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Insulin-like growth factor binding protein 3 accumulates to high levels in culture medium of senescent and quiescent human fibroblasts

(growth regulation/aging)

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ABSTRACT **Insulin-like growth factor binding protein 3 (IGFBP-3) mRNA levels were consistently higher in both senescent normal human diploid fibroblasts (HDFs) at late passage (old cells) and prematurely senescent HDFs from a subject with Werner syndrome (WS) during serum depletion and repletion of growth medium and during proliferation from sparse to high-density inhibited cultures, compared to normal early-passage (young) HDFs. However, IGFBP-3 protein accumulated to higher levels in conditioned medium of old cells than in medium of WS and young cells, in that order, under the same conditions. Insulin-like growth factor I (IGF-I) was not detected in naive medium or in any of the media conditioned by these three cell types, whereas IGF-II was detectable in serum-repleted medium and remained relatively constant. Thus, molar ratios of IGFBP-3/IGF-II were consistently higher in old and WS cells and increased substantially as all three cell types became quiescent, due to either serum depletion or high cell density. These data are consistent with either an adaptive or a causal role for IGFBP-3 protein in the senescent and quiescent growth arrest of HDFs.**

Human diploid fibroblasts (HDFs) have a finite replicative life-span (1), and this system is now widely used as a model for the study of biological aging (2, 3). Thus, the number of cumulative mean population doublings (MPD) prior to senescent arrest of HDFs is inversely proportional to the age of the donor. Moreover, persons with various inherited disorders of premature aging, such as Werner syndrome (WS), give rise to HDFs with an abbreviated replicative life-span (4, 5). The mechanism for loss of HDF replicative ability is unknown but positive and negative growth regulatory factors appear to be involved (6).

A prominent characteristic of senescent HDFs, whether derived from normal donors or from subjects with WS, is unresponsiveness to the action of various polypeptide mitogens present in fetal bovine serum (FBS) including insulin-like growth factor I (IGF-I), platelet-derived growth factor, and epidermal growth factor (7–12). However, no significant alterations have been detected in the number of binding sites or in the binding affinity of IGF-I and these other factors after correction for the increased surface area of enlarged senescent HDFs (ref. 11; see also ref. 3). IGF-I unresponsiveness, therefore, would appear to reside in either a postreceptor mechanism and/or in a prereceptor block that restricts growth factor bioavailability.

The presence in vertebrate sera of binding proteins for the IGF family (IGFBPs) has recently generated considerable interest (13). Six structurally distinct IGFBPs have now been recognized (14). In adult human serum, the major species is IGFBP-3, which exists as part of a ternary, growth hormone-dependent complex of ≈150 kDa. In brief, the components of this complex are an acid-stable glycoprotein (IGFBP-3), which can exist in at least two glycosylated forms of 40–50 kDa; IGF-I or IGF-II, which bind to IGFBP-3 with equally high affinity; and an acid-labile glycoprotein of 85 kDa, which binds to the IGFBP–IGF complex (15, 16). Recent studies on HDFs (17) and bovine fibroblasts (18) indicate that IGFBP-3 can either inhibit or potentiate IGF-I-mediated stimulation of DNA synthesis, depending on experimental conditions.

We have recently identified several overexpressed gene sequences in a cDNA library derived from WS HDFs (19, 20). Among the overexpressed gene sequences was IGFBP-3 mRNA, which was found to be especially abundant in senescent HDFs, both late-passage normal (old) cells and prematurely senescent HDFs derived from a subject with WS, compared to early-passage normal (young) HDFs. The present report concerns a more detailed analysis of IGFBP-3 expression in HDFs and its relationship to IGF concentration and the dynamics of cell proliferation, quiescence, and senescence.

