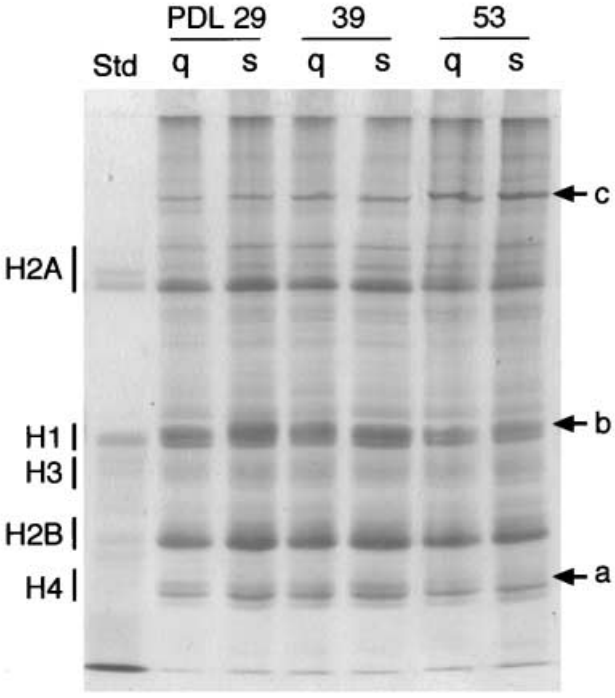

Figure 8. Changes in nuclear proteins due to aging. Basic proteins extracted from nuclei of quiescent (q) or serum-stimulated (s) IMR-90 cells were separated by acid-urea-Triton gel electrophoresis (10 µg proteins/lane). Std, calf thymus histones (type II-A). Arrows indicate apparent changes (see text for details).

be the actin-binding protein caldesmon by proteolysis/MALDI-TOF mass spectrometry and Western blotting. These changes will be analyzed elsewhere.

As to the region of histone H4 (fig. 8), five bands, clear or weak, were visible, most likely corresponding to acetylation variants of the protein [10]. In aged cells, the amounts of the upper three bands (marked 'a' by an arrow; highly acetylated forms) relative to the lower two bands (poorly acetylated forms) were substantially lowered. By densitometric determination, the ratios of those fractions shown in figure 8 were found to be 77 : 100 and 60 : 100 at PDL29, 60 : 100 and 86 : 100 at PDL 39, and 48 : 100 and 34 : 100 at PDL 53. In addition, the ratios were 86 : 100 and 72 : 100 for SV40-transformed cells. The results confirmed that histone H4 acetylation was actually lowered in aged cells. Given little change in the total HDAC activity (not shown) and a decrease in N-HDAC activity (fig. 1) during aging, the above decline in histone H4 acetylation could be ascribable to the reduced HAT activity in aged cells (fig. 7). A recent report mentioned, in the text, the absence of a significant difference in the overall histone acetylation pattern between young and senescent human fibroblasts [25]. Since this is a point of importance, the empirical data from the study should be examined carefully.

Acid extraction of nuclear proteins and their separation with an AUT gel and detection by Coomassie blue staining have been established methods for analysis of histone proteins for more than a decade [26]; nonetheless, nonhistone proteins might migrate to the same region in a gel as histones, which could complicate the protein banding pattern. A method like Western blotting followed by detection with anti-histone antibodies would be useful, if adapted to the AUT gel system. By employing antibodies directed to specific amino acid residues of histones, one could obtain further clues as to which histone residues and which HATs play a role in the aging-related changes in histone modulation.

The reason for the lowered HAT activity in aged cells remains to be analyzed. For the present we might speculate as follows. We have observed a decrease in the amount of subunit A of the ubiquitous transcription factor NF-Y and its activity during aging [8]. Since NF-Y forms a complex, and presumably interacts, with HATs such as PCAF and GCN5, NF-Y dysfunction might impair their normal activities. Similarly, in the case of SV40-transformed cells, SV40 large T antigen interacts with various transcription regulating complex containing HATs and modulates their activities [27, 28], which could eventually lead to transcriptional activation [27] and elevated histone acetylation [29] through not completely identified mechanisms.

