Mapping mechanisms and charting the time course of premature cell senescence and apoptosis: lysosomal dysfunction and ganglioside accumulation in endothelial cells

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Patschan S, Chen J, Gealekman O, Krupincza K, Wang M, Shu L, Shayman JA, Goligorsky MS. Mapping mechanisms and charting the time course of premature cell senescence and apoptosis: lysosomal dysfunction and ganglioside accumulation in endothelial cells Am J Physiol Renal Physiol 294: F100–F109, 2008. First published October 10, 2007; doi:10.1152/ajprenal.00261.2007.—Endothelial cells subjected to glycated collagen I develop premature senescence within 3–5 days, as revealed by increased senescence-associated β-galactosidase activity, decreased proliferation, and an increase in cell size. Here, we analyzed the time course and possible mechanisms of this process. Lysosomal integrity studies revealed a rapid collapse of pH gradient and lysosomal permeabilization, detectable after 30 min, and preceded by the increased production of reactive oxygen species. Measurement of mitochondrial membrane potential after application of glycated collagen demonstrated that depolarization was delayed by 4 h compared with changes in lysosomal pH and permeability. Based on the above findings of lysosomal permeabilization, we hypothesized that the reduced activity of senescence-associated β-galactosidase could be responsible for the cellular accumulation of gangliosides, previously shown to induce cell senescence. After 5 days of exposure to glycated collagen, there was an increase in the levels of gangliosides GM3, GD1b, and GT1b, coincident with development of cell senescence. Treatment of endothelial cells with D-galactosidase could prevent and reverse the occurrence of premature senescence. Advanced glycation end product (AGE)-modified long-lived extracellular matrix proteins (glycated collagen I; GC), a microenvironment emulating the diabetic milieu. Under these conditions we observed a deceleration of proliferation, an increase in cell size, enhanced expression of p16, p21 and p53, and increased number of cells expressing senescence-associated β-galactosidase (SA-β-gal), all suggestive of developing cell senescence. Advanced glycation end product (AGE) formation is considered to be one of the major etiologic factors in the pathophysiology of aging (35). AGE formation is enhanced by hyperglycemia and has been linked to many complications of diabetes (21, 38). AGEs can modify inflammatory events by stimulating production of reactive oxygen species, have a detrimental effects on signal transduction (28), enhance the expression of proapoptotic genes, and stimulate apoptosis in fibroblasts through cytoplastic and mitochondrial pathways (2).

Despite the fact that primary cultures of human umbilical vein endothelial cells (HUVEC) used in our previous experiments were in passages 3–6, the population of senescent cells doubled within 3–5 days of culture in GC lattices (8, 9). However, telomerase activity and length of telomeres showed significant reduction, suggesting that the pathogenesis of stress-induced premature senescence (SIPS) is distinct from that of replicative senescence. Endothelial cell senescence was associated with increased calcium-stimulated synthesis of nitric oxide (NO), despite an increase in the expression of endothelial nitric oxide synthase and increased abundance of 3-nitrotyrosine-modified proteins. In contrast to the replicative senescence, the premature senescence of HUVEC was reversible. Scavenging peroxynitrite with ebselen, supplementing cells with an intermediate in NO synthesis, N^-hydroxy-L-arginine (NOHA), or the addition of a cell-permeable SOD mimetic, Mn-TBAP, prevented and reversed premature senescence. Analysis of senescence-associated β-gal, p53, and p16INKn4 staining of aortas obtained from young Zucker diabetic fat rats confirmed the occurrence of premature senescence of endothelial cells in vivo. Thus premature senescence of endothelial cells appears to be present in an animal model of metabolic syndrome and type 2 diabetes mellitus (5, 7, 14).

