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Decline of Protein Kinase C Activation in Response to Growth Stimulation during Senescence of IMR-90 Human Diploid Fibroblasts

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We have studied the activation of protein kinase C (PKC) during senescence of human IMR-90 fibroblasts by analyzing the phosphorylation of its in vivo substrate MARCKS (myristoylated alanine-rich C kinase substrate). It was found that the extent of TPA-induced phosphorylation of MARCKS was not significantly different between young and old IMR-90 fibroblasts. In contrast, the increase of MARCKS phosphorylation after serum stimulation was 4.5-fold in young fibroblasts as compared to 1.8-fold in old fibroblasts. Analysis of PKC by Western blotting showed that the levels of PKC were not changed during senescence of IMR-90 fibroblasts. However, the generation of diacylglycerol in response to serum stimulation declined in old fibroblasts. These results suggest that the efficiency of signal transduction mediated by diacylglycerol generation and PKC activation during the mitogenic response is age-dependent in human IMR-90 fibroblasts.

Human diploid fibroblasts undergo a finite number of population doublings in tissue culture, which phenomenon has been termed cellular senescence (1). Because the life span of human fibroblast in culture decreases proportionately on the age of the donor (2, 3), it has been suggested that human diploid fibroblast is a useful in vitro model for studying the biochemical basis of cellular aging (4). The

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Abbreviations used: DAG, *sn*-1, 2-diacylglycerol; PKC, protein kinase C; TPA,12-O-tetradecanoylphorbol-13-acetate; MARCKS, myristoylated analine-rich C kinase substrate.

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most prominent biochemical parameter for senescence is the failure to synthesize DNA in response to growth stimulation. It has been demonstrated that the expression of early gene c-fos (5), AP-1 activity (6), cyclins and activities of cdc2 and cdk2 kinases (7, 8) are repressed during senescence of human diploid fibroblasts. These studies point out that the loss of mitogenic responsiveness to growth stimulation in the senescent human diploid fibroblasts is the result of blockage within the early G1 phase of the cell cycle.

Protein kinase C (PKC) is a serine/threonine kinase that is dependent on calcium and phosphatidylserine and is stimulated by *sn*-1,2-diacylglycerol (DAG) and tumor-promoting phorbol esters (9). Upon activation, PKC in cytosol is translocated to membrane (10). The activation of PKC appears to be an important step in the control of many cellular processes including mitogenesis, exocytosis, differentiation and neurotransmission (11). One of the earliest detectable effect of PKC activation is the phosporylation of 80/87 kilodalton cytosolic protein known as MARCKS (12, 13). The level of phosphorylation of this protein is rapidly increased upon addition of PKC agonists or mitogens including platelet-derived growth factor and vasopressin (14, 15). MARCKS has been reported to be a filamentous actin cross-linking protein, whose activity can be inhibited by PKC-mediated phosphorylation, suggesting that MARCKS phosphorylation is involved in the cytoskeleton rearrangement during the mitogenic process (16). In view of the importance of the signal transduction mediated by PKC during cellular growth, here we addressed the question of whether the activation of PKC is altered during cellular senescence by analyzing phosphorylation of MARCKS, translocation of PKC and generation of diacylglycerol in response to growth stimulation in the young and old IMR-90 fibroblasts.

MATERIALS AND METHODS

Cell culture. IMR-90 human embryonic lung diploid fibroblasts (passage number 5, PDL (population doubling level) =12) were obtained from the Institute for Medical Research, Camden, New Jersey. Cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone) at 37°C in 5% CO_2 incubator and subcultured to obtain cultures at higher population doubling levels. Under this experimental condition, IMR-90 cells can reach a PDL value of 60 ± 3. In this study, cells at PDL< 30 and >57 were defined as young and old fibroblasts, respectively.