MATERIALS AND METHODS

Cell Culture. WS8, a HDF strain derived from skin of the arm of a 47-year-old male subject with classical WS, was used in all experiments. These cells, which have a curtailed replicative life-span of 18 MPD (19, 20), were utilized at ≤9 MPD, when the 24-hr [^{3}H]thymidine labeling index (TLI) was 10–20%. Strain J065, HDFs derived from skin of the arm of a normal 56-year-old male, has a replicative life-span of 46 MPD (19, 20). Normal cells designated as young HDFs were at ≤20 MPD (TLI > 90%), whereas late-passage HDFs, designated as old, were from the same strain at 40–42 MPD (TLI ≤ 13%). Cells were routinely propagated in Eagle's minimal essential medium supplemented with 15% FBS (regular growth medium) without antibiotics and prepared for analysis in two ways. In serum depletion/repletion experiments, cells were grown to ≈3/4 confluence in 100-mm Petri dishes containing regular growth medium, rinsed with phosphate-buffered saline, and then incubated for 5 days in medium containing 0.5% FBS (time 0). Cells were then refed with 10 ml of serum-repleted medium containing 15% FBS and replicate dishes were harvested at intervals up to 48 hr. In experiments examining the effects of growth from sparse to high-density cultures, HDFs were subcultured at a 1:7 ratio (young cells), a 1:4 ratio (old cells), or a 1:3 ratio (WS

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Abbreviations: HDF, human diploid fibroblast; IGF, insulin-like growth factor; IGFBP, IGF binding protein; MPD, mean population doublings; WS, Werner syndrome; FBS, fetal bovine serum.

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cells) and then incubated through day 8 without change of medium; one set of replicates was refed at day 8 with fresh growth medium and then allowed to incubate to day 15.

Preparation of RNA and Quantitation of Protein and Cell Counts. Replicate dishes of cells were harvested at intervals for cell counts with a Coulter electronic counter, for protein determination (21) and RNA preparation (22, 23).

Northern Blot Analysis. Total RNA (10 μg per lane) from WS, young, and old HDFs at various intervals after serum depletion/repletion or after subculture were run on agarose/formaldehyde gels, blotted onto nylon filters, and then prehybridized and hybridized as described (22). Filters were probed with the full-length (2.5 kilobase pairs) IGFBP-3 ^{32}P-labeled cDNA insert, washed, and prepared for autoradiography (20). The IGFBP-3 bands on autoradiograms were quantified within the linear range on a model 300A laser densitometer using ImageQuant software (20). Signals were normalized to the strongest band among young, old, and WS IGFBP-3 mRNAs, which was assigned a value of 100%.

Radioimmunoassays. Medium was rapidly collected from dishes and stored at −70°C. IGFBP-3 was quantified as described (24, 25) on thawed growth medium, both naive and after conditioning by exposure to HDF. To calculate molar concentrations, a molecular mass of 43 kDa, determined under reducing conditions (26), was used. IGF-I and IGF-II radioimmunoassays (27, 28) were performed after fractionating growth media by high-performance liquid chromatography under conditions that completely remove IGFBPs (27). Due to the dilution of samples during the chromatography step, the sensitivity of detection for IGF-I and IGF-II was approximately 15 and 30 ng/ml, respectively.

RESULTS

Cell Number and Protein Content After Serum Depletion and Repletion. HDFs undergo progressive enlargement and pack less densely as they approach senescence (1–3, 6, 29). Because we wished to obtain approximately equivalent cell mass in the three cell types, we inoculated fewer old and WS cells than young cells. Thus, at time 0 and after serum repletion, cell numbers were higher in young than in old and WS cultures (Fig. 1*A*) and remained relatively constant for all three cell types up to 18 hr. Thereafter, an upward trend was seen up to 48 hr, reflecting mitotic activity of newly cycling cells. Profiles of cell protein per dish, providing an excellent index of cell mass (29), were similar for all three cell types during the 48 hr (Fig. 1*B*). Thus, the differences observed in cell number per dish were not evident when data were expressed as protein per dish, reflecting the fact that senescent cells have a higher protein content than young cells (e.g., see refs. 2, 3, and 29).

IGFBP-3 mRNA Content After Serum Depletion and Repletion. Steady-state levels of IGFBP-3 mRNA were assessed at the same intervals after serum depletion/repletion (Fig. 1 *C* and *D*). At time 0, IGFBP-3 mRNA levels were substantially higher in old and WS cells compared to young cells. Immediately after serum repletion, mRNA levels fell precipitously in old cells, rebounded by 4 hr, and maintained ≈40% of the peak value throughout the first 24 hr, increasing nearly to the time 0 value at 48 hr. The initial postrepletion decrease in IGFBP-3 mRNA of WS cells was less marked than in old cells, but from 1 to 48 hr similar patterns were maintained in both cell types. In striking contrast, young cells maintained low values from time 0 through 48 hr.

