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High glucose-induced replicative senescence: point of no return and effect of telomerase

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Abstract

Primary human cells enter senescence after a characteristic number of population doublings (PDs). In the current study, human skin fibroblasts were propagated in culture under 5.5 mM glucose (normoglycemia); addition of 16.5 mM D-glucose to a concentration of 22 mM (hyperglycemia); and addition of 16.5 mM L-glucose (osmotic control). Hyperglycemia induced premature replicative senescence after 44.42 ± 1.5 PDs compared to 57.9 ± 3.83 PDs under normoglycemia ($p < 0.0001$). L-Glucose had no effect, suggesting that the effect of hyperglycemia was not attributed to hyperosmolarity. Activated caspase-3 measurement showed a significantly higher percentage of apoptotic cells in high glucose medium. Telomerase overexpression circumvented the effects of hyperglycemia on replicative capacity and apoptosis. The "point of no return," beyond which hyperglycemia resulted in irreversible progression to premature replicative senescence, occurred after exposure to hyperglycemia for as few as 20 PDs. These results may provide a biochemical basis for the relationship between hyperglycemia and those complications of diabetes, which are reminiscent of accelerated senescence.

Keywords: Senescence; Replicative arrest; Skin fibroblasts; Diabetes; Apoptosis; Telomerase; Hyperglycemia; Hyperosmolality

Normal somatic cells have a limited replicative capacity (Hayflick's limit), after which they undergo replicative senescence in vitro [16]. After a characteristic number of cell divisions, the majority of cells in a population fail to divide in response to a variety of growth stimuli. The cells remain alive and are metabolically active, but with an aberrant pattern of gene expression and with an impaired capacity for replication [5]. The latter effect results from the activation of a cell cycle arrest pathway whose final step is characterized by hypophosphorylation of the retinoblastoma protein (RB) [23]. Bypass of this cell cycle arrest pathway allows cells to continue dividing until they reach a state of irreversible replicative incapacity, characterized by a variety of chromosomal aberrations. The relationship between progressive cell division and loss of replicative capacity is not yet fully elucidated. However, each cell division is associated with a predictable loss of DNA at the telomeric ends of chromosomes. Circumvention of progressive telomere length reduction and protection of telomere integrity through the recruitment of telomerase activity increase the replicative capacity of cells in culture and in some cases confer an immortal cellular phenotype [3]. Accordingly, it has been proposed that shortening of telomeres serves as a mitotic clock for the

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counting down of cell divisions toward replicative senescence [1,14]. A recent study has shown that cellular senescence occurs in response to critical shortening of an individual telomere in one of its chromosomes rather than as a response to average telomere length [17]. We have recently demonstrated delayed replication of DNA at telomeric regions, an effect which is associated with incomplete separation of sister chromatids at metaphase in the corresponding telomeres [25].

It has been proposed that replicative senescence is a part of a more general process, which has been termed the senescence phenotype [19]. This phenotype is characterized by an irreversible growth arrest, resistance to apoptosis stimuli (in some cell types such as fibroblasts), and changes in differentiated functions. Controversy exists in the literature pertaining to the susceptibility of senescent fibroblasts to apoptosis. For example, a recent study has reported that Fas-mediated apoptosis occurs in senescent human fibroblasts [33].

Experimental data on the proliferative capacity of cultured skin fibroblasts harvested from diabetic subjects have demonstrated that in vitro replicative life span is reduced when compared with age-matched controls [12,21,37]. This led to the suggestion that a component of the degenerative changes, characteristic of some of the complications of diabetes, arises from accelerated cellular senescence and resembles a form of premature aging [29]. Sibbitt et al. [31] investigated the effect of elevated glucose concentrations on the growth of cultured human fibroblasts harvested from normal donors. In this study, elevated glucose concentration was associated with a reduction in the number of population doublings required to reach replicative senescence. Morocutti et al. [22] investigated this phenomenon in vivo. They demonstrated that senescence of skin fibroblasts from IDDM patients with nephropathy is significantly accelerated compared to that of IDDM patients without nephropathy and normal non-diabetic healthy controls. However, in these studies it was not determined whether the effect of varying glucose concentration mimicked patterns of cellular senescence associated with advanced population doubling in normoglycemic growth conditions. Solini et al. [32] investigated the effect of high glucose concentration on apoptosis in primary fibroblasts at the sixth passage following apoptotic stimulation. Caspase-3 activation (an important regulator of apoptosis) was elevated in fibroblasts maintained at 22 as compared to 5.5 mmol/l glucose.

In the current study, we show that the state of premature replicative arrest induced by high glucose is associated with several features of cellular senescence; is associated with enhanced senescence associated apoptosis; is not reversible, even after cells are restored to normoglycemic conditions after reaching a "point of no return" in hyperglycemic conditions; and does not occur in telomerase overexpressing fibroblasts.

