Identification of a Defect in the Phospholipase D/Diacylglycerol Pathway in Cellular Senescence*

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Normal cells become senescent in culture after a limited number of population doublings becoming unable to respond to mitogens. This raises the possibility of defects in mitogenic signaling pathways in cellular senescence. In contrast to young human diploid fibroblasts (HDF), their senescent counterparts failed to undergo protein kinase C translocation in response to serum stimulation. On the other hand, phorbol 12-myristate 13-acetate was equally active in inducing protein kinase C translocation in young and senescent HDF. This suggested a defect in generation of the endogenous activator of protein kinase C, diacylglycerol. Stimulation of young HDF with serum resulted in 3-4-fold generation of diacylglycerol (DAG). In contrast, senescent cells displayed insignificant DAG formation in response to serum. The mechanism of DAG generation was investigated next. In young HDF, serum induced a 5-fold activation of the phospholipase D (PLD) pathway as measured by the incorporation of exogenous ethanol into phosphatidylethanol, which is a measure of the transphosphatidylethanolation reaction of PLD. In contrast, PLD in senescent cells was not activated by serum. Since senescent cells demonstrate significant elevations in the level of endogenous ceramide, the impact of ceramide on the PLD/DAG pathway was also investigated. A soluble analog of ceramide, C₁₆-ceramide, was found to inhibit serum-stimulated DAG accumulation and PLD activation in young cells. These data demonstrate for the first time a defect in PLD activation in cellular senescence and suggest that ceramide may be responsible for the inhibition of this pathway.

Normal diploid cells undergo cellular senescence in vitro after a limited number of population doublings (1, 2). Senescent cells continue to function metabolically but will not respond to mitogens, i.e. they cannot replicate or undergo DNA synthesis. This phenomenon is related to the aging of organisms in that 1) human diploid fibroblasts (HDF)† taken from younger individuals can undergo more population doublings than those taken from older individuals (3), and 2) cells taken from individuals of a species with a longer potential life span will replicate a greater number of times than those taken from a species with a shorter potential life span (1, 2).

Senescence appears to be a predetermined and dominant process (4, 5). This is supported by experiments where young and senescent fibroblasts were fused and the resultant heterokaryons were unable to undergo DNA synthesis (6). The mechanism by which senescent cells fail to respond to mitogenic stimuli remains, however, poorly determined. Senescent cells in culture are known to have normal numbers of receptors for growth factors, and these receptors appear to have normal binding affinity (7). However, for the most part, post-receptor signal transduction pathways in cell senescence have not been studied.

The AP-1 transcription factor (composed of a heterodimer of c-Fos and c-Jun) is required for cell replication (8). The activation of c-Fos and AP-1 has been shown to be defective in senescent cells (9, 10). AP-1 serves as a downstream target for PKC, and production of this transcription factor requires activation of PKC in response to many, but not all, growth factors (11). PKC is a family of closely related isozymes (12) known to play central roles in mitogenic signal transduction. Activation of PKC requires the generation of endogenous DAG (13), through the action of PI-specific phospholipase C resulting in early and transient DAG formation or the action of a PC-PLD followed by PA phosphohydrolase resulting in delayed and more sustained DAG production (12, 14).

In this study, we examined the possibility that defects in the DAG/PKC pathway may underlie the mitogenic defect in cell senescence. We provide evidence for a defect in PLD activation in senescent cells in response to serum stimulation. This defect results in obliteration of DAG production and PKC translocation, accounting for the failure to induce AP-1. These studies demonstrate for the first time an early defect in cellular senescence, in an important signaling pathway central to mitogenesis. We also investigated a possible connection between the ceramide pathway and the PLD/DAG pathway. In other studies we have shown a significant elevation in ceramide levels in senescent HDF.‡ Here, we show that exogenous ceramide applied to young HDF inhibits PLD activity and DAG accumulation. We propose that senescent cells have a prominent defect in PLD, resulting in no DAG production and no PKC activation. We also propose that the elevation in endogenous ceramide in senescent cells inhibits proliferation by inhibiting PLD activation.

EXPERIMENTAL PROCEDURES

Materials—Normal HDF (WI-38 human fetal lung, AG06814E) were obtained from the NIA, National Institutes of Health. d-Erythro-C₁₆-ceramide was synthesized as described (15). Radiochemicals were purchased from New England Nuclear.

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The abbreviations used are: HDF, human diploid fibroblast(s); PLD, phospholipase D; PC, phosphatidylcholine; PE, phosphatidylethanol; PI, phosphatidylinositol; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; DAG, sn-1,2-diacylglycerol; PA, phosphatidic acid; DMEM, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's phosphate-buffered saline.