IGFBP-3 and IGF-II Levels and Molar Ratios After Serum Depletion and Repletion. When cells were exposed to 0.5% FBS-supplemented medium, IGFBP-3 protein levels increased from undetectable levels initially to 4.2 nmol per liter (0.18 μg/ml) for young, 23.7 nmol per liter (1.02 μg/ml) for old, and 23.3 nmol per liter (1.00 μg/ml) for WS cells after 5

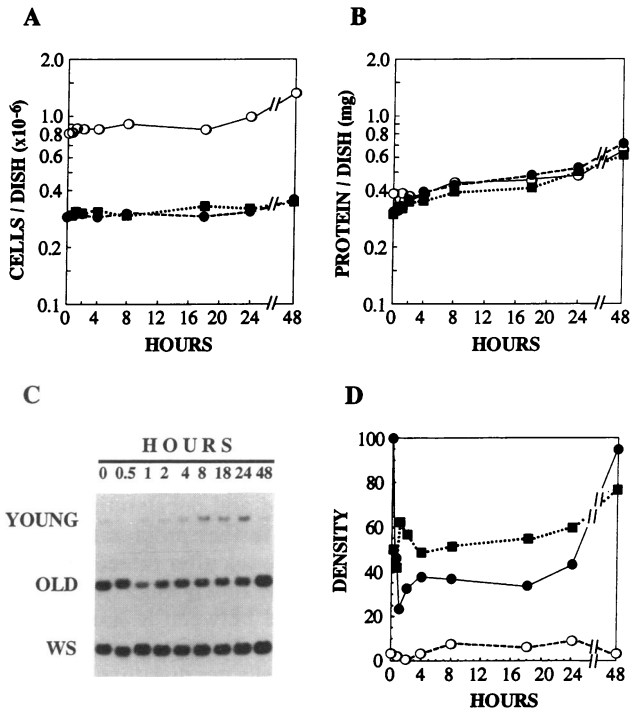

FIG. 1. Cell numbers, total protein content, and IGFBP-3 mRNA levels of young, old, and WS HDFs after serum depletion and repletion. Replicate dishes of cells were maintained in 100-mm dishes containing medium supplemented with 0.5% FBS for 5 days, repleted in regular growth medium (15% FBS-containing medium), followed by determination at intervals for cell counts (*A*), protein (*B*), and IGFBP-3 mRNA by Northern analysis (*C*) and by densitometric analysis (*D*). Data depicted here were derived from one of two separate experiments, which gave virtually identical results. Ethidium bromide-stained gels photographed before Northern transfer demonstrated equivalent RNA sample loading and integrity, as judged by 28S and 18S ribosomal RNA bands, and autoradiographs of RNA bands in Northern blots probed with 28S rDNA showed even transfer and relatively constant expression (data not shown). Northern blot utilized here and in Fig. 3*C* is representative of three separate Northern analyses from the two experiments. ○, Young HDFs; ●, old HDFs; ■, WS HDFs. RNA was not available for young cells at 0.5 hr.

days in this low-serum medium. Neither IGF-I nor IGF-II achieved detectable levels (2 and 4 nmol per liter, respectively) in 0.5% FBS medium, whether naive or conditioned for 5 days by any of these three cell types. Thus, although the precise molar ratios of IGFBP-3/IGF-I or IGFBP-3/IGF-II at time 0 could not be determined, these ratios clearly increased in conditioned 0.5% FBS medium during quiescence of all three types of HDF, particularly old and WS cells. Cumulative IGFBP-3 levels after repletion with regular growth medium (Fig. 2*A*) were also highest in conditioned medium of old and WS cells compared to young cells, at early and late time points, reaching values of 55.6, 28.6, and 6.3 nmol per liter (2.39, 1.23, and 0.27 μg/ml, respectively) at 48 hr. IGF-I was undetectable in any of these media. In contrast, IGF-II levels were wholly contributed by 15% FBS at the time of repletion (Fig. 2*B*) at ≈13 nmol per liter and remained relatively constant in all three cell types during the 48 hr thereafter. Plotting the molar ratios of IGFBP-3/IGF-II (Fig. 2*C*) revealed a hierarchy of old > WS > young HDFs, which was maintained throughout the 48 hr. In all three cell types, after serum repletion, the ratio increased from $<<1$ in the first 18 hr to a peak at 48 hr, at which time these ratios were 3.5 in old, 2.0 in WS, and 0.5 in young cells.

