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A positive role of phosphatidylinositol 3-kinase in aging phenotype expression in cultured human diploid fibroblasts

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Abstract

In order to detect the role that phosphatidylinositol 3-kinase (PI3K) plays in the aging of human diploid fibroblasts, we analyzed cellular inositol phospholipids and expression of PI3Ks. In aged cells a decrease in phosphatidylinositol 3,4-bisphosphate ($PI3,4P_2$) was notable, while phosphatidylinositol 3-phosphate (PI3P) and phosphatidylinositol 4,5-bisphosphate ($PI4,5P_2$) decreased slightly. On the other hand, the messages of PI3K IIα, Vps34, and p110δ decreased and that of PI3K IIβ increased during aging. These changes might relate to the aging phenomena, with the PI3K subspecies functioning differentially. Consistently, a PI3K inhibitor LY294002 greatly suppressed enlargement and flattening of cell body and nucleus as well as cell proliferation, both phenotypes being typical of aged cells. An oxidative stress, pulse exposure to hydrogen peroxide (H_2O_2), induced these senescent cell-like phenotypes, which LY294002 was also able to abolish. Upon examining three different cell systems (HL-60, N1E-115, and PC-12 cells) we found clear parallelism in a cellular event between the dependence on a PI3K activity and the sensitivity to H_2O_2. On the analogy of these relationships, we could hypothesize that expression of an aging phenotype such as the morphogenesis is positively promoted by some PI3K subspecies, if such a phenotype as cell cycling is negatively affected by attenuation of another PI3K function in the course of cellular aging.

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Keywords: Cellular aging; Human diploid fibroblasts; Phosphatidylinositol 3-kinase; Hydrogen peroxide; Cellular morphology

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1. Introduction

Aging is an irreversible alteration of the biological system. Although the mechanisms for aging have yet to be established, accumulation of damages due to oxidative stresses has been proposed to be critical to induction of senescence in cultured human cells (Beckman and Ames, 1998; Finkel and Holbrook, 2000). When normal cells undergo a finite number of cell divisions, to the end of their life span, they gradually exhibit a decrease in replicative capacity and changes in a variety of phenotypes, including cell shape. Enlargement and flattening of cell body and nucleus are typical of the aged cells; however, such morphology can also be induced by oxidative stresses such as exposure to hydrogen peroxide (H_2O_2; Chen and Ames, 1994; Matuoka and Chen, 2002) or hydroxyl radical (Bazso-Dombi et al., 2000). Furthermore, oxidative stresses have been found to bring a cell to a state resembling the normal aging (Chen et al., 1998; Bazso-Dombi et al., 2000). On the other hand, H_2O_2 is known to transiently elevate the activity of phosphatidylinositol 3-kinase (or phosphoinositide 3-kinase; PI3K; Konishi et al., 1999; Van der Kaay et al., 1999).

PI3K is a large family of enzymes, which functions for a wide variety of cellular phenomena, including cell cycle regulation, cytoskeletal organization, and cell survival signaling (Wymann and Pirola, 1998; Cantrell, 2001). Its involvement in the aging process has also been suggested by the fact that loss-of-function mutations in the insulin/PI3K signaling pathway extend the life span of nematode *C. elegans* (Morris et al., 1996). On the contrary, LY294002 (LY) and Wortmannin, both specific inhibitors of PI3K, have been reported to induce senescent cell-like phenotypes (Tresini et al., 1998; Collado et al., 2000). Only insufficient information has been provided on this issue, thus far; however, given universal changes in biological functions accompanying aging, PI3K is a potential candidate as a regulator acting in the progress of aging. Therefore, it is of importance to detect relationship of PI3K to the aging phenomena.

In the present study, we have analyzed changes in inositol phospholipids and PI3K molecules during the aging of human diploid fibroblasts and then effects of PI3K inhibition on the aging phenotypes. The results indicate a decrease in the amount of phosphatidylinositol 3,4-bisphosphate ($PI3,4P_2$), a PI3K product, and a decrease in the message of PI3K IIα and an increase in that of PI3K IIβ in aged cells. Inhibition of PI3K with LY has been found to reduce cell proliferation and to abolish expression of the morphology associated with aging or induced by H_2O_2. Since we have observed that the dependence of a cellular event on a PI3K function parallels with its enhancement by oxidative stress, the above findings strongly suggest an essential role of PI3K in, at least some facets of, the cellular aging.

2. Materials and methods

2.1. Cell culture

Cell strains IMR-90 (human fibroblast with in vitro life span of population doubling level or PDL 50-55; in the present report, IMR-90 cells before PDL 40 are referred to as 'young' or 'presenescent' and those after PDL 45 as 'old' or 'aged'), SV40-transformed IMR-90, A-431 (human epidermoid carcinoma), HT-1080 (human fibrosarcoma), Saos-2 (human osteosarcoma), HeLa (human cervical adenocarinoma), HL-60 (human leukemia), N1E-115 (mouse neuroblastoma), and PC-12 (rat pheochromocytoma) were received from Japanese Collection of Research Bioresources (formerly, Japanese Cancer Research Resources Bank; Tokyo, Japan). Unless otherwise stated, cells were cultured in Dulbecco's modified MEM (DME) supplemented with 10% fetal bovine serum (FBS) under the standard conditions (Matuoka and Chen, 2000). For chemical treatments, cells were inoculated at a split ratio of 1:8, received LY (50 mM stock solution in EtOH; Calbiochem) and/or nicotinamide (NAA; 1 M stock solution in water; Sigma) on the next day, and cultured for 3–4 days, and finally fixed and stained with Giemsa solution (Sigma). As to H_2O_2 treatment of IMR-90 cells, confluent cells were exposed to 300 μM H_2O_2 for 2 h, washed, cultured in fresh medium for 3–4 days, subcultured to a density of approximately 2×10^3 cells per cm^2, cultured for 3 days, and then fixed for Giemsa staining or senescence-associated β-galactosidase (SA-gal; Dimri et al., 1995). In an experiment, cells were treated with LY either for 6 h before or for 3 days after H_2O_2 treatment. For differentiation experiments, cells were cultured in RPMI medium 1640 supplemented with 10% FBS. HL-60 cells were inoculated by 1:20 dilution and incubated for 2 h. Then H_2O_2 was added to the indicated final concentrations and, 2 h later, cells were washed and cultured in a fresh medium for 2 days, followed by further treatment with 12-O-tetradecanoyl phorbol-13-acetate (TPA) and/or LY for 24 h (for control cultures, DMSO and EtOH were added to the corresponding concentrations). In the same manner as IMR-90 cells as described above, N1E-115 and PC-12 cells were treated or untreated with H_2O_2 and then subcultured. On the next day N1E-115 cells received a medium containing 10% or no FBS and with or without LY and were cultured for 3 days. Similarly, PC-12 cells were cultured for 3 days in a medium with or without nerve growth factor and/or LY.