The mechanistic basis of SIPS in endothelial cells has not been determined. Although this phenomenon displays features distinct from the replicative senescence, other features of...
replicative senescence [e.g., cell staining with SA-β-gal (13), cell cycle arrest and induction of p16 and p53, increase in cell size] are recapitulated (9). In both cases, the detection of cell senescence is based on the SA-β-gal assay, the molecular basis of which is obscure. β-Gal (EC 3.2.23) is a lysosomal hydrolase cleaving β-linked terminal galactosyl residues from gangliosides, glycoproteins, and glycosaminoglycans. Therefore, we sought to investigate the role of lysosomes and gangliosides in premature senescence and programmed cell death.

It has recently been demonstrated that p53 destabilizes and permeabilizes lysosomes, effects that occur before p53 induction of mitochondrial permeability transition and commitment to apoptosis (41). Our previous observations on the early induction of p53 in the course of premature senescence led us to focus our investigation on lysosomal integrity and mitochondrial membrane potential (ΔΨm) in the course of developing premature senescence.

MATERIALS AND METHODS

Reagents. Acidine orange, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetrachloro-
benzimidazocarbocyanine iodide (JC-1), dihydroethidium (DHE) (Hoechst 33342, dextran Texas red (10,000 MW, amine fixable), and an Imagen Green C12-FDG laZ Gene Expression Kit were obtained from Invitrogen/Molecular Probes (Eugene, OR). Collagenase type II was purchased from GIBCO (Grand Island, NY). A CaspaTag Caspase Activity Kit was from InterGen (Purchase, NY). The following antibodies were used: Lamp-1 (H4A3, Developmental Studies Hybridoma Bank), FITC-conjugated goat anti-mouse IgG (Jackson Immunoresearch), and chloroquine diphosphate salt (MP Biomedicals).

Cell culture. HUVEC and EGM-2 growth medium were purchased from Clonetics (Walkersville, MD). HUVEC were used between passages 3 and 6 and maintained at 37°C in a 95% air-5% CO2 humidified atmosphere. For experiments, HUVEC were grown to 80% confluence. ECV304 cells, a vascular endothelial cell line, were cultured and analyzed for glycosphingolipids as previously reported (31). Primary cultures (31) of mouse aortic endothelial cells (MAEC) from α-galactosidase A null mice, a model of Fabry disease, were isolated and cultured as previously described (32, 33).

Preparation of GC- and collagen-coated dishes. GC and native collagen (NC) were prepared as previously described (8). In brief, 500 µg/ml collagen (Vitrogen; Cohesion, Palo Alto, CA) was prepared in 2X PBS (pH 7.4) containing 500 mM n-glucose. The solution was sterilized by using a 0.22-µm filter and incubated at 37°C for 6 wk. The pH value of the solution was monitored weekly and adjusted as necessary to pH 7.4. The solution was then dialyzed against 2X PBS, pH 7.4, twice overnight. The protein concentration was calculated from the absorbance at 280 nm. GC prepared by this protocol contains 21 µmol CML and 90 µmol fructosamine/g of collagen. In contrast, the NC used in our experiments contains 4 µmol CML and 11 µmol fructosamine/g of collagen. To better emulate the in vivo pathological microenvironment found in diabetes, GC was additionally used in a 1:3 dilution with NC (1:3 GC) to achieve a clinically relevant AGE concentration with CML and fructosamine as gauges (8). Glycated or native collagen was directly added to the cell culture medium at a concentration of 100 µg/ml. In control groups 1X PBS was added to the cell culture medium.

Senescence associated β-galactosidase activity. We used the fluorogenic substrate C12-FDG to measure β-galactosidase activity by flow cytometry (24). This compound is a membrane-permeable, nonfluorescent substrate of β-galactosidase, which after hydrolysis of the galactosyl residues emits green fluorescence and remains confined within the cell. High levels of acid lysosomal β-galactosidase present in HUVEC of all replicative ages would mask the detection of senescence-dependent activity in live cells. Therefore, Kurz et al. (17) used chloroquine, a weak base which concentrates in the lysosomes, to raise their pH to ~6. At pH 6 the specific activity of the enzyme is very low, whereas senescent cells show positive staining. Parallel cultures of HUVEC were treated with NC, GC, or a mixture of both (1:3 GC) at a final concentration of 100 µg/ml for up to 5 days. Cells were treated with 300 µM chloroquine for 2 h to induce lysosomal alkalization (20). C12-FDG (33 µM) was then added to the pretreatment medium, and the incubation was continued for 4 h. At the end of the incubation, cells were treated with collagenase (2 mg/ml) for 2 min at 37°C. Cells were washed with ice-cold PBS, trypsinized, and resuspended in ice-cold PBS with 2% FBS. After centrifugation at 500 g, cells were immediately analyzed using a FACSScan flow cytometer (Becton Dickinson). The C12-fluorescein signal was measured on the FL-1 detector, and β-galactosidase activity was estimated using the median fluorescence intensity (MFI) of the population. At least three independent experiments were performed.