Materials and methods

Cell culture. Foreskins were obtained from healthy newborn infants at the time of ritual circumcision after obtaining parental informed consent. The study was approved by the Institutional Ethics Review Board. The foreskin sample was minced in tissue culture medium with a surgical scalpel and, subsequently, digested by exposure to trypsin. Culture medium consisted of Eagle's minimal essential medium (MEM), supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 12.5 U/ml nystatin. Glucose concentration in this medium was 5.5 mM. Explants were incubated at 37 °C in room air with 5% supplemental CO_2 until reaching confluency. Subsequently, the adherent fibroblasts were washed with PBS, then removed by brief incubation in 0.25% trypsin, washed twice in tissue culture media, resuspended, and reseeded on a new tissue culture plate. Cells were subsequently passaged in this manner each time they reached confluency. Beginning with the third passage, fibroblasts from each donor were passaged under three different growth conditions as follows: (1) baseline medium containing 5.5 mM glucose (normoglycemia), (2) addition of supplemental D-glucose to a concentration of 22 mM (hyperglycemia), and (3) supplemental addition of 16.5 mM L-glucose (osmotic control). Explants from three separate individuals were used and propagated under each of these three conditions until replicative arrest (except L-glucose—two foreskins). Each of the three growth conditions for each of the explants was studied in quadruplicate. Replicative arrest was defined as the absence of cell division for up to 28 days in culture, under conditions of twice-weekly enriched medium change (20% fetal calf serum). In two foreskins (each in quadruplicate cultures), fibroblasts propagated for a varying number of population doublings (15, 20, 25, 30, 35, and 40) in high-glucose concentration were subsequently switched to normoglycemic medium and grown in these new conditions until reaching replicative senescence. BJ fibroblasts stably transfected so as to constitutively express the rate-limiting catalytic component (hTERT) of the human telomerase complex, and shown to maintain high telomerase activity [3], were provided by Dr. Woodring Wright and propagated in culture in high glucose medium. We used a telomerase repeat amplification protocol (TRAP) assay, as previously described in our laboratory [35] to confirm high telomerase activity (data not shown).

Determination of mean population doubling. At each cell culture passage, following trypsinization and suspension, an aliquot was counted in a hemocytometer. Trypan blue exclusion was used to assess cell viability. In this way, the number of cells plated and harvested at each passage was determined, to calculate the number of population doublings (PDs), as previously described [15]. Each determination of cell count was performed in triplicate. The cumulative number of population doublings (CPD) at the time of replicative senescence was designated as the replicative life span [11].

Effect of various hyperosmotic media on fibroblast proliferation. In two foreskins (each in quadruplicate cultures), fibroblasts propagated for 20–22 population doublings in normal glucose concentration were either continued to be cultured in normal glucose concentration (5.5 mM, measured osmolarity—307 mosm/l) or switched to four different hyperosmotic media. The four different hyperosmotic agents were: D-glucose (22 mM, measured osmolarity—323 mosm/l), urea (5.5 mM glucose + 16.5 mM urea, 323 mosm/l), mannitol (5.5 mM glucose + 16.5 mM mannitol, 322 mosm/l), and L-glucose (5.5 mM D-glucose + 16.5 mM L-glucose, 322 mosm/l). Population doublings were determined in these cells during 275 h.

Nuclear incorporation of bromo-deoxyuridine (BrdU). Cells were incubated for 1 h in the presence of 10^{-5} M BrdU. After the incubation, cells were harvested, treated with hypotonic KCl solution (0.075 M) for 10–20 min at 37 °C, and fixed with cold methanol–glacial acetic acid (3:1). The cells were washed with fixative, dropped on cold slides and air dried. Denaturation was carried out by immersing the slides in boiling water for 2 min and then slides were dehydrated by a

series of ice-cold ethanol washes (70%, 80%, 90%, and 100% for 2 min each). The cells were air-dried, incubated in a blocking solution (3% BSA in 4× SSC) for 30 min at 37 °C, and then incubated for 30 min at 37 °C with mouse anti-BrdU (Pharmingen; 1:150 dilution) in 50 μl of 0.1% Tween 20/1% BSA in a humid chamber. Cells were then washed three times for 5 min with PBS, incubated for 30 min with goat anti-mouse AMCA (Jackson Immuno Research Laboratories, 1:50 dilution) in a humid chamber, washed three times for 5 min in PBS/0.1% Tween 20, and counterstained with propidium iodide. Slides were visualized by conventional fluorescence microscopy.

Cell cycle distribution of senescent cells. To determine the cell cycle distribution at replicative senescence, an aliquot of cells from each cellular growth condition was stained with propidium iodide and analyzed by fluorescent activated cell sorter (FACS), using a FACS Calibur, Becton–Dickinson Immunocytometry Systems, San Jose, CA, USA. Cells defined as having reached replicative senescence were trypsinized and reseeded on a new tissue culture plate for 30 h prior testing, to avoid confluency.