Two dimensional gel electrophoresis of the ^{32}P-labeled MARCKS.
The examination of MARCKS phosphorylation was performed as previously described (Wolfman and Marcara, 1987). Confluent IMR-90 fibroblasts were serum-starved in DMEM for 24 hours. Prior to ^{32}P-labeling, cells were incubated in a phosphate-free medium for 30 min, washed and refreshed with the same medium plus 250μCi ^{32}P-orthophosphate (Du Pont-New England Nuclear) for 2 hr. Afterward, TPA (12-O-tetradecanoylphorbol-13-acetate, Sigma), or 10% fetal bovine serum was added to the cultures for 15 and 45 min. After washing, trichloroacetic acid (TCA, 10%) was then added to the cells on ice for 30 min and then aspirated off. The cell debris was rapidly washed three times with 5 ml ice-cold 20 mM phosphate buffer, pH 7.6, and transferred to Eppendorf tubes. After centrifugation at 12,000xg for 20 min, the insoluble pellets were washed 2 times with ice-cold acetone, followed by evaporation to eleminate residual acetone. The pellets were then suspended in 100μl of lysis buffer containing 0.2% SDS and modified to contain ampholines in the following ratio: pH 3-5, 60%; pH 5 to 7, 20%; pH 3.5 to 10, 20%. The soluble samples containing the same TCA insoluble counts were loaded simutaneously on the first dimension IEF as described by O'Farrell (17). The second dimensions were run on 10% SDS-PAGE.

Translocation of PKC. Confluent IMR-90 fibroblasts in the 10-cm dish were serum-deprived and treated with TPA or fetal bovine serum as described above. Cells were rinsed in 1X homogenizing buffer (20 mM Tris-HCl, pH 7.5, containing 2mM etylenediaminetetraacetic acid (EDTA), 0.5 mM ethylene glycol bis(β-aminoethyl ether)-N, N, N', N'-tetraacetic acid (EGTA), 2mM phenylmethylsulfonyl fluoride (PMSF), 25μg/ml leupeptin and 0.33 M sucrose), and sonicated in this buffer directly. The homogenates were then centrifuged for 30 min at 12,000 xg to yield cytosol and particulate membrane fractions. The particular membrane fractions were rinsed once in the homogenizing buffer and resuspended in this buffer containing 0.2% Triton X-100 and resedimented for 30 min at 12,000xg. For the experiment concerning the readily reversible translocation, cells after serum stimulation were rapidly washed with ice-cold PBS, rinsed with 1μM Ca^{+2}, and scraped in 100μl of the latter solution. After sonication, one tenth volume of 10X homogenizing buffer was added, followed by the immediate separation of cytosol and membrane fractions as described above. The level of PKC extracted from the particulate membrane fraction or the cytosol fraction was subsequently assessed by Western blot analysis.

Western blot analysis of PKC. The equal amounts of proteins were separated on 10% SDS-polyacrylamide gel, followed by electrophoretically transferred to PVDF membrane (Millipore). After blocking in 5% powdered milk, the membrane was incubated with

monoclonal antibody against αPKC(1:200, Amersham) for 4 hours, followed by incubation with alkaline phosphatase (AP) conjugated goat anti-mouse IgG antibody. The AP color development was performed according to vender's instruction (Promega).

Measurement of diacylglycerol. For determination of diacylglycerol production, cells were serum-deprived for 16 hours, and then labeled with 5μCi of [U-^{14}C]glycerol (Amersham, spec. radioactivity 141 mCi/mmol)/ml for 24 hours. Afterward, cells were treated with fetal bovine serine or TPA. The cells were then scraped into 0.5 ml of ice-cold methanol and extracted with 0.25ml of chloroform containing 20 μg of 1-stearoyl-2-arachidonyl glycerol (Sigma) and processed by the method described by Bligh adn Dyer (18). Lipids were fractionated by thin-layer chromatography by using the following solvent system: hexane/diethylether/acetic acid (60:40: 1, v/v/v). DAG was visualized by exposing TLC plates to iodine vapor and autoradiographed. Spots were then cut for radioactivity measurement.