2.2. Analysis of inositol phospholipids

The composition of cellular phospholipids was analyzed as described (Itoh et al., 2000). Briefly, IMR-90 cells were inoculated at a split ratio of 1:8, cultured for 7 days and then fed with medium containing either 10% (confluent samples 'c') or 0.1% (stimulation samples 's') FBS for 2 days. The cells were rinsed twice with phosphate-free DME (Gibco #11971-025), cultured for 40 h in phosphate-free DME containing 10% FBS (pre-dialyzed against saline) and 75 μCi/ml of [^{32}P]orthophosphate (9000 Ci/mmol; NEN) and harvested for lipid extraction and 2-dimensional thin-layer chromatography with solvent systems chloroform/methanol/25% ammonia/water

(15:11:2:2) and 1-butanol/acetic acid/water (30:5:5) (Hiroyama and Takenawa, 1999). Normally an aliquot of each sample corresponding to 150 000 dpm was loaded on a plate. In another experiment, confluent (7 days after 1:8 inoculation) or growing (2 days after 1:8 inoculation) cells were rinsed twice with inositol-free DME (Gibco #11968-021), cultured for 40 h in inositol-free DME, 10% FBS (pre-dialyzed against saline), and 5 μCi/ml of *myo*-[2-^{3}H]inositol (20 Ci/mmol; Amersham). Lipids were extracted, deacylated and analyzed by liquid chromatography with a SAX anion exchange column (Serunian et al., 1991). Samples corresponding to a radioactivity of approximately 5×10^6 dpm were loaded each time and the results were normalized accordingly.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

Cells were cultured for 5 days after 1:8 inoculation, fed with DME + 0.1% FBS for 2 days, and incubated with DME containing either 10% ('+' samples) or 0.1% ('−' samples) FBS for 18 h. Some cell samples were cultured for 2 days after 1:8 inoculation ('gr' samples). RNA preparation and RT-PCR were carried out as described (Matuoka and Chen, 2000). For PCR, thermal program was 94 °C, 30 s, 25–35 cycles (as indicated in Table 1) of [94 °C, 30 s, 55 °C, 30 s and 72 °C, 1 min] and 72 °C, 5 min. The primers used are listed in Table 1. PCR products were analyzed by agarose gel electrophoresis, ethidium bromide staining and densitometry with the program NIH IMAGE for Macintosh.

3. Results

The pattern of phospholipids extracted from young and old IMR-90 cells were compared (Fig. 1A). There was an increase in phosphatidic acid upon serum stimulation in both young and old cells, consistent with the notion that phosphatidic acid relates to cell proliferation (Strum et al., 1997). With regard to cellular aging, no remarkable difference was observed. However, when analyzed by ion exchange column/liquid chromatography, in aged cells there were a slight decrease in phosphatidylinositol 3-phosphate (PI3P) and phosphatidylinositol 4,5-bisphosphate ($PI4,5P_2$) and a notable decrease in $PI3,4P_2$ with phosphatidylinositol 4-phosphate (PI4P) being unchanged (representative chromatograms for confluent cells are shown in Fig. 1B). In the present experimentation we failed to detect phosphatidylinositol 3,4,5-trisphosphate ($PI3,4,5P_3$), presumably due to a very low content of this phosphoinositide. Other studies (e.g. Konishi et al., 1999; Van der Kaay et al., 1999) also showed the amount of $PI3,4,5P_3$ to be small (~ 1/300 of $PI4,5P_2$ or ~ 1/100 of PI3P). Such a level of radioactivity was unable to be detected as a peak. Whether the cells were growing or confluent, the differences between young and old cells were essentially identical (Table 2), indicating that these changes are not subordinate to the growth activity of cells. On the other hand, comparison of inositol phospholipids between confluent and growing cells, a slight decrease in PI3P and a substantial decrease in $PI3,4P_2$ were observed in both young and old cells. As judged