SA-galactosidase (β-Gal) staining. Assays were performed in standard plastic six-well dishes (Corning) containing subconfluent cultures of approximately 60,000 cells/well. Cells were washed twice in PBS, fixed for 10 min (room temperature) in 0.5% glutaraldehyde, washed again in PBS, and incubated at 37 °C (5% CO_2) with 1 ml fresh senescence associated β-Gal (SA-β-Gal) stain solution containing 1 mg/ml of 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) (final concentration) as described by Dimri et al. [9]. The assay was performed at pH 6.0 (specific for SA-β-Gal activity) and 4.0 (specific for lysosomal β-galactosidase) as a control. Staining was evident within 2–4 h and was maximal at 12–16 h. Following incubation of 12–16 h, cells were washed with room temperature PBS, then covered with 5 ml ice-cold PBS, and incubated at 4 °C for 48 h. This last incubation was found to enhance the fibroblast-staining efficiency at any staining solution pH. Cells were then counted using phase-contrast microscopy at 150-fold magnification and the total number of cells and number of positively stained cells per field were recorded. Counts were performed at five random locations in the same well and every field was counted three times for determination of the mean percentage of positively stained cells.

Assessment of apoptosis. Confluent cultures of skin fibroblasts cultured in normal and high glucose media and telomerase positive overexpressing BJ fibroblasts cultured in high glucose medium at different PDs were maintained for 30 days in the quiescent state. Replicative arrested normal and high glucose fibroblasts (defined as the absence of cell division for up to 28 days in culture, under conditions of twice weekly enriched medium change) were then maintained in culture for an additional 30 days. Cells were then trypsinized and seeded in two-well chamber slides for 24 h. The cells were fixed with 4% paraformaldehyde for 20 min at room temperature, washed, and permeabilized by 0.5% Triton-X in PBS for 5 min. After blocking with 5% goat serum + 0.1% Triton-X, the cells were incubated with affinity purified rabbit anti-human caspase-3 antibody (R&D Systems, UK) for 3 h. The cells were washed three times and incubated with rhodamine red-X-conjugated affinity purified goat anti-rabbit IgG (Jackson Immuno Research Laboratories, USA) for 1 h. The cells were washed and then stained with DAPI. The slides were examined by fluorescence microscopy. For each set of fibroblasts and growth conditions, quadruplicate counts were performed and at least 450 cells were counted in different zones of each well. The extent of apoptosis was determined by the percentage of the cells that stained positively for activated caspase-3.

Statistical analysis. Population doublings for each culture at different growth conditions, compared to "normal glucose" growth condition, was determined using a paired two-tailed *t* test.

Results

A comparison of results obtained under different growth conditions, using fibroblasts from the same

Table 1
Population doublings of skin fibroblasts cultured in various media

Foreskin[a]	Normal glucose (NG)[b]	High glucose (HG)	High L-glucose (HLG)	*p* value NG vs. HG[c]	*p* value HLG vs. HG[c]	*p* value HLG vs. NG[c]
A1	53.00	44.36	–	<0.001	–	–
A2	52.60	42.90	–			
A3	55.12	43.78	–			
A4	54.08	41.87	–			
B1	55.27	41.52	64.31	<0.001	<0.001	<0.03
B2	54.99	40.48	60.12			
B3	56.12	40.60	59.55			
B4	55.38	43.16	62.24			
C1	62.10	50.16	56.29	<0.003	<0.003	=0.21
C2	63.30	46.90	61.62			
C3	64.90	49.10	58.88			
C4	60.94	50.21	62.71			
BJ1		216.59				
BJ2		223.95				
BJ3		201.96				
BJ4		212.47				

[a] Letters represent the three different foreskins, numbers represent the replicates for each foreskin. BJ refers to the telomerase positive fibroblasts cultured in high glucose only. Since proliferation of BJ continued unabated, the number of PDs for BJ1-4 refers to the point at which the study was discontinued.

[b] All skin fibroblasts were cultured in MEM-Eagle's medium but with different glucose/L-glucose concentrations as indicated. Normal glucose (NG)-medium contains 5.5 mM glucose; high glucose (HG)-medium contains 22 mM glucose; high L-glucose (HLG)-medium contains 5.5 mM glucose plus 16.5 mM L-glucose (osmotic control).

[c] Two-tailed paired *t* test.

Table 2
The effect of various hyperosmotic media on the number of population doublings in two foreskins during 275 h (means ± SD)

Medium	NG	HG	Urea	Mannitol	HLG
Foreskin B (PD 22)	3.04 ± 0.18	2.47 ± 0.18[a]	3.05 ± 0.24	3.08 ± 0.14	3.01 ± 0.16
Foreskin B (PD 20)	2.27 ± 0.06	1.96 ± 0.05[b]	2.19 ± 0.16	2.25 ± 0.3	2.33 ± 0.16

[a] $p = 0.006$ vs. the rest groups.
[b] $p = 0.009$ vs. the rest groups.

donor, minimizes the contribution of interindividual variability.