DNA synthesis. The incorporation of [^{3}H]thymidine into acid-insoluble material was used to estimate the rate of DNA synthesis. Cells were serum-deprived 48 hours and then stimulated by 10% fetal bovine serum. At indicated time intervals after serum stimulation, [^{3}H]thymidine (5 μCi/ml) was added to the cultures , and the metabolic incorporation was carried out at 37°C for 1 hours. The amount of radioactivity incorporated into 10% trichloroacetic acid-insoluble material present in homogenates was determined by scintillation counting.

RESULTS

Phosphorylation of 87-kilodalton substrate of protein kinase C in young and old fibroblasts.

As a marker of protein kinase C activation, the acidic 80/87 kDa PKC substrate, named as MARCKS, has been detected in many tissues and is identified by its characteristic migration on two-dimensional gels (13). After serum-deprivation for 24 hours, the phosphorylation of protein at molecular weight of 87 kilodalton, pI ranging from 4.30 to 4.45, could be observed in both IMR-90 fibroblasts at PDL 23 and at PDL 59. Treatment of cells with TPA for 15 min, a significant increase in phosphorylation of 87-kDa protein occurred in both young and old cells. In contrast, serum stimulation for 15 and 45 min, the increase of MARCKS phosphorylation was more significant in young cells as compared to that in old cells (Figure 1A). By excising the spots corresponding to MARCKS protein on 2-D gels