Table 1
Polymerase chain reaction primers and amplification conditions

Gene (database entry name)[a]	Sequence	Amplification[b]
PI3K p110α (HSPHI3K)	5′-ACAATGCCTCCAAGACCATCATC-3′ 5′-CATACATTGCTCTACTATGAGGTG-3′	30 cycles
PI3K p110β (S67334)	5′-ATGTGCTTCAGTTTCATAATGCCTC-3′ 5′-ATTCATATTAGGAGACACTTGAAAGCT-3′	30 cycles
PI3K p110γ (HSIDEM)	5′-GGCATGGAGCTGGAGAACTATAAAC-3′ 5′-ATCCATCAGAGATTTCTTCTTGGC-3′	30 cycles
PI3K p110δ (HSP110DEL)	5′-CAACGCAGGATGCCCCCTGG-3′ 5′-CTGCCCTGGATGAGCTCGATG-3′	30 cycles
PI3K IIα (HSPHOSI3K)	5′-ATGGCTCAGATATTTAGCAACAGCG-3′ 5′-ATTTTGGAATGCAGCCTGTTTAC-3′	35 cycles
PI3K IIβ (HSC2PI3K)	5′-CCTCACCATGTCTTCGACTCAGG-3′ 5′-CTTCTAGGATCCGATGCTCTAGCAG-3′	35 cycles
PI3K IIγ (HSC2PI3KI)	5′-AAAAAATGGCATATTCTTGGCAAACG-3′ 5′-GTGCTCCAGATCTTCCCAGAATTG-3′	35 cycles
Vps34 (HSPITR1)	5′-CGGTGCGATGGGGGAAGCAGA-3′ 5′-ATCCAGCCAATCTACTTTCACCATGTG-3′	30 cycles
PI3K p85α (HUMP13KIN)	5′-TGCAAACATGAGTGCTGAGGGGTA-3′ 5′-AATTCTGCCAGGTTGCTGGAGCTC-3′	30 cycles
PI3K p85β (HSPHOSINK)	5′-AACCATCCAGACCCCACCCCACT-3′ 5′-GATCCACGTCGCTCAGGGACCAG-3′	30 cycles
p55γ (D88532)	5′-CGCGATGTACAATACGGTGTGGA-3′ 5′-ATTCAGGCGTTTCTGTCTCACTC-3′	30 cycles
p150 (SP150)	5′-TGCCATTATGGGAAATCAGCTTGCT-3′ 5′-TCTATAAGCCAAAAGTTGAGAGAGATC-3′	35 cycles
p101 (AF128881)	5′-TCCTACCAGAGACTGGTGAGGGCT-3′ 5′-CACAGTGCCCGTCAGTTTCCAAG-3′	35 cycles
β-actin (HUMACCYBB)	5′-GGGCCGTCTTCCCCTCCATCGTGG-3′ 5′-CCGTGGCCATCTCTTGCTCGAAGTC-3′	25 cycles
Shc p66 (HSU73377)	5′-CCCTTGCCCAAACTGGCAGGG-3′ 5′-ATTCCAGCAAATTTCAGGTTACTCCT-3′	35 cycles
Shc p46/p52 (HSSHC)	5′-GCGGAGACTCCATGAGGCCCTG-3′ 5′-ATTCCAGCAAATTTCAGGTTACTCCT-3′	25 cycles
TK (HUMTKRA)	5′-AGCACAGAGTTGATGAGACGC-3′ 5′-GCTTCCTCTGGAAGGTCCCAT-3′	25 cycles
P53 (HSP53G)	5′-CGGACGATATTGAACAATGGTTCAC-3′ 5′-GCCCATGCAGGAACTGTTACACAT-3′	25 cycles

[a] Entries in the GeneBank/EMBL/DDBJ databases.
[b] See Materials and Methods for the other amplification conditions.

from the magnitude of all these changes, it seems that the most affected step was conversion of PI4P to $PI3,4P_2$, a function of PI3K.

Next we wanted to learn which PI3K subspecies related to the above changes in inositol phospholipids and, therefore, carried out RT-PCR to examine if there is any fluctuation of the mRNAs of PI3K molecules. Some aging- and growth-related molecules were also examined as reference. The results are shown in Fig. 2 (electrophoretograms of RT-PCR products) and Fig. 3 (densitograms thereof,

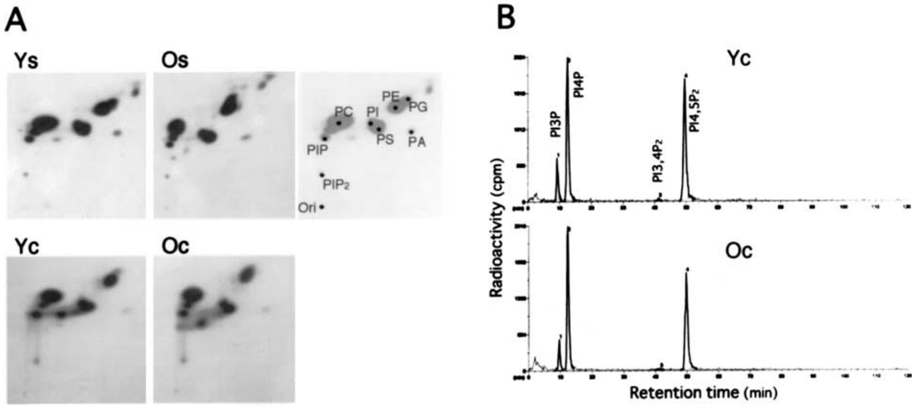

Fig. 1. Changes in cellular phospholipid composition due to aging. (A) IMR-90 cells were cultured in the presence of [^{32}P]orthophosphate. Phospholipids extracted from cell layers were analyzed by thin-layer chromatography. Ys, PDL 33.5, serum-stimulated; Os, PDL 50.5, serum-stimulated; Yc, PDL 37.5, confluent; Oc, PDL 49.5, confluent; Ori, origin of development; PIP_2, phosphatidylinositol bisphosphate; PIP, phosphatidylinositol phosphate; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PA, phosphatidic acid. Difference in the phospholipid pattern was not apparent between young and old cells or between stimulated and confluent cultures, except some increase in PA in stimulated cells. (B) IMR-90 cells were cultured in the presence of *myo*-[2-^{3}H]inositol. Cell-associated inositol phospholipids were analyzed by liquid chromatography. Each load contained a lipid sample corresponding to a radioactivity of 5×10^6 dpm. Yc, PDL 28.5, confluent; Oc, PDL 45, confluent. PI4P, phosphatidylinositol 4-phosphate; PI3P, phosphatidylinositol 3-phosphate; PI4,5P_2, phosphatidylinositol 4,5-bisphosphate; PI3,4P_2, phosphatidylinositol 3,4-bisphosphate. Representative chromatograms are shown (see Table 2 for the full results). In old cells PI3P, PI3,4P_2, and PI4,5P_2 decreased, whereas, PI4P was unchanged.