Alternatively, SA β-gal expressed in HUVEC was analyzed according to the protocol described by Dimri et al. (13). In brief, subconfluent HUVEC were stained in the working buffer (pH 6) at 37°C overnight. Stained cells were viewed under an inverted microscope at ×200.

Detection of apoptosis. A CaspaTag Caspase Activity Kit (InterGen) was used to detect active caspases in living cells through the use of a carboxyfluorescein-labeled caspase inhibitor, which irreversibly binds to active caspases. Fluorochrome-labeled inhibitors of caspases detecting early events associated with caspases activation are specific and convenient markers of pro-apoptotic cells (30). FAM-VAD-FMK, a carboxyfluorescein (FAM) derivative of a potent general inhibitor of caspase activity, benzylxoxazolyl valanalys aspartic acid fluoromethyl ketone (zVAD-FMK), was employed. FAM-VAD-FMK enters the cells and irreversibly binds to activated caspases (caspase-1, -2, -3, -4, -5, -6, -7, -8 and -9). The staining was performed according to the manufacturer’s specifications. Cells were finally resuspended in PBS containing 1 µg/ml propidium iodide (PI; Molecular Probes, Eugene, OR). Cell green (FAM-VAD-FMK) and red (PI) fluorescence was measured by FACSAn. In addition, quantification of apoptotic cells was performed using Hoechst, and annexin-V.

Detection of reactive oxygen species. Endogenous formation of superoxide was monitored by the oxidation of dihydroethidium (DHE). Cells were incubated with DHE (10 µM) in Krebs-HEPES buffer (pH 7.4) for 30 min at 37°C. The cells were illuminated under an inverted fluorescent microscope at the wavelength of 480 nm; emission was detected at 610 nm. For quantitative analysis, cells were illuminated with 30-ms light pulses at 120-s intervals using an automatic shutter (Lambda 10–2, Sutter Instruments) interfaced to Image-1-Fluor software (Universal Imaging). Because DHE is photoactivatable, the duration of illumination was kept to a minimum. Images were collected using a Nikon TE-2000U microscope equipped with a Spot Insight camera (Diagnostic Instruments) with an appropriate dichroic mirror, stored, and analyzed using Image-1 software.

Mitochondrial generation of superoxide was detected using MitoSOX. HUVEC were cultured in 48-well plates and studied at confluence. For analysis of mitochondrial superoxide production, cells were washed with warm Hank’s balanced salt solution with calcium and magnesium (HBSS/CA/Mg) and incubated in 200 µl of MitoSOX working solution (5 µM in HBSS/CA/Mg) for 10 min at 37°C, protected from light. After recording of the baseline fluorescence intensity, cells were challenged with NC or GC (50 µg/ml), and the fluorescence intensity (510/580 nm) was monitored using a fluorescence plate reader (Mithras LB940, Berthold Technologies, Bad Wildbad, Germany) and fluorescence microscopy.
Monitoring lysosomal pH. HUVEC were exposed to the lysosomotropic weak base acridine orange. Due to proton trapping, this vital dye accumulates mainly in the acidic vacuolar apparatus, preferentially in lysosomes. Acridine orange is also a metachromatic fluorophore (3). HUVEC were cultured in 48-well cell culture clusters (Corning) and incubated with NC or GC (100 μg/ml) for various periods of time. HUVEC were loaded by incubation with 1 μM acridine orange (Molecular Probes) for 15 min at 37°C. The unbound dye was removed by washing with PBS. Fluorescence intensity was measured using a Berthold fluorescence plate reader (Mithras LB 940) using the following filter settings: excitation at 485 ± 20 nm and emission at 535 ± 20 nm. The background fluorescence from dye-free incubations was subtracted from data reported. Chloroquine (300 μM) was used as a positive control. For the single-cell observations, cells were stained according to above protocol and imaged using a Nikon TE2000U microscope equipped with a Spot Insight camera (Diagnostic Instruments).