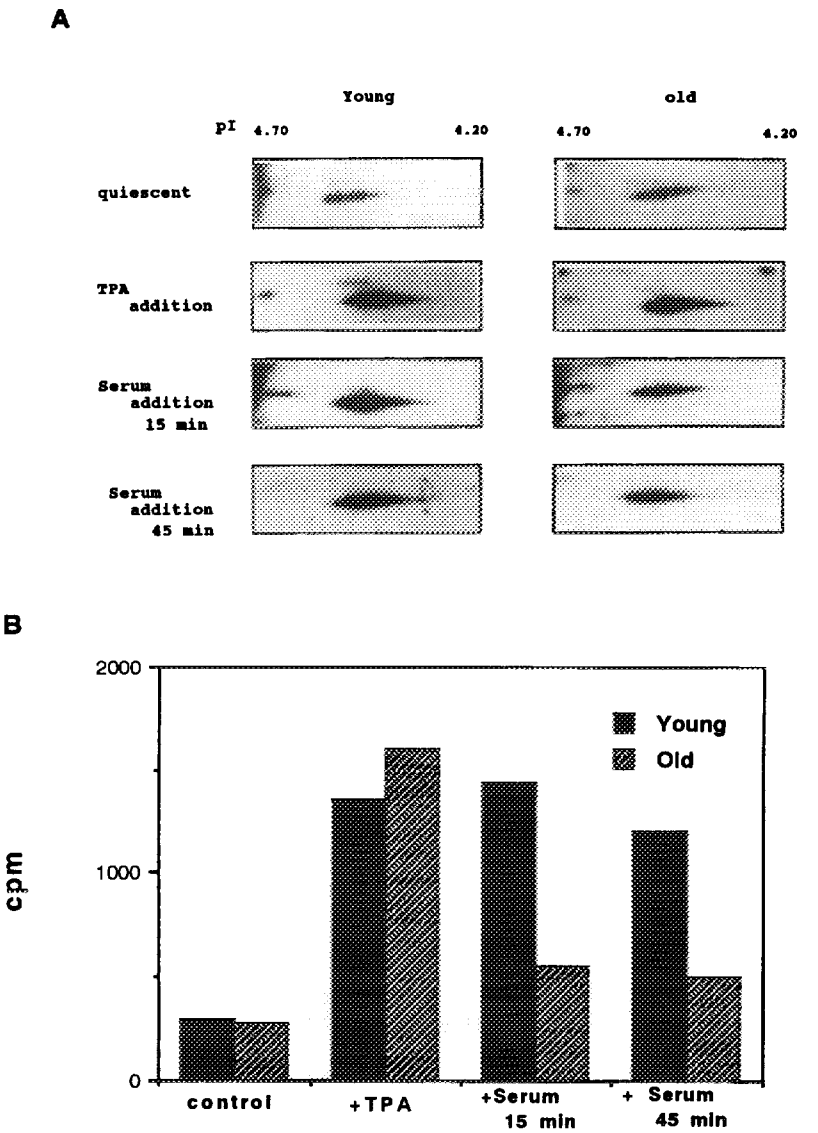

Figure 1. Induction of MARCKS phosphorylation by TPA and serum in human IMR-90 fibroblasts.
Confluent monlayers of IMR-90 fibroblasts at PDL 23 and 59 * were serum-deprived for 24 hours and labeled with ^{32}P-orthophosphate. Cells were then treated with 35 nM TPA for 15 min or 10% fetal bovine serum for 15 and 45 min at 37^{0}C and harvested for 2D electrophoresis analysis. (A) Shown are regions of the autogradiogram covering the area of migration of MARCKS ranging pH 4.2 to 4.7. The phosphorylated MARCKS was detected as a diffuse spot of pI approximately 4.4 at the molecuclar weight of 87 kilodalton. (B) Quantitative anlysis of the extent of MARCKS phosphorylation. The gel regions containing phosphorylated MARCKS were excised for radioactivity measurement. The data represented the average cpm from three different sets of experiments.

for radioactivity measurement, it showed that the increase of MARCKS phosphorylation by serum stimulation was more than 4-fold in the young cells and about 1.8-fold in old cells, respectively (Figure 1B). TPA treatment essentially gave approximately 4-fold increase of MARCKS phosphorylation in both young and old cells. Since the phosphorylation of acidic 87 kDa protein is an indicator of activation of PKC in vivo, these data clearly suggest that PKC activation via the pathway of growth stimulation becomes less efficient during cellular senescence. However, PKC activation by its agonist, phorbol ester, is age-independent in IMR-90 fibroblasts.

PKC levels remained unchanged in young and old IMR-90 fibroblasts.

As the decrease of MARCKS phosphorylation in old cells in response to serum-stimulation could result from defect in translocation of PKC, we then examined the levels of PKC in membrane and cytosolic fractions isolated from young and old fibroblasts with treatments of TPA and serum stimulation by Western blot. It is well established that TPA rapidly intercalates into the membrane lipid bilayer and is slowly metabolized, thus maintaining PKC in stable association with the membrane. Our Western blot analysis detected PKC with the molecular weight of 80 kDa, which clearly translocated on the membrane fraction after TPA-treatment for 30 and 60 min in both young and old IMR-90 cells (Figure 2 A). However, with serum stimulation for 30 min and 60 min PKC was not well detected in the membrane fractions (Figure 2B). The activation of PKC by serum stimulation is known to be mediated by the generation of diacylglycerol, which in turn is responsible for the association of kinase with membranes. However, this process may be more readily reversed in the cell during the isolation of subcellular fractions due to the rapid degradation of diacylglycerol by endogenous lipases. We, therefore, isolated the membrane fraction in the presence of 1 μM of Ca^{+2} without chelator for the experiment of serum stimulation (Figure 2C). It appeared that under this assay condition the levels of PKC associated with membrane fraction remained constant during the period of stimulation in the young cells as well as in the old cells. Moreover, the level of PKC did not appear to be less in old cells, either. Based on this result, it is evident that a large portion of PKC normally is present in IMR-90 fibroblasts in weak, calcium-dependent assoication with membranes. This also indicates that our method of analyzing the translocation of PKC was not able to