Foreskin explants from three different donors were established and each of these was then propagated as four separate primary culture lines for each incubation condition. As shown in Table 1, proliferative life span determined as CPD was significantly lower in high glucose culture conditions, when compared to corresponding normal glucose and high L-glucose growth conditions. The L-glucose osmotic control was tested in quadruplicate cultures derived from two of the foreskin explants and also revealed a significantly higher CPD compared to high D-glucose. Measurements of the population doubling time of cells derived from two of the foreskin explants propagated for 275 h in physiologic D-glucose concentration and in hyperosmotic cultures with D-glucose, urea, mannitol, and L-glucose showed that cells growing in high D-glucose divided more slowly than cells growing in other hyperosmotic agents (Table 2). In contrast, telomerase overexpressing BJ fibroblasts continued to proliferate in high glucose conditions for more than 200 passages in culture.

By morphological inspection we observed that, as previously described [28], senescent human fibroblasts increase in size and display a flatter morphology compared to early passage cells.

We used the measurement of nuclear incorporation of BrdU to provide confirmation that the senescent cells are growth-arrested. BrdU incorporation patterns determined in counts of more than 1500 nuclei from each growth condition showed that prematurely arrested cells in hyperglycemic media were non-replicating, as indicated by <1% BrdU positive nuclei, compared to >35% BrdU positive nuclei in early passage cells (results not shown).

We next sought to compare the cell cycle distribution pattern of high glucose-induced premature replicative arrest with the typical senescence pattern observed in late passage cells grown in normal glucose conditions. As shown in Fig. 1, both conditions of replicative arrest showed an identical pattern of cell cycle arrest in G1.

We used the measurement of SA β-galactosidase activity to determine whether the premature replicative arrest observed with growth in hyperglycemic conditions mimicked the corresponding parameters, characteristic

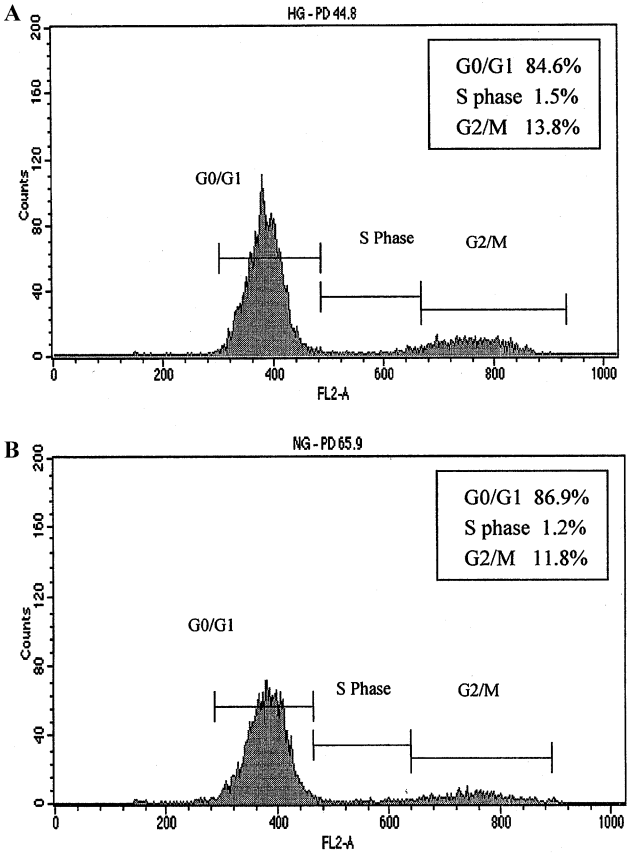

Fig. 1. Cell cycle distribution pattern in high glucose-induced premature replicative arrested fibroblasts (PD 44.8) (A) and in senescent fibroblasts propagated in normal glucose concentration (PD 65.9) (B).

of normal senescence under euglycemic conditions. As reported by Dimri et al. [9], replicative senescence was associated with a marked increase in the cytochemically detectable SA-β-Gal activity at pH 6.0 in subconfluent cultures, while little or no staining was observed in subconfluent cultures at early passages (Fig. 2). At pH 6.0, a homogeneous perinuclear SA-β-Gal staining was detected in senescent cells and was nearly absent in early passages. In contrast, a punctate pattern of SA-β-Gal staining at pH 4.0 due to lysosomal activity was detected in young as well as in senescent cells (Fig. 2). For fibroblasts grown in high glucose medium, SA-β-Gal positive staining appeared in less than 5% of cells for approximately the first 35 PDs and after 35 PDs rose to a peak of