Effects of NAA on gene expression

Finally, we were interested whether exposure of aged cells to NAA restores, if partially, the gene expression pattern of young cells. The expression of a number of genes changes during aging [4, 30]. Many of them, for example, p16, p21, EF1α, procollagen α1(III), and fibronectin, exhibit a drastic change in expression at the terminal phase of cell aging [31–34]. NAA appears to be effective at broad stages of aging rather than on terminal-phase cells. We analyzed changes in gene expression by RT-PCR. The quality of RNA preparations and RT products were checked by agarose electrophoresis, which showed the synthesized cDNAs extending to 7.7 kb (not shown). We studied two aging-related genes: the expression of the TK gene is primarily coupled with cell cycling [35], while that of the PI3K IIβ (a subtype of the PI3K family lipid/protein kinases [36]) is upregulated by aging and downregulated by growth stimulation [Matuoka et al., unpublished data]. β-Actin (abundant, < 2-kb message) and PI3K IIα (rare, ~ 5-kb message) were used as PCR controls for TK (abundant, < 2-kb message) and PI3K IIβ (rare, ~ 7-kb message), respectively. Upon NAA treatment, TK gene expression rose only slightly in both young and aged cells, suggesting that growth stimulation due to NAA is marginal (fig. 9). On the other hand, NAA treatment substantially reduced PI3K IIβ gene expression in aged cells to the level of young cells, while that in young cells changed little. PI3K, being a multifunctional enzyme family, is involved in structural and functional

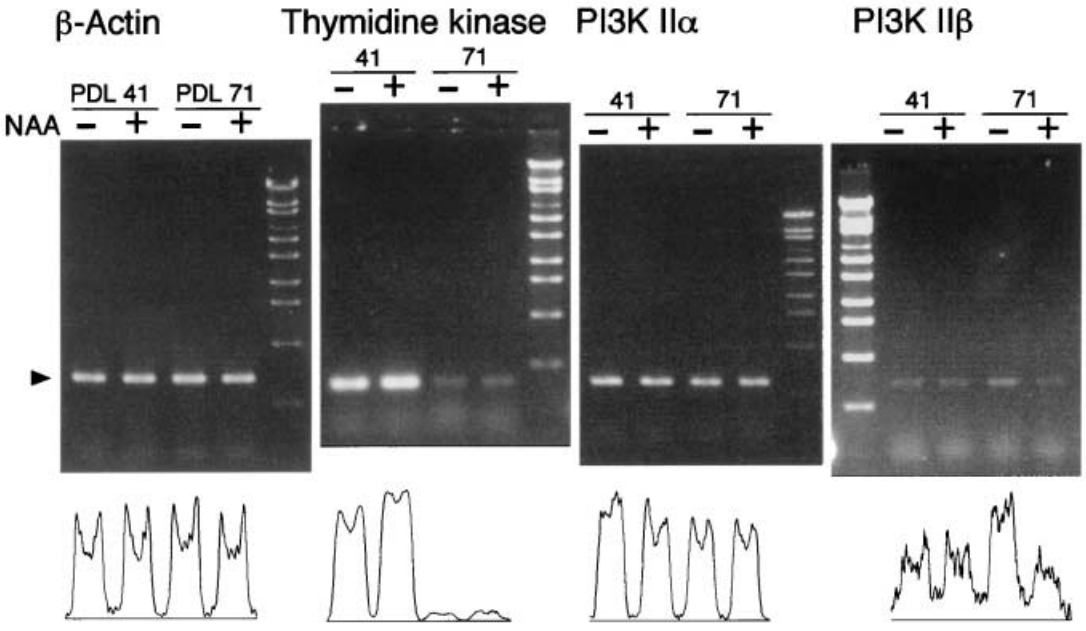

Figure 9. Changes in gene expression due to NAA treatment. BJ cells were treated with 3 mM NAA for 3 days. mRNAs were analyzed by RT-PCR. Densitograms show relative amounts of the respective products (arrowhead).

regulation of the cell and has also been implicated in aging [36]. Analyzing the effects of NAA on these and other aging-related genes in detail would serve to better define its biological action.

In conclusion, the rejuvenation by NAA is specific. There have been few observations of cellular rejuvenation thus far, besides phenotypical reversion after long-term culture with carnosine [17]. The NAA effect is rapid, reversible, and substantially separate from the cellular replicative system. These features suggest that an NAA-triggered signal acts directly on an event pivotal for expression of various aging phenotypes rather than on the cell replication machinery. Thus, NAA action would be a useful means to differentiate the aging of non-dividing cells from that of dividing cells, or the aging of cell replication from that of other cell functions and, therefore, to control the aging process without the risk of cell immortalization [37]. Since the key events are likely to relate to the genes influenced by NAA-induced modulation of histone acetylation, a global search for the genes reacting to NAA treatment would be worthwhile.

Histone modulations, including H4 acetylation, have been shown to be an important regulatory mechanism for gene expression [38]. Given that changes in gene regulation are critical in the aging process [6, 13, 30], the decline in histone acetylation in aged cells (fig. 8) would influence cellular aging. The activation of HAT by NAA could cause a, if partial, recovery from altered gene regulation during the aging of human fibroblasts.

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