Cell Number and Protein Content After Subculture. Cell counts and protein levels after subculture are shown in Fig.

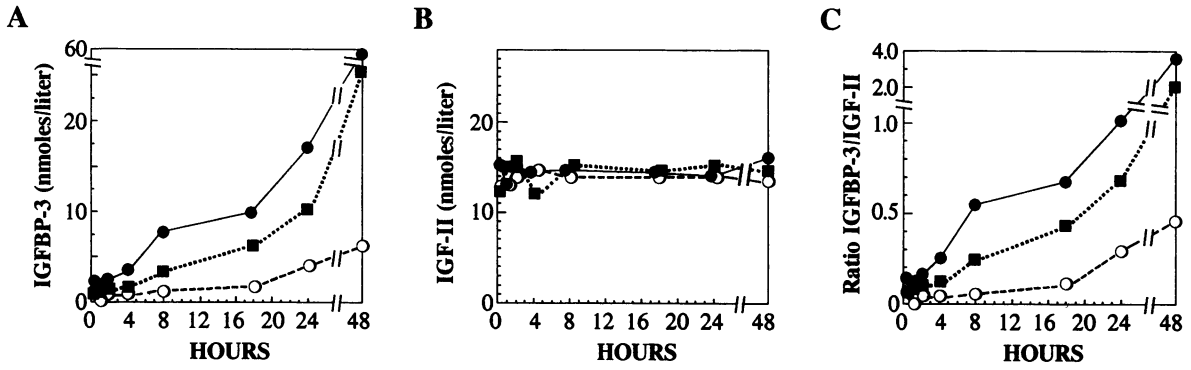

FIG. 2. Levels of IGFBP-3 and IGF-II and the IGFBP-3/IGF-II ratio in conditioned medium of young, old, and WS HDFs after serum depletion/repletion. Medium exposed to cells in Fig. 1 was collected and assayed for IGFBP-3 and IGF-II. Symbols are the same as in Fig. 1.

3 *A* and *B*. Young cells showed rapid exponential growth from low density between days 0 and 4 and decelerating growth between days 4 and 8, with some further proliferation in the postconfluent interval—that is, 1 week after refeeding to day 15. Old and WS cells grew more slowly than young cells through day 8 but also increased between 8 and 15 days. Protein content per dish was highest in young cultures after day 2. The greater differences in cells per dish than in protein per dish again reflect the elevated protein content of senescent compared to young cells.