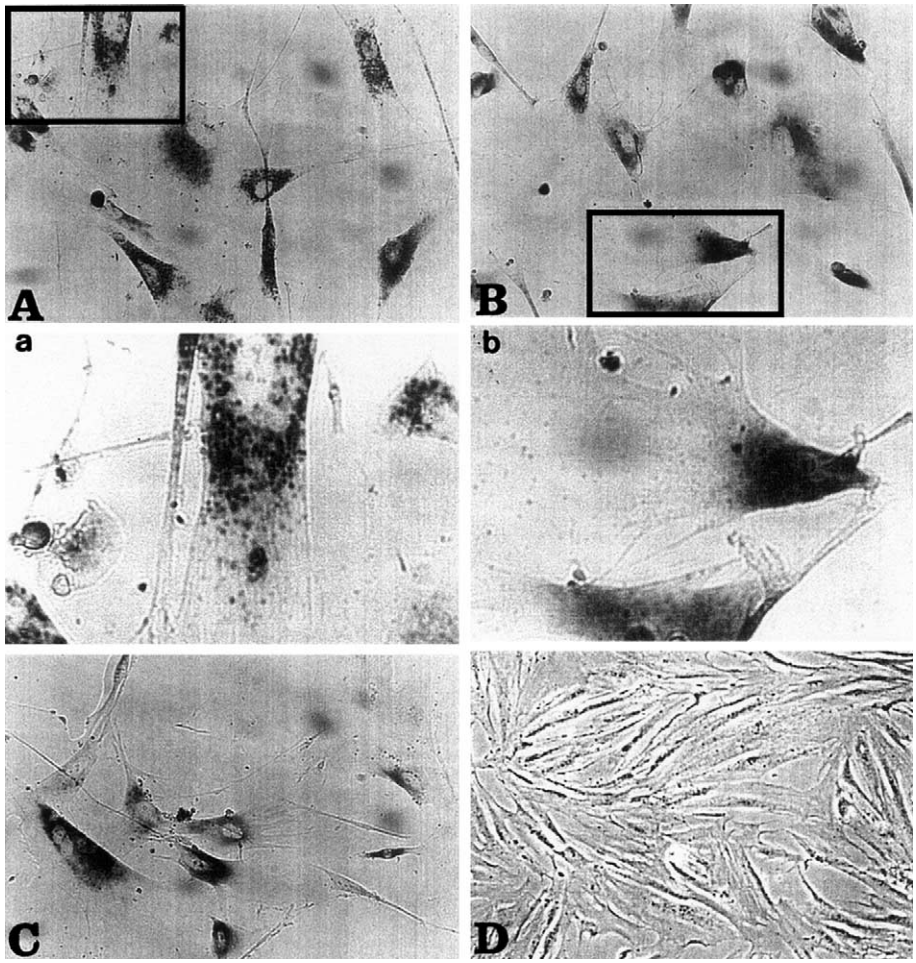

Fig. 2. SA-β-Gal assay. (A) Punctate pattern of SA-β-Gal staining due to lysosomal activity (pH 4.0) in normoglycemic cells at PD 40 (magnification 20×) with the highlighted fields shown at higher magnification (60×) in corresponding panel (a). (B) Homogeneous perinuclear SA-β-Gal staining characteristic of cellular senescence, in normoglycemic cells at PD 60 (magnification 20×) with the highlighted fields shown at higher magnifications (60×) in corresponding panel (b). (C) SA-β-Gal staining at pH 6.0 in cells grown under hyperglycemic conditions at PD 44. (D) Near absence of SA-β-Gal staining (pH 6.0) at PD 9, in cells grown under normoglycemic conditions.

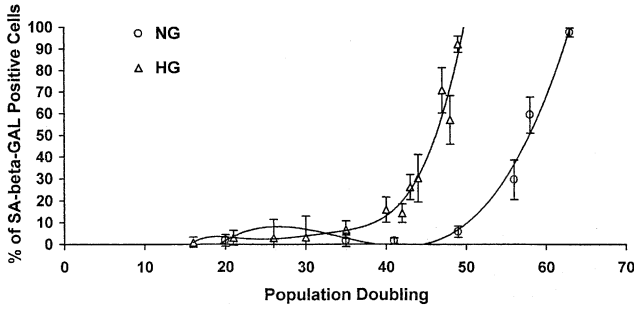

Fig. 3. Time course of the appearance of SA-β-Gal positive cells in foreskin C fibroblast cultures grown in different media (quadruplicate). NG, glucose normal (5.5 mM glucose); HG, glucose high (22 mM glucose).

80% positively staining cells by 47 PDs (Fig. 3). This was in contrast to cells propagated in normal glucose medium, which remained with 5% of cells staining positively for SA-β-Gal up to approximately 50 PDs and only then began to show a progressive rise in staining reaching greater than 80% cells staining positively for SA-β-Gal at approximately 60 PDs. For telomerase-expressing BJ fibroblasts grown in high glucose medium for greater than 200 passages, SA-β-Gal positive staining at pH 6.0 appeared in less than 1.5% of cells.