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chased from DuPont NEN. Solvents were from Mallinckrodt (analytical grade). Fetal bovine serum was from Life Technologies, Inc. Cells were grown in high glucose DMEM from Whittaker Biological Supply. β-Oc-tylglycophoranoside was from Calbiochem. Dioleoylphosphatidylglyc- erol, phosphatidylethanolamid, and dioleoylglycerol were from Avanti Polar Lipids, Inc. ATP was from Pharmacia Biotech, Inc. All other reagents were analytical grade from Sigma.

### Cell Culture—WI-38 HDF were maintained in 10-cm plates in DMEM containing 10% fetal bovine serum and subcultured at a 1:5 ratio. Young cells are defined as having more than 35 population doublings remaining, whereas senescent cells are unable to undergo population doubling for up to 3 weeks after seeding, i.e. they enter a terminal, non-replicative but viable stage. Senescent cells incorporated less than 2% the amount of [3H]thymidine incorporated by young cells in response to serum in a 48-h period. Cells were seeded at 4 x 10^5 (young) or 8 x 10^5 (senescent) cells/well in 12-well plates for DAG measurements and PLD assays. Cultures were seeded at 8 x 10^5 (young) or 1.6 x 10^6 (senescent) cells in 10-cm plates for AP-1 and PKC analysis.

### PKC Translocation—WI-38 HDF cells were treated with either 100 nM PMA or 20% serum for 20 min at 37° C. Cells were placed on ice, and the medium was aspirated. The cells were washed twice with phosphate-buffered saline, scraped into 0.5 ml prechogenization buffer, sonicated two times for 30 s, and centrifuged in a T1L-100.3 rotor at 40,000 rpm for 40 min. The supernatant was removed and diluted 1:1 with 2 x sample buffer. The membrane pellet was reuspended in an equal volume of homogenizing buffer, mixed 1:1 with 2 x sample buffer. Samples were boiled and analyzed by Western blot analysis. Protein was estimated by Bradford analysis (16).

### Western Blots—Samples (50 μg of protein from senescent cells or 100 μg of protein from young cells) were run on 10% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose at 4° C overnight. Blots were then washed with 5% nonfat dry milk in DPBS for 1 h at 20° C to block nonspecific binding sites. Blots were incubated with PKC isoenzyme-specific antisera at a dilution of 1:500 at 1:1000 with or without competing peptide (20-40 μg) for 2 h at 20° C. These isoenzyme-specific antisera have been characterized previously and have high affinity for their respective antigens (17). The blots were washed three times with 5% nonfat dry milk in DPBS for 15 min at 20° C and then once with 1 x DPBS. The blots were incubated with secondary antiserum (goat anti-rabbit linked to horseradish peroxidase) for 30 min at 20° C, then washed three times with 1 x DPBS. Blots were developed using ECL under conditions described by the manufacturer (Ameraham Corp.).

### Lipid Analysis—Incubations were stopped by aspiration of medium and addition of 1 ml methanol on ice. Cells were scraped, transferred to a glass tube, and extracted (18). Total lipid phosphate was quantitated by the procedure of Rouser et al. (19). Lipids extracted from cells labeled with [3H]myristic acid were analyzed by thin layer chromatography using solvent A:chloroform:methanol:acetic acid:H2O (50:25:8:4) or solvent B:upper phase of a mixture of ethyl ch1oroform:acetone:methanol:acetic acidwater (80:50:20:100) (20). DAG was quantitated using DAG kinase by a transphosphatidylation reaction, and it has been well established that low molecular mass primary alcohols such as ethanolic ethanol (PEt) produced is metabolically stable and can be easily separated from the natural lipids.

### RESULTS

A key defect in senescent cells appears to be the inability to turn on the c-fos protooncogene and activate AP-1 (9, 10) in response to mitogenic stimuli such as serum. We confirmed that there is a defect in AP-1 activation in senescence using a gel retardation assay (data not shown). Since PKC is a proximal effector of this pathway (8), we next studied the cellular PKC response to serum stimulation in low passage and in senescence HDF. Initially we performed PKC isoenzyme analysis by Western blots of extracts from these cells. WI-38 HDF contained a significant amount of PKC-α, trace amounts of PKC-δ, and undetectable amounts of PKC-β1, -β2, -γ, -ε, or -ζ. Moreover, there was no difference in isoenzymes distribution or abundance between young and senescent cells (data not shown). Therefore, in all subsequent studies we concentrated on PKC-α. Cells were made quiescent by incubating for 24 h in 0.5% serum 5 days after the last feeding. Quiescent cells were treated with 10% serum for 20 min. Cell extracts were analyzed for PKC-α by Western blot. Fig. 1 shows that, unlike young HDF, senescent cells were unable to translocate PKC in response to serum, whereas senescent cells appeared to contain equal or slightly higher levels of PKC-α than young cells. This supports the results of De Tata et al. (22), who found defective translocation of PKC activity in cell senescence. In order to determine whether the defect lies in PKC or in endogenous signaling pathways resulting in activation of PKC, we next evaluated the effects of PMA on PKC translocation. The addition of PMA caused PKC translocation in both young and senescent cells (Fig. 1). Therefore, senescent cells are competent to translocate PKC and the defect in senescence appears to reside in the mitogenic signal transduction pathways upstream of PKC.