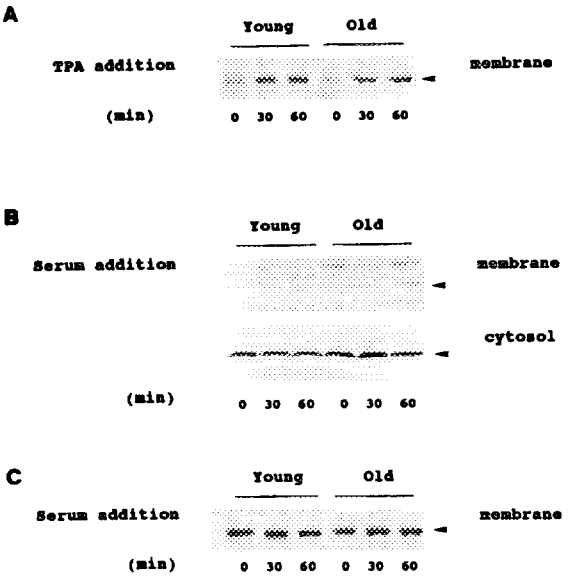

Figure 2. Effects of TPA or serum treatment on PKC translocation in IMR-90 fibroblasts.
IMR-90 fibroblasts at PDL 20 and 60 were serum-deprived for 36 hours and incubated with either no addition or TPA (35 nM) or fetal bovine serum (10%) for 30 and 60 min. Cells were then homogenized, and cytosol and particulate membrane fractions were obtained as described in Materials and Methods. Equal amounts of protein were electrophoretically separated and processed by immunoblotting using the α-PKC antibody. The arrows indicate the immunoreactive α-PKC with the apparent molecular weight of 80 kDa.
(A) The membrane fractions (50μg of protein) of cells treated with TPA. (B) The cytosolic (80 μg of protein) and membrane (80μg of protein) fractions of cells treated with fetal bovine serum. The harvesting and homogenizing methods for (A) and (B) were the same. (C) The membrane fractions (50μg of protein) isolated from cells treated with fetal bovine serum were prepared in the presence of 1 μM Ca^{+2} solution as described in Materials and Methods.

distinguish DAG-mediated membrane association of PKC during serum stimulation. Neverthelss, these results suggests that the steady-state levels of PKC remain relatively unaltered during senescence of IMR-90 fibroblasts.

Generation of diacylglycerol decreased in the old IMR-90 fibroblasts.

Since PKC activation induced by TPA treatment was well detected in both young and old fibroblasts, it is very unlikely that the lesser extent of MARCKS phosphorylation was the result of decrease

in the expressions of PKC and MARCKS. It is well known that the external stimuli to the cells can cause the level of *sn*-1,2-diacylglycerols (DAG) to increase; DAG then functions as a second messenger by binding to and activating protein kinase C. We then turned to measure the cellular concentrations of DAG during serum stimulation. The young and old IMR-90 cells were labeled with ^{14}C-glycerol for 24 hours to reach isotopic equilibrium during 36-hr period of serum deprivation. The intracellular DAG was isolated and analyzed by TLC. After serum stimulation for 1 hour, DAG level increased 2-3 fold relative to the control in the young cells, whereas there was only 1.5 fold increase of DAG level in the old cells (Table 1). After 2 hr of serum stimulation, the levels of DAG became similar in young and old cells. Thus, the transient increase of DAG generation afer serum stimulation was more prominent in young IMR-90 fibroblast, suggesting that the mechanism mediated DAG release

Table 1. DAG production in young and old IMR-90 fibroblasts in response to serum stimulation

Serum addition (hr)	% DAG	% of control
	Young cells	
0	0.83 ± 0.08	100
1	2.22 ± 0.25	267
2	1.30 ± 0.10	150
	Old cells	
0	0.90 ± 0.03	100
1	1.40 ± 0.08	150
2	1.35 ± 0.10	140

IMR-90 fibroblasts at PDL 22 (young) and 60 (old) serum-deprived and equlibrated with [U-^{14}C]glycerol were either untreated or stimulated with fetal bovine serum (10%) for different times, after which DAG levels were determined, normalizing to total ^{14}C-labeled lipid. Results are the mean ± SD of three independent experiments.

during serum stimulation also become less efficient during cellular senescence. This result is well correlated with the observation that the extent of MARCKS phosphorylation upon serum stimulation was much lower in old cells.

DNA synthesis in young and old fibroblasts in response to serum stimulation.