Measurements of lysosomal permeability. HUVEC (~10⁴/well) were seeded on circular microscope coverslips (Fisher), precoated with fibronectin (final concentration 10 μg/ml), UV light-sterilized, and incubated with cell culture medium (control), NC, a mixture of NC and GC (1:3 GC), and GC.

**Fig. 1.** Time course of developing premature senescence. Representative flow cytometric histograms of companion cultures of human umbilical vein endothelial cells (HUVEC) treated with native collagen (NC; green), glycated collagen (GC; red), or a mixture of both (1:3 GC; blue) at a final concentration of 100 μg/ml for 1–5 days are shown. The dashed line represents unstained cells.

**Fig. 2.** Time course of caspase activation. A: flow cytometric analysis of HUVEC stained with FAM-VAD-FMK, a carboxyfluorescein (FAM) derivative of a potent general inhibitor of caspase activity, benzoyloxycarbonyl valylaspartic acid fluoromethyl ketone (zVAD-FMK), and propidium iodide (PI). During the final 1 h of culture, cells were labeled with 10 μM FAM-VAD-FMK, rinsed twice with PBS, and resuspended in PBS containing 1 μg/ml PI. Displayed are dot blots of HUVEC incubated for 16 h with cell culture medium (control), NC, a mixture of NC and GC (1:3 GC), and GC. B: proportion of caspase-positive and PI-negative HUVEC showing a maximal rate of apoptosis at 16 h incubation with GC. *P < 0.05 vs. NC.
mitochondria. HUVEC were cultured in 48-well plates and treated for 24 h (26).

and placed into 24-well tissue culture plates (Falcon). At 80% confluence, cells were incubated with 0.5 mg/ml amine-fixable Texas red dextran (MW 10,000) for 24 h followed by a washout period for 120 min in 120-s intervals using an automatic shutter. After 10-min baseline recording, GC or NC was added and the results were continually recorded for 2.5 h. Note that in HUVEC stimulated by GC, a rapid elevation of superoxide production was documented.

Assessment of Ψm. Detection of Ψm was performed usingJC-1, a cationic dye, which exhibits a potential-dependent accumulation in mitochondria. HUVEC were cultured in 48-well plates and treated for the indicated periods of time with NC, GC, or 1:3 GC (100 μg/ml). Cells were incubated with JC-1, at a final concentration of 5 μg/ml, for 15 min at 37°C and then washed twice with PBS at room temperature. A fluorescence plate reader (Bertold Mithras LB 940) was used for detection of JC-1 aggregates with excitation/emission filters of 530 ± 40/620 ± 40 and 485 ± 20/528 ± 20 nm for detection of monomers. Data were analyzed using a red/green fluorescence ratio (JC-1 aggregate/monomer), and fluorescence ratios were normalized to values obtained in experiments with NC.

Fig. 3. Superoxide production in HUVEC exposed to GC and NC. HUVEC cultured on 35-mm glass bottom dishes (MaTek) were stained with dihydrochloromethane and placed into 24-well tissue culture plates (Falcon). At 80% confluence, cells were incubated with 0.5 mg/ml amine-fixable Texas red dextran (MW 10,000) for 24 h followed by a washout period for 120 min in 120-s intervals using an automatic shutter. After 10-min baseline recording, GC or NC was added and the results were continually recorded for 2.5 h. Note that in HUVEC stimulated by GC, a rapid elevation of superoxide production was documented.