Table 2
Changes in inositol phospholipids due to aging

	PI4P	PI4,5P_2	PI3P	PI3,4P_2
Amount (cpm)[a]				
Young, growing	7689 ± 157	9910 ± 156	2371 ± 112	256 ± 45
Old, growing	7922 ± 118	6953 ± 108	2298 ± 77	98 ± 1
Young, confluent	9703 ± 169	10 234 ± 182	2064 ± 102	127 ± 21
Old, confluent	9737 ± 157	8823 ± 105	1644 ± 56	49 ± 24
Relative amount (%)				
Old vs. young (growing)	103	70	97	38
(confluent)	100	86	80	39
Confluent vs. growing (young)	126	103	87	49
(old)	122	126	71	50

[a] Normalized for a sample load of 5×10^6 dpm; mean ± S.E. ($n = 3$ or 4). Young, IMR-90 PDL 30.5; old, PDL 46 (see Materials and Methods and the legend to Fig. 1).

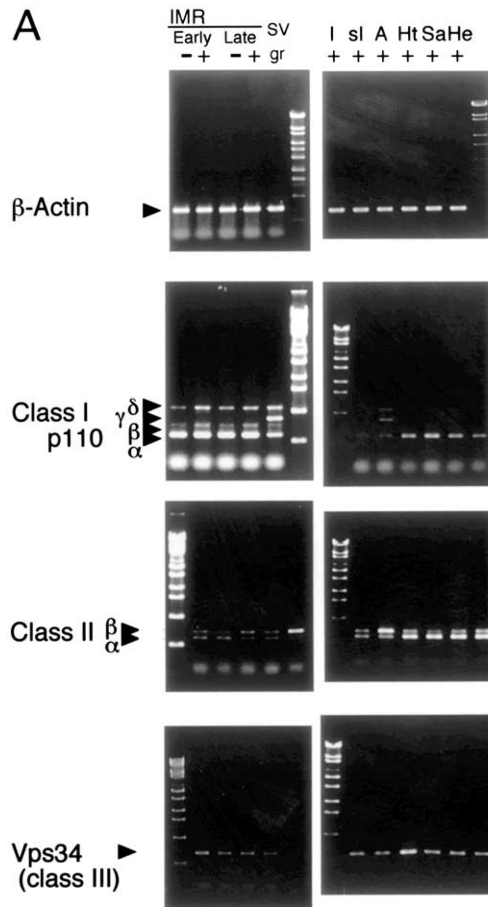

Fig. 2. Changes in expression of mRNAs due to aging, serum stimulation, and cell transformation: (A) PI3K catalytic units, (B) PI3K regulatory units, and (C) aging-related molecules. RNA samples were analyzed by RT-PCR. IMR, IMR-90; early, PDL 29; late, PDL 51; SV, SV40-transformed IMR-90; I, IMR-90; sI, SV40-transformed IMR-90; A, A-431; Ht, HT-1080; Sa, Saos-2; He, HeLa; −, serum-deprived; +, serum-stimulated; gr, growing. The amounts of RT-PCR products were estimated by densitometry as shown in Fig. 3.

showing some notable changes). With regard to PI3K IIγ and p101, we could not amplify their signals by RT-PCR, presumably due to no or low expression of their mRNAs in fibroblasts (Stephens et al., 1997; Rozycka et al., 1998); another possible cause might be inadequacy of the primers used, even though we tried a few sets of them. With PI3K p110γ, we did not detect its signal in IMR-90 cells and some tumor cells, either. In this case, however, we found SV40-transformed IMR-90 cells exhibit a strong signal for the molecule, showing the primers for p110γ working efficiently.

As summarized in Table 3, there were some unique changes in the mRNA expression for PI3K catalytic units due to aging and cell proliferation, whereas, we

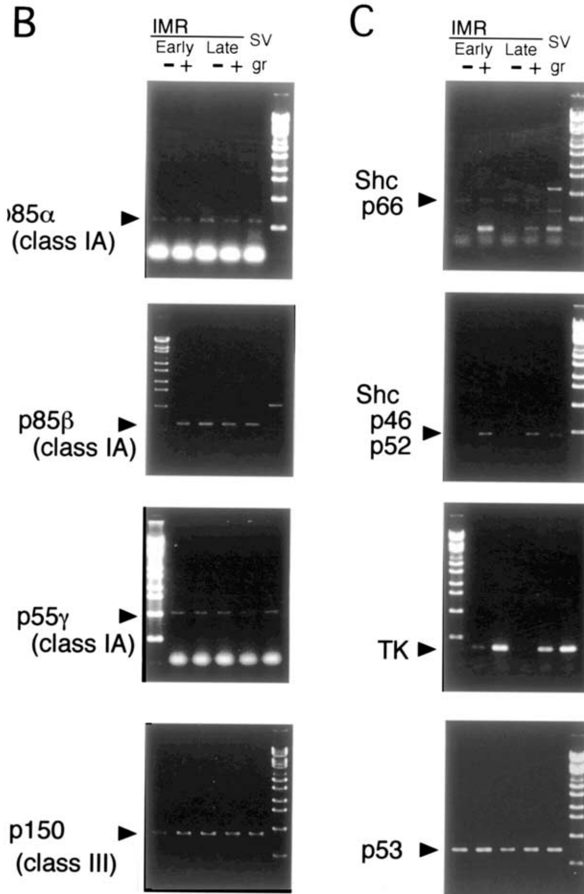

Fig. 2 (*Continued*)