We sought to determine whether serum stimulation of senescent cells elicits the DAG response necessary for PKC activation and translocation. To do this, we first established the normal profile of the DAG signal in young quiescent HDF. Fig. 2A shows the DAG response in cells stimulated with 10% serum. DAG levels did not change in the first 2 min of serum stimulation. By 10 min, DAG levels increased from 2.1 ± 0.1 pmol/nmol of phospholipid (base-line level) to 7.2 ± 1.7. Senescent cells (Fig. 2B) were found to contain higher basal levels of DAG (3.6 ± 0.72 pmol/nmol of phospholipid) but produced only a 30% increase. This contrasted with the 340% increase in DAG in young cells in response to serum. Therefore, the DAG signal is defective in senescent cells.

Studies of the DAG response have shown DAG to be produced primarily by PI-phospholipase C and/or by PC-PLD (12). We wanted to determine which mechanism was involved in the fibroblast mitogenic response. We did not see an early wave of DAG production (within 2 min; Fig. 2A, inset) that is characteristic of PI turnover. In addition, when we labeled cells over-night with [3H]arachidonic acid and stimulated with serum, we did not detect significant hydrolysis of the PI-labeled pool at early or later times (data not shown). These results are consistent with minimal activation of PI-phospholipase C. Moreover, calcium signaling, a product of PI turnover, appears to be normal in senescent cells (23). Since we observed high and delayed DAG levels and since PLD activity is widely believed to be responsible for the higher and more prolonged accumulation of DAG, we next examined the PLD pathway. PLD catalyzes a transphosphatidylation reaction, and it has been well established that low molecular mass primary alcohols such as ethanol can compete with water in hydrolysis (14). The phosphati-dylethanol (PEt) produced is metabolically stable and can be easily separated from the natural lipids.
Fig. 2. Defect in serum-stimulated DAG production in senescent cells.

DAG levels in response to serum stimulation in young (A) or senescent (B) WI-38 HDF cells were grown in 12-well plates. Cells were made quiescent 5 days after last feeding using 0.5% serum in DMEM. After 24 h, 10% serum was added for the indicated times. The medium was aspirated, 1 ml of methanol was added, and cells were placed on ice. Cells were scraped using an additional 1 ml of methanol, and lipids were extracted (18). Lipids were analyzed for total lipid phosphate (19) and DAG content (21). Data represent the mean ± S.E. of four (senescent) separate experiments. Inset in A is for short term (seconds) serum stimulation of young HDF.

Fig. 3 shows that serum stimulated the PLD pathway in young HDF, with PEt increasing from a basal level of 2000 ± 297 to 11,300 ± 2150 dpm at 20 min. That PEt accumulation began leveling off by 2 min, preceding DAG production, further supports this mechanism. In contrast, senescent cells showed almost no response to serum, with PEt levels increasing only from 1500 ± 465 to 3200 ± 715 dpm at 20 min. Therefore, the defect in DAG production in response to serum stimulation in cellular senescence could be accounted for by the absence of PLD activity.

In order to determine if PLD was defective in senescence or if a signal from serum stimulation to PLD activation was missing, we elected to evaluate PLD activation by a different mechanism. Conricode et al. (24) found that PLD can be activated by PMA. We therefore used PMA to evaluate the responsiveness of PLD to this mediator. PMA was found to be an effective agonist in young cells (Fig. 4), and the effect of PMA was additive with serum in young cells. PMA also slightly stimulated PLD activity in senescent cells, even though these cells were unresponsive to serum. The degree of PMA-induced activation, however, was less than 2-fold over base-line level compared to over 4-fold in young cells. This suggested that PLD may be functional in senescent cells but that its activity may be inhibited.

Previous studies indicate that ceramide is a growth regulatory lipid (25, 26). In other studies we have found that ceramide is elevated 4-fold in senescent cells to 14.6 pmol of ceramide/nmol of total phospholipid and that ceramide can induce a senescent phenotype when added to young cells. Since DAG generation and PLD activity are defective in senescence, we examined the possible role of ceramide. To study this we used a soluble analog of ceramide (N-hexanoylsphingosine or C16- ceramide). C16-ceramide was added to young quiescent HDF for the indicated times. Cells were then stimulated with serum and analyzed for DAG production as described under "Experimental Procedures." Treatment with C16-ceramide (10 μM at 3, 24, and 48 h prior to stimulation) nearly completely inhibited the cells' ability to produce DAG in response to serum (Fig. 5A).