Effects of inhibitor of ganglioside synthesis D-threo-ethylendioxy-phenyl-2-palmitoylamino-3-pyrrolidinopropanol. HUVEC were pre-treated with an inhibitor of glucosoceramide-based glycosphingolipid synthesis, including ganglioside synthesis, D-threo-ethylendioxyphenyl-2-palmitoylamino-3-pyrrolidinopropanol (D-threo-EtDOP4), 10–100 nM, or its inactive analog, D-erythro-ethylendioxyphenyl-2-palmitoylamino-3-pyrrolidinopropanol (D-erythro-EtDOP4). Cells were subjected to GC, and the proportion of apoptotic and senescent HUVEC was quantified 3 days later. SA-β-gal-stained cells were viewed under HPTLC.

Fig. 4. Analysis of mitochondrial superoxide production using MitoSOX Red. The relative fluorescence intensity (510/580) of oxidized MitoSOX in HUVEC was recorded by a fluorescence plate reader after stimulation with GC or NC. The representative fluorescence images (top) showed the oxidized MitoSOX fluorescence signal monitored at 30 min. Peroxynitrite (ONOO−) was used as a positive control. *P < 0.05 (n = 4) for control compared with NC, GC GC1:3, and ONOO−. **P < 0.05 (n = 4) compared with NC.
an inverted microscope. The percentage of SA-β-gal-positive cells was determined by counting the number of SA-β-gal-positive cells under brightfield illumination and the total number of cells in the same field under phase contrast. At least eight random fields were counted for each culture dish.

Apoptotic cells were detected using Hoechst and annexin V. The data presented were obtained by counting the number of apoptotic cells per 500 cells using fluorescence microscopy. In each experiment, at least 15–20 randomly chosen fields were examined.

Statistical analysis. All experiments were repeated at least three times. Values are given as means ± SE. ANOVA was used for multiple comparisons. P values <0.05 were considered significant.

RESULTS

Time course of premature senescence of endothelial cells: commencement at the late phase of incubation with GC. SA-β-galactosidase has been defined as a pH 6 hydrolytic activity manifested in situ when senescent cells are incubated with the chromogenic substrate X-Gal (13). Kurz et al. developed an alternative approach to measure this activity, a FACS assay that detects the hydrolysis of a fluorogenic β-gal substrate in living cells (20). Using this approach, no increase in β-gal activity after incubation of HUVEC for 24 and 48 h with either NC or GC was detected, but measurements on days 3–5 of treatment showed an increase in fluorescence intensity of cells treated with the 1:3 GC and GC compared with NC (Fig. 1).

Time course of endothelial cell apoptosis: increase at the early phase of incubation with GC. We incubated HUVEC for 6, 16, and 26 h or 2, 3, 4, and 5 days with either GC or 1:3 GC and compared the apoptotic rate of cells treated with NC or control cells. The percentage of apoptotic cells, defined as caspase-positive but PI negative (Fig. 2A), was maximal by 16 h of incubation with GC. After that, the rate of apoptosis declined (Fig. 2B). The number of PI-positive cells remained relatively constant throughout the duration of experiments. Collectively, the above data demonstrated that HUVEC exposed to GC are induced toward one of two cell fates: an early apoptotic program and delayed development of premature senescence. The time course of these events appeared to be amenable to in vitro studies.

GC induces an early oxidative stress. We next determined whether GC can induce oxidative stress, which is known to affect lysosomal function (36). Quantitative fluorescence microscopy of DHE in HUVEC treated with 50 μg/ml GC showed an increase in fluorescence intensity, which occurred within 10 min of application (Fig. 3). No increase in fluorescence intensity was detectable in cells treated with NC.