did not observe any fluctuation with the regulatory molecules. Aged cells decreased in the expression of PI3K IIα, Vps34, and p110δ, while there was an increase in the PI3K IIβ message. Although we described a significant decrease in p110α in aged TIG-1 cells (another human fibroblast strain) (Matuoka and Takenawa, 1998), we observed only a slight decrease therein in the present study. We do not have a definite explanation for the discrepancy but a possibility could be that, since in the previous experiment a weak band of the PCR product for p110α was obtained only after 45–50 cycles of amplification, the primers used in that study might not be optimal for quantitative analysis. Thus, we consider that the present result from an efficient amplification of the p110α message should be more reliable and override the previous one. Another observation was that, despite the implication of PI3K in cell cycle progression, only the message of PI3K p110β was elevated by growth stimulation; instead, that of PI3K IIβ decreased thereupon. With regard to malignant transformation, most of the tumor cells examined exhibited enhanced

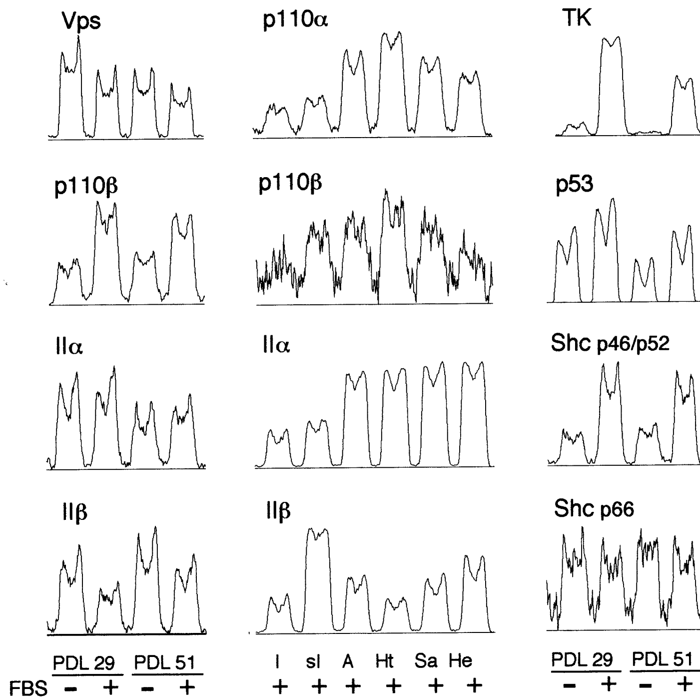

Fig. 3. Densitometric presentation of the results in Fig. 2. RT-PCR products were run in an agarose gel, stained, and photographed, followed by densitometry. Some representative changes are shown. See Table 3 for the full results. The symbols and abbreviations are as in Fig. 3.

expression of some PI3K catalytic units (e.g. p110α, p110β, p110δ, and IIα), although these fluctuations varied from cell type to cell type (in particular, SV40-transformed IMR-90 cells).

We examined expression of some aging-related signaling molecules for reference. The messages of thymidine kinase (TK) and p53 were lower in aged cells, in agreement with other reports (Chang and Huang, 2001; Kim et al., 2002), showing that the RT-PCR analysis performed in this study worked sufficiently to examine the mRNA levels. Thus, the above observations on the PI3K expression are strong suggestion that these PI3K subtypes are regulated individually and play their own unique roles in the cell regulation. In addition, we also observed that the expression of TK, p53, and Shc p42/p52 was enhanced by growth stimulation and that of Shc subtypes p66 and p46/p52 affected little with aging.

Now, we attempted to address a question as to how PI3K relates to aging. As a first step, we treated aged cells with LY (Fig. 4). Besides its growth inhibitory effect (Tresini et al., 1998; Matuoka and Chen, 2002), LY induced a dramatic change in cellular morphology: LY greatly reduced aging-associated enlargement and flattening of the cell body and nucleus. Elongated cell shape was also prominent with both young and old cells under LY treatment. As we found recently (Matuoka et al.,

Table 3
Changes in expression of PI3Ks due to cellular aging, growth stimulation, and transformation: summary

Molecule (subtype)	Aging	Growth stimulation	Transformation
Catalytic units			
Class IA p110α	0/−	0	++ (SV-IMR 0)
p110β	0	++	++
p110δ	−	0	+/++
Class IB p110γ	Low	Low	Low (SV-IMR ++)
Class II IIα	−	0	++ (SV-IMR 0)
IIβ	+	−	0/+/++
IIγ	Low	Low	ND
Class III Vps34	−	0/−	0 (A431+)
Regulatory molecules			
p85α (class IA)	0	0	ND
p85β (class IA)	0	0	ND
p55γ (class IA)	0	0	ND
p101 (class IB)	Low	Low	ND
p150 (class III)	0	0	ND

As compared with respective control counterpart (young, unstimulated, or untransformed cells): ++, elevated greatly; +, elevated; 0, changed little; −, decreased. ND, not determined; Low, no or low expression detected; SV-IMR, SV40-transformed IMR-90.