Untreated cells showed an 82 ± 28% increase in DAG compared to a 2 ± 9% increase in ceramide-treated cells. Significant inhibition was also seen with 24-h treatment (not shown). We also looked at ceramide's effects on PLD activity. As shown in Fig. 5B, cells treated with C16-ceramide were unable to activate PLD in response to serum or PMA. Ceramide reduced the ability of cells to respond to serum from a 73 ± 4% increase to 10 ± 6% and reduced the PMA response from 547 ± 15% to 76 ± 5%.

These data show that ceramide can inhibit the production of DAG through PLD in response to mitogenic signals.

DISCUSSION

These studies demonstrate that senescent HDF, unlike their young counterparts, cannot respond to serum-induced activation of PLD. This results in their inability to generate a sustained diacylglycerol signal, which accounts for their inability to translocate PKC and to transcribe c-fos and activate AP-1. This does not appear to be due to an intrinsic defect in PKC since PKC is able to translocate upon PMA stimulation. This inability to activate PLD appears to be a consequence of the elevated levels of ceramide in senescent cells.

These findings have several important implications. First, they locate a defect in a central signal transduction pathway in...
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Togenic pathway involving PC hydrolysis by PLD with PA generation in young fibroblasts. A, was added in triplicate. Data are representative of three separate experiments performed for 5 min with 10% serum or 100 nM PMA in 2 μl of MeSO in the presence of 2% ethanol. Data represent the mean ± range of single determinations from two separate experiments.

Fig. 4. Regulation of PLD by PMA in young and senescent HDF. Samples were treated as in Fig. 3, except that cells were stimulated for 5 min with 10% serum or 100 nM PMA in 2 μl of MeSO in the presence of 2% ethanol. Data represent the mean ± range of single determinations from two separate experiments.

Fig. 5. Ceramide inhibition of DAG generation and PLD activation in young fibroblasts. A, low passage cells were prepared, treated for 20 min, and analyzed as in Fig. 2, except that Ceramide was added in 1 μl of ethanol at 3, 24, and 48 h prior to serum treatment. Data represent the mean ± S.E. of two separate experiments performed in duplicate. B, samples were treated for 30 min (which accounts for the higher stimulation seen by PMA as compared to Fig. 4, where stimulation was for 5 min) and analyzed as in Fig. 3, except that Ceramide was added in 1 μl of ethanol at the indicated time prior to serum treatment. Data are representative of three separate experiments performed in triplicate.

cellular senescence. This pathway is a well characterized mitogenic pathway involving PC hydrolysis by PLD with PA generation (which may act as a potent mitogen, reviewed in Ref. 27). This is followed by DAG generation via a PA phosphohydrolyase. DAG then activates PKC, leading to increased c-fos transcription and AP-1 activation. The distal arm of this pathway, i.e., c-fos transcription and AP-1 activation, is known to be unresponsive in senescent HDF (9, 10), and we are now able to trace the defect to an upstream target, i.e., PLD. Consequently, DAG is not produced and PKC cannot be activated. The mechanism by which PLD in turn is activated is not completely understood. PLD has been shown to be activated by PMA in a PKC-dependent manner (24). Recently PLD has also been shown to require a cytosolic factor, namely ADP-ribosylation factor for activation (28, 29). In senescence, PLD activity is greatly diminished but is still partially responsive to PMA, indicating that PLD is present and that intracellular messages (such as ADP-ribosylation factor or a yet undescribed factor) could be missing or altered. That PKC is missing is less likely, inasmuch as we and others show no change in PKC amounts or ability to translocate in response to PMA in young and senescent cells.

Another mechanism by which PLD could be inactivated in senescence may involve the presence of a "dominant" inhibitor of PLD. A dominant factor has been implicated in inducing cellular senescence by several investigators (4, 30). Such an inhibitor could be ceramide or a target of ceramide that inhibits PLD. Therefore, a second and significant implication pertains to the role of ceramide in inhibiting this mitogenic pathway. Evidence for this role is now emerging. First, senescent HDF contain significantly elevated levels of ceramide when compared with young HDF (4-fold). Second, the addition of exogenous ceramide results in inhibition of PLD activation and DAG generation by serum. While this manuscript was in preparation, Gomez-Munoz et al. (31) showed that ceramide inhibits PLD activation in NIH 3T3 mouse fibroblasts, thus further supporting a role for ceramide in modulating PLD. Third, ceramide is a potent inhibitor of growth (25) and DNA synthesis; activities closely associated with PLD and DAG (14, 32). Therefore, we can now begin to delineate a target for ceramide action, i.e., PLD, and this pathway may constitute a critical component in the determination of cell senescence.

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