To investigate the potential involvement of mitochondrial superoxide production, experiments were conducted using HUVEC loaded with MitoSox. As shown in Fig. 4, application of NC resulted in a minor elevation of MitoSox fluorescence. In contrast, application of GC led to a dramatic and rapid increase in fluorescence in HUVEC, the magnitude of which was comparable to that of peroxynitrite, used as a positive control. These data indicate that GC-induced generation of reactive oxygen species is in part due to superoxide production by mitochondria.

Collapse of lysosomal pH gradient after treatment with GC. In resting endothelial cells, acridine orange was detectable as a red punctated fluorescence signal with minimal green cytosolic fluorescence. When cells were presented with GC, the punctated pattern of red fluorescence nearly disappeared in the span of 2 h, in parallel with the increased intensity of green fluorescence over the cytosol. The baseline fluorescence pattern remained preserved in cells treated with NC (Fig. 5A). Quantitative fluorescence microscopy of cultured endothelial cells confirmed these observations. After incubation of HUVEC loaded with 1 μM acridine orange, the intensity of green fluorescence of GC-treated cells increased compared with cells treated with NC. This effect was statistically significant for cells incubated with GC (100 μg/ml) as early as 1 h after application, and the difference persisted for at least 24 h (Fig. 5B). Cells incubated with a lower concentration of 1:3 GC showed no changes in fluorescence pattern for the first 4 h of incubation, but after 5 h the acridine orange fluorescence intensity increased significantly compared with cells incubated with NC (P < 0.05). This increase in green fluorescence intensity lasted for at least 24 h. Chloroquine, known to dissipate the lysosomal pH gradient, was used as a positive control and showed an increase in fluorescence intensity after 2 h of incubation, which persisted for at least 5 h (Fig. 5B).
Untreated control cells showed a stable fluorescence ratio, similar to HUVEC treated with NC. These data demonstrated that GC dissipates the lysosomal pH gradient.

**GC permeabilizes lysosomes early after treatment.** To further investigate the effect of GC on lysosomal integrity, we employed an additional experimental approach to study lysosomal permeability to macromolecules. The endocytosis of the fluorochrome-conjugated dextran-Texas red, its subsequent traffic through the endocytotic compartments, and accumulation in lysosomes after 20 h in a dextran-Texas red-free culture medium were used for the loading of the lysosomal compartments (4). Labeled HUVEC were treated with GC, fixed at different times postchase, and costained with antibodies against a lysosomal-associated membrane protein, Lamp-1. Colocalization of both markers, as seen in control cells, attested to the integrity of the lysosomal membrane (Fig. 6A) (26). There was no significant difference between untreated control cells and cells incubated with NC. In contrast, within 30 min of incubation with GC and, even more so, 1:3 GC, the colocalization was significantly diminished compared with cells treated with NC (NC: 95.23 ± 2.07%, GC: 69.67 ± 7.07%, 1:3 GC 61.1 ± 6.6%, P < 0.01). This decreased

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**Fig. 6.** Lysosomal permeability after incubation with glycated collagen as assessed by decreased colocalization of dextran-Texas red with Lamp-1. HUVEC were incubated for 24 h in culture medium containing dextran conjugated to Texas red (MW 10,000) followed by a 24-h chase in a dextran-free culture medium for accumulation in lysosomes, as detailed in MATERIALS AND METHODS. Cells were incubated with GC, NC/GC (1:3 GC), NC, or remained untreated (control) and studied at the indicated times. Treatment with chloroquine (300 μM) was used as a positive control. Cells were fixed, permeabilized, and stained with an antibody to Lamp-1 and a nuclear stain, Hoechst 33342. Shown is a summary of colocalization of dextran-Texas red and Lamp-1. There was no significant difference in colocalization in control cells vs. cells treated with NC. Beginning at 30 min of incubation with GC and 1:3 GC, there was a significant decrease in colocalization, indicative of a permeabilization of lysosomes, which continued for up to 24 h (*P < 0.05).
significant changes in incubation with a mixture of NC and GC (1:3 GC) showed no after 4 h incubation with GC ( \( P < 0.05 \) ) (Fig. 7). The incubation with a mixture of NC and GC (1:3 GC) showed no decrease in \( \Psi_m \) compared with cells treated with NC. Control cells showed the fluorescence ratio to be higher than that in all treatment groups (Fig. 7). At 5 and 50 \( \mu \)M, FCCP, used as a positive control, significantly decreased mitochondrial \( \Psi_m \) ( \( P < 0.01 \) ). Hence, the data demonstrated that GC triggered the collapse of \( \Psi_m \). Importantly, lysosomal leakiness predated these changes in \( \Psi_m \).