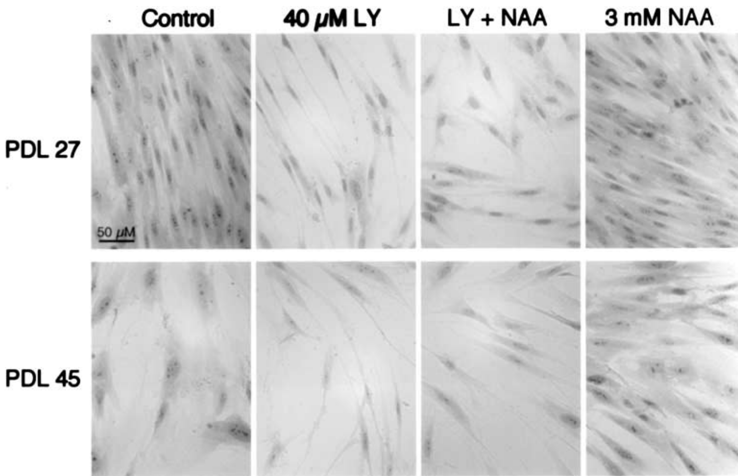

Fig. 4. Effect of aging and chemical treatments on cellular morphology. IMR-90 cells were treated with LY294002 and/or NAA. See Materials and Methods for details. Control, 0.08% EtOH. LY and NAA prevented aged cells from enlarged and flattened cell morphology as well as gigantic nuclei. On the other hand, in LY-treated cells elongation of cell body was prominent, the shape being suppressed by NAA.

2001), NAA suppressed the aging morphology without affecting cellular replicative capacity (Fig. 4). When cells were treated with a combination of LY and NAA, all of the above phenotypes (i.e. cell growth, senescence morphology, and elongated cell

shape) were suppressed. This observation indicates that (1) the expression of the senescence morphology is under regulation of a signaling event requiring a PI3K function and that (2) the LY-induced cell elongation involves machinery susceptible to the action of NAA.

To further confirm that PI3K plays a role in the aging-associated phenotypical changes, we employed H_2O_2 induction of premature senescence (Fig. 5; Chen and Ames, 1994; Matuoka and Chen, 2002). Young cells pulse-exposed to H_2O_2 (2 h) underwent 'aging process' and, therefore, even after removal of H_2O_2 and subculture, exhibited the same morphology as aged cells and SA-gal activity specific to aged cells (Dimri et al., 1995; Fig. 5A). Next, the cells were treated with H_2O_2 similarly but, at that time, with pre- and/or postincubation with LY. With both pre- and postincubation with LY, the expression of aging morphology (Fig. 5B) and SA-gal activity (not shown) were both suppressed. LY pretreatment alone was not effective, suggesting that a cellular event evoked after H_2O_2 exposure may involve a PI3K activity and be critical for induction of the aging phenotypes. In this regard, we observed SA-gal expression within 24 h after H_2O_2 treatment (not shown). It has

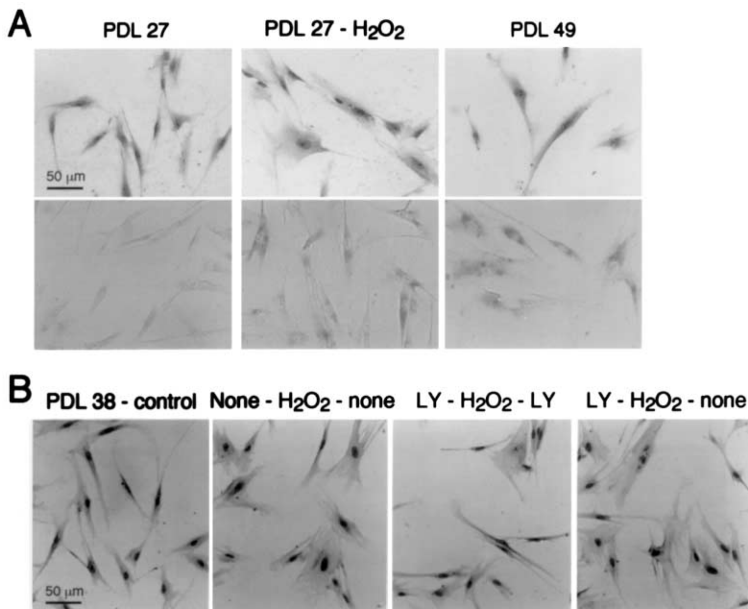

Fig. 5. Effect of H_2O_2 and PI3K inhibitor on expression of senescence phenotypes. (A) IMR-90 cells treated or untreated with 300 μM H_2O_2 were stained with Giemsa (upper panels) or for SA-gal (lower panels). See details under Materials and Methods. (B) IMR-90 cells were exposed to H_2O_2 as in (A) except that some cultures were incubated with 40 μM LY294002 either for 6 h before H_2O_2 treatment or for 3 days after H_2O_2 treatment. Control, no H_2O_2 (0.08% EtOH); None-H_2O_2-none, no LY treatment; LY-H_2O_2-LY, LY treatment both before and after H_2O_2; LY-H_2O_2-none, LY treatment only before H_2O_2. All the cells were subcultured after the chemical treatment and cultured in the absence of H_2O_2 or LY; thus, the phenotypical change seen here is ascribable to an irreversible and persistent aftereffect of the chemicals rather than their direct effect.

also been suggested that H_2O_2 exerts its effects only after conversion to hydroxyl radicals (Chen et al., 1998).