It is now well known that final common pathway effectors of apoptosis are represented by a family of intracellular cysteine proteases known as caspases. Cytochrome *c* release from mitochondria activates a cascade of caspases, with caspase-3 being prominent among them [13]. When varying concentrations of staurosporine were tried to induce apoptosis, we noted that many cells were no longer adherent and as a result disappeared from the slide. Accordingly, we proceeded to determine the level of spontaneous apoptosis occurring in the absence of inducing agents in the proliferating and in the replicative senescent fibroblasts at normal and high glucose growth conditions, as well as in the telomerase positive BJ fibroblasts cultured in high

glucose and passaged more than 200 times. We stained the cells with anti-active caspase-3 antibody and counted the percentage of positively staining cells in the samples. We found a clear and progressive increase in the percentage of apoptotic cells, as indicated by active caspase-3 immunodetection in fibroblasts cultured in normal glucose medium, beginning with determinations at 5 PDs (0.8%) through 2.5% and 3.6% of fibroblasts at 17 and 42 PDS, respectively, and reaching 7.9% at replicative senescence (64 PDs, Fig. 4). Furthermore, this phenomenon was found to be more pronounced in fibroblasts propagated in high glucose medium, with an increase to 9.6% of apoptotic cells at 43 PDs, the point of premature senescence. In contrast, the rate of spontaneous apoptosis in telomerase positive BJ fibroblasts cultured in high glucose medium was only 0.9%, despite having been propagated in culture for more then 200 passages.

We next went on to determine whether there is a consistent "point of no return," beyond which exposure to a hyperglycemic environment would result in irreversible progression to premature replicative arrest, even if cells were subsequently restored to a low glucose environment. These experiments were conducted using cells derived from two foreskin explants with four replicates each. Cells were grown in high glucose conditions for the number of PDs indicated in Fig. 5 and then reverted and maintained in normal glucose medium until replicative arrest. Replicative arrest in cells exposed to high glucose for 10, 15, and 20 PDs occurred after 56.0 ± 0.9, 55.2 ± 1.6, and 52.2 ± 2.1 ($n = 8$) PDs, respectively, vs. 57.9 ± 3.8 PDs for cells grown consistently in normal glucose medium and 44.4 ± 3.15 PDs in cells grown consistently in high glucose medium. Cells exposed to high glucose medium for >20 population doublings all progressed to early replicative arrest, even though they were reverted to normal glucose medium for the subsequent duration of cell culture. Premature replicative arrest occurred at almost the same number of PDs in all cell lines that had been exposed to high glucose medium for the initial 20 PDs (45.8 ± 2.2 PDs for all cells grown for 20 or more PDs in high glucose medium; Fig. 5). These results define a rather consistent in vitro "point of no return" of 20 PDs, beyond which exposure to high glucose medium results in irreversible progression to premature replicative arrest, which cannot be recovered.

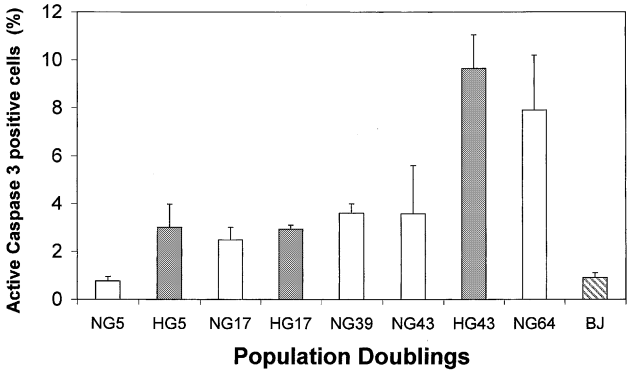

Fig. 4. Mean (±SD) percentage of active caspase-3 positive cells at different population doublings in fibroblasts cultured in high and normal glucose media. NG, 5.5 mM glucose; HG, 22 mM glucose; BJ, telomerase overexpressing fibroblasts cultured in 22 mM glucose. The numbers after the abbreviations indicate the number of the population doublings.