To further confirm whether the change of DAG generation is also correlated with the mitogenic response of IMR-90 fibroblasts. DNA synthesis was measured in the young and old cells that were serum-deprived for 48 hr followed by serum stimulation. After the addition of serum for 18 hr, DNA synthesis was increased approximate 25-fold in young cells and 10-fold in old cells (Table 2). Clearly, the decrease of DNA synthesis in response to growth stimulation is associated with old IMR-90 fibroblasts.

DISCUSSION

In this report, we presented the evidence that PKC activation at the early response to serum stimulation declined during cellular senescence of IMR-90 fibroblasts and such an alteration was relevant to the lower level of DAG generation in the old IMR-90 fibroblasts.

Table 2. DNA synthesis in young and old IMR-90 fibroblasts in response to serum stimulation

Serum addition (hr)	Young cells	Old cells
	(cpm/ 35 mm dish)	
0	550 ± 50	370 ± 17
8	3,280 ± 120	580 ± 89
18	12,350 ± 910	4,030 ± 360
22	15,870 ± 1,060	4,220 ± 420

IMR-90 fibroblasts at PDL 22 (young) and 60 (old) were serum-deprived for 48 hr in Dulbecco's medium, followed by stimulation with fetal bovine serum (10%). Cells were in vivo labeled with [^{3}H] thymidine at various time points for DNA synthesis. Results are the mean ± SD of three independent experiments.

Given the fact that PKC functions as a critical component of the signal transduction pathways that cells utilize to recognize and respond to a variety of extracellular agents, our results suggest that the impairment of cellular growth pathway mediated by PKC activation may also contribute to the loss of growth-responsiveness during senescence of IMR-90 fibroblasts.

Our Western blot analysis provided evidence that the amount of PKC protein did not show significant difference between the young and old IMR-90 fibroblasts. We also performed histone PKC activity assay with the cell extracts used for Western blot analysis, and reached similar conclusion (data not shown). The translocation of PKC protein and the phosphorylation of MARCKS protein after TPA induction were both well detected in the yound and old cells, indicating no inherent age-assoicated functional defect in PKC and MARCKS proteins. The difference in the magnitude of DAG production after serum stimulation between young and old IMR-90 fibroblasts well matched the degree of the decrease of MARCKS phosphorylation in the old IMR-90 fibroblasts. As cellular senescence is characterized by the loss of the growth response, these data may imply that the level of DAG generation accompanied with PKC activation are the important element in determining the early mitogenic responsiveness in human IMR-90 fibroblasts.

Our results raised the question of whether the difference in DAG generation upon serum stimulation between young and old fibroblasts is due to the changes occurring during lipolysis or lipid distribution. The early increase in the amount of DAG following serum stimulation is mainly mediated by PLC (phospholipase C). It has been demonstrated that PLCγ1 activity is decreased in IMR-90 cells during senescence in response growth stimulation (19). PLCγ1 is known to be responsible for DAG generation from phosphatidylinositol-4,5-bisphosphate (PIP_2) of the plasma membrane (20). Together with our data, it would be very likely that the mitogenic pathway via PLCγ1 that linked to PKC activation became altered during senescence of IMR-90 fibroblasts. Consistent with this notion, study in growth-stimulated murine T lymphocytes has also shown that PKC translocation, levels of Ca^{+2} and inositol triphosphate appeared to be reduced in the the cells isolated from the older donor (21).

The reduced expression of cell cycle dependent genes such as c-fos (5), PCNA, thymidylate synthetase, ribonucleotide reductase (22), thymidine kinase (23, 24), cyclin A and activities of cdc2 , cdk2 kinases (7, 8) is now considered to play the major role in the diminishment of the cell cycle progression during cellular senescence. The constant maintenance of Rb protein in unphosphorylated form (25) in the senescent fibroblasts has also been shown to be responsible for the defect in proliferation at the onset of senescence. Taken together, senescent phenotype is a result of alterations in an array of biochemical events including the decline of DAG generation and PKC activation during the early response to growth stimulation.

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