Accumulation of gangliosides in senescent endothelial cells.

Next, we addressed the possibility that the loss of lysosomal acidic pH optimum would result in the accumulation of uncleaved products, such as gangliosides (20) and asked the question of a role of gangliosides in determining cell fate after exposure to GC. Initial studies were performed to establish the technique and compare results obtained in cultured HUVEC with other endothelial cell lines, such as those obtained from Fabry-MAEC or ECV304. Data presented in Fig. 8A show that major classes of gangliosides were present in HUVEC, albeit at a lower abundance than in Fabry-MAEC, the prototypical lysosomal disorder. The repertoire of gangliosides in HUVEC was similarly represented when lipids were extracted from methanol-fixed and trypsin-released HUVEC. No significant changes in the abundance of individual gangliosides were detected after 3 days of incubation with GC. When lipid extraction was performed after 5 days of culture on GC-I, but not after 3 days, there was a consistent increase in the abundance of GM3 (C16/C18), GD1b, and GT1b compared with the cells cultured on NC-I (B).

Fig. 8. Ganglioside abundance after treatment with GC. A comparative analysis of major gangliosides in HUVEC, ECV304, and Fabry cells is shown. Data show that major classes of gangliosides were present in HUVEC, albeit at a lower abundance compared with Fabry mouse aortic endothelial cells (MAEC; A). The repertoire of gangliosides in HUVEC was similarly represented when lipids were extracted from methanol-fixed and trypsin-released HUVEC. When lipid extraction was performed after 5 days of culture on GC-I, but not after 3 days, there was a consistent increase in the abundance of GM3 (C16/C18), GD1b, and GT1b compared with the cells cultured on NC-I (B).

Premature senescence and apoptosis of endothelial cells: effect of inhibitor of ganglioside synthesis d-erythro-EtDOP4.

To elucidate the role of accumulation of degraded gangliosides in cell reprogramming toward apoptosis or senescence, endothelial cells were pretreated with an inhibitor of glucosceramide-based glycosphingolipid synthesis, including ganglioside synthesis, d-threo-EtDOP4 (10–100 nM) or its inactive analog, d-threo-EtDOP4 (10–100 nM) (1, 22). Cells were subjected to GC, and the proportion of apoptotic and senescent HUVEC was quantified at 16 h or 3 days later. While inhibition of ganglioside synthesis did not appreciably affect cell senescence (Fig. 9B), it clearly prevented development of apoptosis in both GC-treated and in control cells (Fig. 9A). An effect similar to that with GC could be reproduced with lysosomal permeabilization using chloroquine (not shown). These find-
ings suggested the scenario whereby accumulation of gangliosides acts as a proapoptotic, rather than a prosenescence, signal, even under basal conditions.

**DISCUSSION**

The data presented herein demonstrated that a long-lived extracellular matrix protein, collagen I, modified by nonenzymatic glycation results in lysosomal dysfunction, as judged by the collapse of the pH gradient and permeability to macromolecules, later collapse of \( \Psi_m \), and further delayed accumulation of SA-\( \beta \)-gal and degraded gangliosides GM3 (C16/C18), GD1b, and GT1b in cultured early-passage human endothelial cells. The latter event was coincident with the development of premature senescence. Additionally, apoptosis, but not senescence, could be prevented by inhibiting ganglioside accumulation.