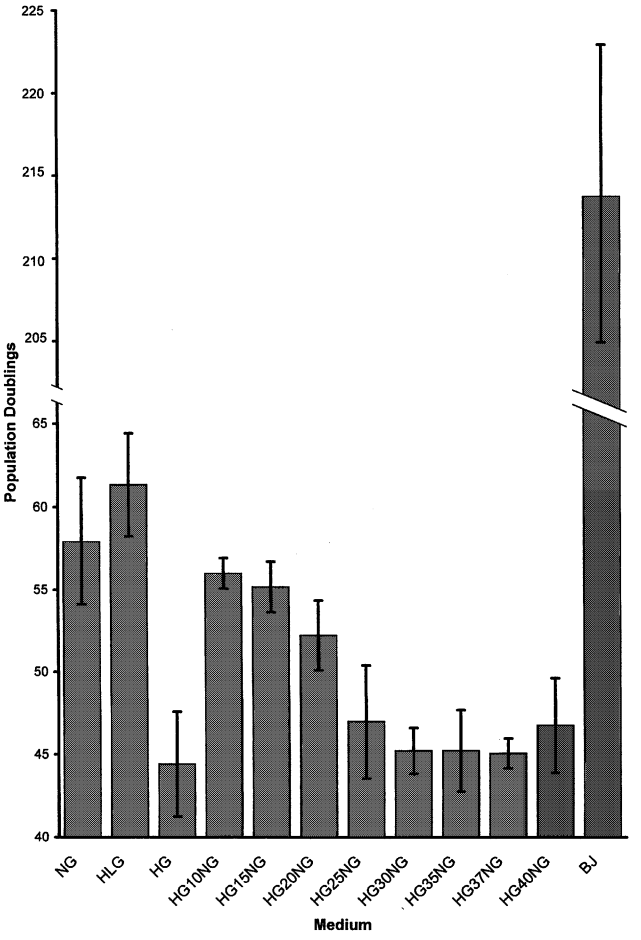

Fig. 5. Mean population doublings (±SD) to senescence of foreskin explant fibroblasts from two different donors each grown in four replicate cultures, in different media. NG, 5.5 mM glucose; HLG, 5.5 mM glucose + 16.5 mM L-glucose; HG, 22 mM glucose; HG15NG, fibroblasts cultured in HG medium (22 mM) for 15 population doublings and then switched to growth in NG medium (5.5 mM); the same nomenclature is applied to HG20NG, HG25NG, etc.; BJ, telomerase overexpressing fibroblasts cultured in 22 mM glucose showed unabated proliferation as 213.74 ± 9.18 PDs, at which point the experiment was discontinued.

Discussion

In primary human cells in which telomerase activity is not detected, the telomere length decreases with the

increasing number of cell divisions in vitro and with age in vivo. Primary human cells enter senescence after a characteristic number of population doublings in culture.

The results of the current study, as well as those in a previous study [31], indicate that varying glucose concentrations per se is sufficient to modulate the rate and number of population doublings required to reach replicative senescence. Thus, premature loss of replicative capacity was evident in cells incubated in high glucose medium, compared to those propagated in medium containing physiologic glucose concentrations. In the current study, we confirmed that this apparent premature replicative arrest also shared various features, characteristic of cellular senescence. Cells underwent morphological changes, characteristic of senescent cells [28]. DNA replication was markedly reduced, as indicated by very low levels of BrdU incorporation. Flow cytometry revealed an identical pattern in the distribution of cells across the cell cycle under both conditions of replicative arrest. Furthermore, a similar pattern of abundant SA-β-Gal staining at pH 6.0 was observed at replicative arrest under all of the varying glucose growth conditions. SA-β-Gal staining at pH 6.0 has been used by Dimri et al. [9] and others [7,26] as a biochemical marker characteristic of cellular senescence in cell culture and in tissue sections obtained from aged donors. The use of SA-β-Gal as a universal marker for aging has been questioned by Severino et al. [30]. However, there is general agreement that cytochemically detectable SA-β-Gal is elevated in cells at replicative senescence and that this is a useful technique for estimation of replicative age in vitro. It is clear that there are potential limitations to SA-β-Gal as a marker for senescence, particularly in vivo. In this study, it was shown that skin fibroblasts cultured to senescence displayed a high percentage of cells with homogeneous perinuclear SA-β-Gal staining, characteristic of cellular senescence at pH 6.0. At a pH of 4.0, β-galactosidase was positive in both early and late passage fibroblasts and is considered to result from non-specific lysosomal β-galactosidase activity [9]. In contrast, when performed at pH 6.0, a high percentage of staining is confined to late passage cells. The simplest explanation is that elevated lysosomal activity in senescent cells [4,8] may increase the lysosomal β-galactosidase activity to a level, which becomes detectable, even at a pH of 6.0. However, it is not yet known whether there is true β-galactosidase activity, which becomes evident in senescent fibroblasts at this higher pH or whether the pH 6.0 staining is the consequence of an as yet unknown senescence associated substance which mimics β-galactosidase activity [6,20]. As shown in the current study, the morphology of staining is strikingly different at pH 4.0 and 6.0. While SA-β-Gal (pH 6.0) shows a pattern of homogeneous perinuclear staining, lysosomal activity associated β-galactosidase (pH 4.0) shows quite a distinct punctate pattern throughout the cytoplasm and overlying the nucleus as well (see Fig. 2). In the current study, we also examined the temporal relationship between the number of PDs and the percentage of SA-β-Gal (pH 6.0) positively staining cells. Of interest, in both normal and high glucose conditions, there was not a smooth and steady increase, but rather an abrupt "spurt" in the percentage of cells with this activity beginning at approximately 6 PDs, prior to replicative senescence.