Then, we wanted to learn how the above action of H_2O_2 and the function of PI3K relate to each other. For this purpose we employed cell differentiation systems which have been analyzed well (Klesse and Parada, 1999; Sarner et al., 2000; Kim et al., 2001; Fig. 6). HL-60 human leukemia cells were pretreated with H_2O_2 and, after washing and culturing, stimulated with TPA for monocytic differentiation. H_2O_2 substantially promoted their differentiation, while LY was capable of abolishing it. The results indicate that the monocytic differentiation involves both a PI3K function and an event positively affected by H_2O_2. With N1E-115 mouse neuroblastoma cells, neurite outgrowth triggered by serum deprivation was slightly promoted by H_2O_2 pretreatment and partially inhibited with LY. PC-12 rat pheochromocytoma cells were stimulated with NGF to exhibit neurite outgrowth. In this case, H_2O_2 pretreatment did not promote their differentiation; nor did LY suppress it. The

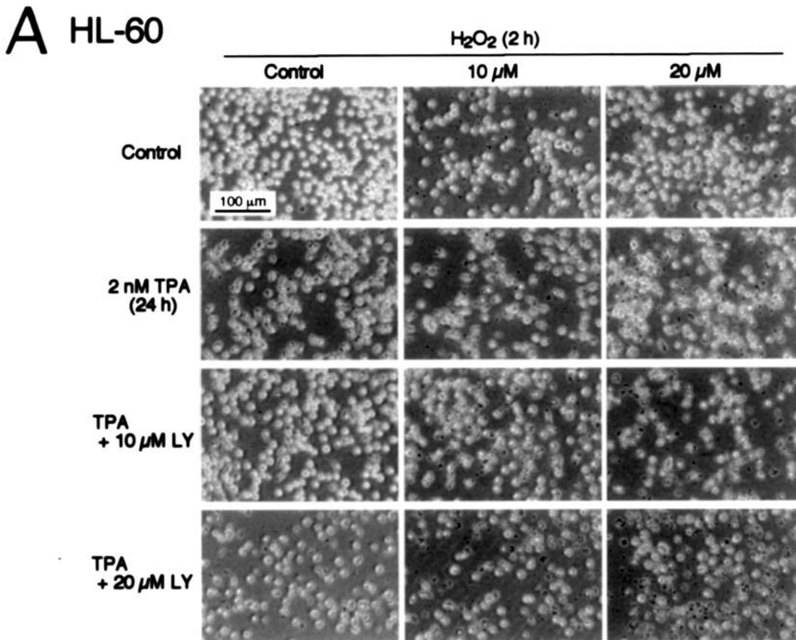

Fig. 6. Effect of H_2O_2 and LY294002 on cellular differentiation. (A) HL-60, (B) N1E-115, and (C) PC-12 cells. Cells were pretreated or not with H_2O_2 and then stimulated for differentiation in the presence or absence of LY. See Materials and Methods. For each cell strain, several doses were examined, among which an appropriate (i.e. effective but not too damaging) dose was employed. N.B.: With HL-60, H_2O_2 pretreatment enhanced induction of (but was unable to induce by itself) oblong and rough adherent cell phenotypes dose-dependently and LY abolished these changes dose-dependently. H_2O_2 pretreatment enhanced neurite formation in N1E-115 cells to some extent (and only weakly induced the change by itself). LY was capable of only partially suppressing N1E-115 neurite formation. In contrast, NGF induction of neurite formation in PC-12 cells was not affected by either H_2O_2 pretreatment or LY treatment.

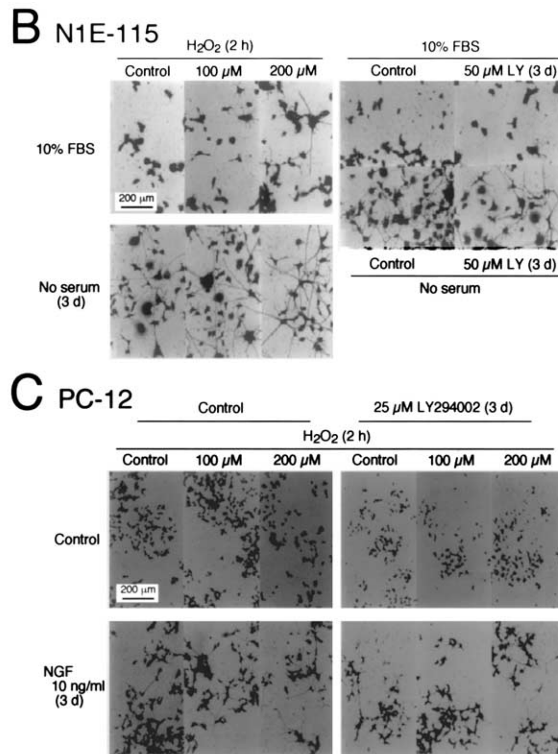

Fig. 6 (*Continued*)

observation indicates that neither a PI3K activity nor an H_2O_2-triggered event is involved in the differentiation of PC-12 cells. With these 3 cell systems, the parallelism is evident between the stimulatory effect of H_2O_2 and the inhibitory effect of LY.

4. Discussion

PI3K is an enzyme family which functions at diverse facets of cellular physiology, including regulation of proliferation and morphology. Being accompanied with alterations of both growth and morphology, aging involves a PI3K function as well (Krasilnikov, 2000). Then, how is PI3K committed to this phenomenon? In order to address this question we have analyzed (1) whether the activity of PI3K changes during aging; (2) whether the expression of PI3K changes during aging; (3) whether inhibition of PI3K affects aging phenomena; (4) whether inhibition of PI3K affects induction of ‘aging’ by oxidative stress; (5) whether the PI3K function and the oxidative stress-evoked event are interrelated.