We investigated the time course and plausible cellular and molecular mechanisms leading to premature senescence and apoptosis in endothelial cells. In our experimental setting, the earliest event detected on exposure to GC was an increase in reactive oxygen species followed by lysosomal permeabilization with the collapse of lysosomal pH. An increase in the production of reactive oxygen species occurred within 10 min after application of GC. It has previously been shown that AGE-induced activation of its cognate receptor, RAGE, leads to the generation of reactive oxygen species (40), and the data presented here are in good agreement with those findings.

Although aging affects many cellular components, perhaps, the most remarkable changes occur in mitochondria and lysosomes (29). An early event after exposure of endothelial cells to GC, beginning 30 min after application, is the collapse of the lysosomal pH gradient and lysosomal permeabilization. Several characteristics of aged cells and organisms may result from the reduced lysosomal protein degradation pathways, including the increased cellular protein content of senescent cells and the accumulation of proteins with inappropriate posttranslational modifications (12). An increase in the volume and fragility of lysosomes are common findings in tissues from senescent organisms (11). Compared with other intracellular membranes, lysosomal membranes are overly sensitive to free radical damage (19). The change in lysosomal pH is most relevant to the course of premature senescence, since reduced proteolytic activity may contribute to age-associated pathologies. For example, reduced proteolytic activity has already been implicated in the intralysosomal aggregation of amyloidogenic peptides and formation of amyloid deposits (10). Moreover, the appearance of abnormally increased SA-\( \beta \)-gal activity is currently used as a biomarker of senescence.

Moderate release of lysosomal enzymes has been shown to induce apoptosis (6). In our experimental system of premature senescence, lysosomal changes predate mitochondrial damage, a phenomenon first described by Brunk et al. (37) and conceptualized as “the lysosomal-mitochondrial axis” theory of replicative senescence. We detected mitochondrial depolarization only after 4 h of exposure to GC, the delay of at least 3 h compared with the lysosomal permeabilization and loss of pH gradient. This observation is in accord with reported data on aging being associated with decreased activity of the citric acid cycle, \( \beta \)-oxidation, and oxidative phosphorylation enzymes (15). As a result, mitochondria of aged postmitotic cells have a decreased \( \Psi_m \) and reduced ATP production (6, 29). Oxidant-induced mitochondrial damage, resulting in progressive loss of cellular ATP, degeneration, and eventual cell death, is believed to play a key role in aging (6, 17).

It has been argued that the loss of acidic pH optimum would result in the accumulation of uncleaved products, such as gangliosides (20). The data presented in this study confirm this prediction. The levels of several gangliosides [GM3 (C16/C18), GD1b, and GT1b] were increased in HUVEC subjected to GC-I. Our findings further the concept of gangliosides as...
biomarkers of cellular aging and apoptosis (23), albeit GD3 ganglioside does not seem to be involved in the events initiated by GC-I. Previous studies of effects of AGEs on retinal microvascular endothelial cells and pericytes also revealed that all glycosphingolipids were increased, except for the GD3, which was even decreased in pericytes (27). This raises the question of the possible role of the accumulated cytosolic SA-β-gal, normally responsible for the degradation of gangliosides, in the pathogenesis of cell senescence. We addressed this question by blocking ganglioside synthesis while exposing the cells to GC. Surprisingly, however, such a blockade did not prevent development of senescence in HUVEC. These data argue against the possibility that accumulation of SA-β-gal and undergraded gangliosides GM3 (C16/C18), GD1b, and GT1b are causatively involved in premature senescence, at least in the experimental setting reported here. On the other hand, inhibition of ganglioside synthesis resulted in the reduction of apoptosis. This unexpected phenomenon was observed not only under stress conditions, but it also occurred under resting conditions. This intriguing observation needs further mechanistic elucidation.

In conclusion, endothelial cells exposed to an extracellular milieu containing nonenzymatically GC-I succumb to either apoptosis, with peak rates at 16 h, or premature senescence, peaking at 3–5 days. Lysosomal dysfunction accompanies treatment with GC and appears to trigger apoptosis. Although senescence occurs in synchrony with the accumulation of nondegraded gangliosides, the latter are not causatively involved in its development.

GRANTS

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