Having confirmed this relationship between glucose concentration and replicative capacity, and the observation that expression of active telomerase can abolish this effect, it is of interest to consider the potential relationship to telomere length. We have carried out extensive analyses using telomere restriction fragment (TRF) length blots and the results of these studies do not reveal a clear association of premature senescence with accelerated telomere shortening. In particular, the TRF length of high glucose-induced premature senescence was generally greater (mean TRF length ranged from 6.1 to 9.0 kb) than that observed in normal glucose advanced passage senescence (mean TRF length ranged from 4.5 to 6 kb) (data not shown). However, these results do not shed additional insight into or sharpen our ability to draw unambiguous conclusions, because TRF blots reflect only an average telomere length, and it has been recently shown that critical shortening of even a single telomere may be sufficient to signal premature senescence [17]. In general, two potential formulations can be suggested for the relationship between high glucose-induced premature senescence to telomere shortening. According to one model, higher glucose concentrations might be associated with accelerated shortening of one or more critical telomeres, without affecting the overall or average TRF length. For example, hyperoxic cell culture growth conditions have been reported to accelerate telomere shortening. In addition, accelerated telomere shortening has been reported in some but not all clinical progeria syndromes. On the other hand, if there is no direct effect of glucose concentration on the rates of telomere shortening with cell division, then measured TRF would be predicted to reflect the number of PDs that have elapsed at the time of replicative arrest. Our observations based on the overall TRF length favor the latter formulation. This is also consistent with a number of recent models, regarding the relationship between telomere length and replicative arrest. In particular, it has recently been suggested that there may be a varying threshold for telomere length, at which replicative arrest occurs [36]. Under such conditions, different physiologic and biochemical factors can modulate this threshold. Our findings are consistent with this formulation and suggest that varying glucose concentration may serve as an important physiologic and possibly pathophysiologic modulator in this regard.

No significant changes were observed in replicative senescence timing between skin fibroblasts cultured in normal glucose and in high concentrations of L-glucose, suggesting that the early replicative senescence under hyperglycemic conditions was attributable to the high D-glucose concentration per se rather than to hyperosmolality. This finding is consistent with previous studies using different osmotic controls [31]. The significant difference in the effect of D-glucose vs. other osmotic agents on population doubling time demonstrates further the effect of D-glucose on fibroblast cell division.

An imbalance between cell renewal and apoptosis contributes to aging in various tissues in vivo [40]. An increase in apoptosis at senescence in vitro was observed in cultured human vascular smooth muscle cells derived from atherosclerotic plaques [2] and in human endothelial cells [18,38]. Young quiescent human skin fibroblasts in vitro have been shown to undergo apoptosis two weeks after withdrawal of serum from the culture medium, as evident by DNA fragmentation [39]. In another study, caspase-3 activation was evident in young quiescent human skin fibroblasts in vitro [32]. Recent publications have addressed the question of apoptosis in senescent fibroblasts. Some reports have shown that growth arrested human fibroblasts are resistant to apoptosis (defined by DNA fragmentation) in response to serum withdrawal [39]. In contrast, it has been recently shown that Fas-induced apoptosis occurs in senescent human fibroblasts, as evidenced by activation of caspase-8 and caspase-3 [33]. We examined spontaneous apoptosis and also used as a marker what is now considered to be a very reliable measure of activation of the apoptotic cascade—namely, measurement of activated caspase-3. Our results clearly show that senescent fibroblasts undergo spontaneous apoptosis. Our results are in accordance with the recent suggestion of Ferbeyre and Lowe [10] that senescence parallels apoptosis as a cellular response to stress.

Multiple physiological and pharmacological stimuli induce cell death by apoptosis. High glucose (but not L-glucose) has been shown to cause a significant increase in apoptotic cells in cultured human endothelial cells [24]. Young quiescent fibroblasts grown in medium with high glucose concentration (22 mM) also displayed enhanced apoptosis and caspase-3 activation compared to corresponding cells grown in normal (5.5 mmol/l) glucose or high mannitol (5.5 mmol/l glucose + 16.5 mmol/l mannitol) media [32]. We have used caspase-3 to compare spontaneous apoptosis in early and late passage cells under normal and high glucose growth conditions. We report here for the first time an increase in the percentage of spontaneous apoptosis in contact-inhibited quiescent human skin fibroblasts culture in vitro, as CPD increases, and a high percentage of spontaneous apoptosis in fibroblasts at replicative senescence. This spontaneous apoptosis is much more pronounced in the fibroblasts cultured in high glucose medium. Telomerase expression appears to protect the fibroblasts from the glucose effect on apoptosis.

Having confirmed this relationship between glucose concentration and replicative capacity, it was of interest to determine whether there is a "point of no return" for hyperglycemia-induced premature replicative senescence. We found that the effect of high glucose is reversible up to 20 PDs, but not beyond. Such reversibility adds further physiologic relevance to the phenomenon and may have eventual clinical implications. Many biochemical and pathophysiologic factors may modulate this threshold, but the effect of hyperglycemia may be of particular interest in terms of its potential pathophysiologic significance. Reduced replicative capacity has been observed in cells harvested from diabetic subjects and this finding has been correlated with some of the degenerative complications of poor glucose control in diabetic subjects. It is now well established that the level of glucose control is of paramount importance in preventing many of the complications of both type I and type II diabetes mellitus [27,34]. The "point-of-no-return" findings in the current study might also indicate that it is of particular clinical importance to avoid an early or critical period of sustained hyperglycemia, which might induce irreversible effects on cellular replicative capacity.

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