We found a substantial difference in the composition of cellular inositol phospholipids between young and old fibroblastic cells. Among them $PI3,4P_2$ was markedly low in aged cells (Table 2). Since we observed a similar change with both growing and confluent cell cultures, the decline was aging-related rather than growth-coupled. Although we were unable to analyze a change in $PI3,4,5P_3$ due to a detection limit in these experiments, the results imply a decrease in polyphosphoinositides, which are synthesized by the action of class I or class II PI3K enzymes (Wymann and Pirola, 1998). Consistently, by RT-PCR analysis we observed an aging-related decrease in the expression of PI3K IIα and p110δ (Table 3). It is interesting that the message of PI3K IIβ was elevated in aged cells. On the other hand, growth stimulation elevated the p110β message, suggesting a role of this molecule in the cell cycling regulation. Since the inhibition of PI3K caused a decrease in cell growth (Fig. 4), a PI3K function seems required for proliferation. However, the pattern of the changes in PI3K molecules (Table 3) clearly shows that the aging-related growth reduction is distinct from the modulation by serum deprivation. A change in mRNA expression of a gene does not necessarily indicate that in the amount or in the activity of its gene product; nonetheless, all the above observations, taken together, strongly suggest differential regulation of individual PI3K subtypes, which may result in diversion of cellular functions. In this sense, one could hypothesize that in aged cells a decline in the expression of PI3K IIα or p110δ relates to their lowered replicative capacity, while an elevation of that of PI3K IIβ relates to their aging morphology. Consistent with the idea, a PI3K inhibitor greatly suppressed that cell shape (Fig. 4).

Other observations include: (1) Little change was observed with the PI3K regulatory molecules examined (Fig. 2), suggesting that, at the transcription level, PI3Ks are regulated through their catalytic units rather than the regulatory units; (2) a marked increase in expression of various PI3K subspecies, e.g. p110α, p110β, p110δ and IIα, was observed in the tumor cells examined (Fig. 3), supporting the notion of overstimulation of tumor cell proliferation by overproduction of polyphosphoinositides (Roymans and Slegers, 2001).

It has been shown that a pulse exposure of fibroblasts to H_2O_2 evoked irreversible changes similar to the cellular aging (Fig. 5A; Chen and Ames, 1994; Matuoka and Chen, 2002). Importantly, when those phenotypes were examined, H_2O_2 had been washed off and cells had been subcultured; therefore, the premature senescence could not be ascribable to a direct effect of H_2O_2 on the cell but to a change which H_2O_2 had already brought about in the cell. Now, we found that a PI3K inhibitor was capable of abolishing the effect of H_2O_2 (Fig. 5B). This indicates that the H_2O_2 induction of cell aging involves a PI3K function. In order to further analyze their relationship, we employed three different cell systems (Fig. 6). Monocytic differentiation of HL-60 human leukemia cells requires a PI3K function, which then activates a protein kinase system (Kim et al., 2001). Thus, PI3K inhibition abolishes the differentiation. The signal for neurite outgrowth of N1E-115 mouse neuroblastoma cells is transduced to PI3K via Ras and then to Cdc42 and Rac1 (Sarner et al., 2000). In this case, however, the PI3K system does not control the entire pool of these Rho family GTP-binding proteins (Cantrell, 2001) and,

accordingly, PI3K inhibition exerts only partial suppression of the differentiation. With PC-12 rat pheochromocytoma cells, their neurite outgrowth depends on the MAPK/ERK cascade, independent of the PI3K function (Klesse and Parada, 1999). Thus, PI3K inhibition does not affect the differentiation of PC-12 cells. As to the action of H_2O_2, we observed the following: H_2O_2 activated HL-60 cells effectively, N1E-115 cells to some extent, and PC-12 cells little (Fig. 6). In other words, the more negatively sensitive an event was to the PI3K inhibitor, the more positively sensitive it was to H_2O_2. Here is a clear parallelism between the effectiveness of H_2O_2 pretreatment and the susceptibility to the PI3K inhibition.

On the analogy of the above cell differentiation systems, it is conceivable that the aging process, at least that concerning cell structure, depends on a PI3K function. Among a variety of functions, the PI3K products 3′-phosphoinositides stimulate PKB/Akt (directly or via PDK1), which promotes cell cycling (Roymans and Slegers, 2001). On the other hand, 3′-phosphoinositides stimulate Vav and other guanine nucleotide exchange factors, which activate small GTPases regulating subcellular structure (Roymans and Slegers, 2001). One could speculate that the latter function of PI3K is upregulated during aging, while the former is downregulated or attenuates, both contributing to expression of the respective phenotypes.

The subsequent question would be which PI3K subspecies is involved in that process. It would also be of importance to identify the molecule or structure on which the oxidative stresses engrave damages and which is liable for the above modulation of the PI3K function. Although shortening of the telomere regions at the ends of chromosomes has been proposed as the cause of aging, that does not apply, at least, to the H_2O_2-induced premature senescence (Chen et al., 2001; Matuoka and Chen, 2002). An alternative possibility would be oxidative modification of nuclear and mitochondrial DNAs (Beckman and Ames, 1998; Finkel and Holbrook, 2000) such as accumulation of oxo-8-deoxyguanosine (Chen et al., 1998).

Finally, the findings in this study imply not only some of the PI3K activities diminishing with cellular aging but also a positive role of the PI3K function in the aging process. In this connection, inhibition of PI3K activity has been reported to induce senescent cell phenotypes (Tresini et al., 1998; Collado et al., 2000). However, we observed otherwise (Fig. 4). Besides, one could observe that the effect of LY294002 is temporary and reversible (Tresini et al., 1998) and that the PI3K inhibitor-treated cells appear damaged or aberrant rather than aged (Collado et al., 2000). In addition, in contradiction to their notion, a loss-of-function mutation of nematode PI3K confers an extension of life span (Morris et al., 1996). Here we would hypothesize that individual PI3K subspecies assume their respective roles in the aging phenomena. Given the situation, further studies are urgent to establish the functions of PI3Ks in cellular aging.

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