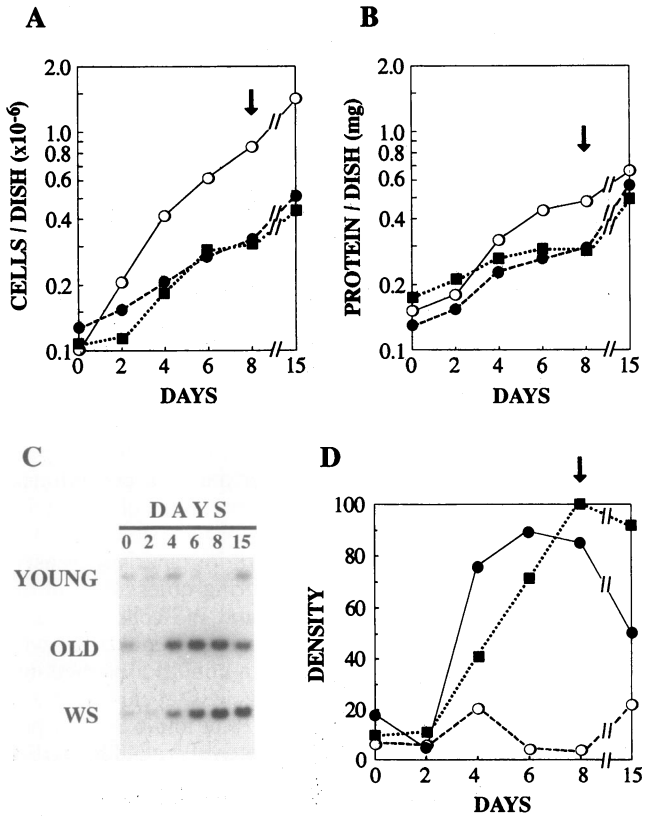

FIG. 3. Cell numbers, total protein content, and IGFBP-3 mRNA levels of young, old, and WS HDFs after subculture. Confluent cultures of HDFs were subdivided into replicate 100-mm dishes containing regular growth medium (15% FBS) at a 1:7 split ratio (young HDFs), a 1:4 ratio (old HDFs), or a 1:3 ratio (WS HDFs) followed by determination at intervals for cell counts (*A*), protein (*B*), and IGFBP-3 mRNA (*C* and *D*). Cells were refed with regular growth medium at day 8 (arrow). Data depicted here were derived from one of two separate experiments, which gave virtually identical results. Symbols are the same as in Figs. 1 and 2.

IGFBP-3 mRNA Content Before and After Subculture. IGFBP-3 mRNA levels before subculture (time 0; Fig. 3 *C* and *D*) were low in all three cell types and remained at this level at day 2 after subculture. At days 4–8, IGFBP-3 mRNA in WS and old cells increased to much higher levels than in young cells, with a downward trend in old cells at day 15. IGFBP-3 mRNA levels in young cells remained relatively low through day 8, with moderate peaks at the midexponential phase of growth (day 4) and at day 15.

IGFBP-3 and IGF-II Levels and Molar Ratios After Subculture. Cumulative IGFBP-3 concentrations in the medium conditioned by old cells were substantially higher than concentrations in media of WS and young cells after subculture (Fig. 4*A*). IGFBP-3 levels in WS medium were higher than in young cell medium at days 8 and 15. In all three cases, however, IGFBP-3 accumulated progressively to day 8 and again accumulated in the 7 days after complete medium replacement to day 15, with the peak value in old HDFs of 227.2 nmol per liter (9.79 μg/ml). Once again, IGF-I could not be detected in any of the media. However, initial IGF-II values of ≈13 nmol per liter (Fig. 4*B*) fell in all three cell types with different time courses and appeared to recover by day 15 (after medium replacement at day 8) in young and WS cells. It appears, therefore, that addition of fresh regular growth medium and high cell density are both required to elicit IGF-II secretion by young and WS cells, as reported for IGF-I secretion in adult rat hepatocytes (30). The molar ratio of IGFBP-3/IGF-II was <1 in all three cell types at day 2 (Fig. 4*C*). However, this ratio increased the fastest and to the highest levels in old cells, attaining a value of >5 at day 4 and a peak ratio of 27 at day 15. In contrast, the IGFBP-3/IGF-II molar ratio in medium of both young and WS cells increased more gradually, exceeding a ratio of 1 at day 6 and reaching peaks of 2.9 at day 8 in young cells and 4.6 at day 15 in WS cells. The ratio fell slightly at day 15 in young cells, apparently due to secretion of IGF-II.

DISCUSSION

We have demonstrated that senescent HDFs, particularly late-passage (old) cells from normal donors, and, to a lesser extent, cells derived from WS of premature aging, express high levels of IGFBP-3 mRNA and IGFBP-3 protein compared to early-passage (young) normal HDFs. Although a major regulatory component of IGFBP-3 gene expression is transcriptional (31), we cannot discern from the present studies, which detected increased steady-state levels of IGFBP-3 mRNA in senescent cells, whether enhanced gene transcription, processing of initial RNA transcripts, or stabilization of IGFBP-3 mRNA are involved, singly or in concert. Nonetheless, it would appear from the frequent disparities between IGFBP-3 mRNA levels (Fig. 1 *C* and *D*

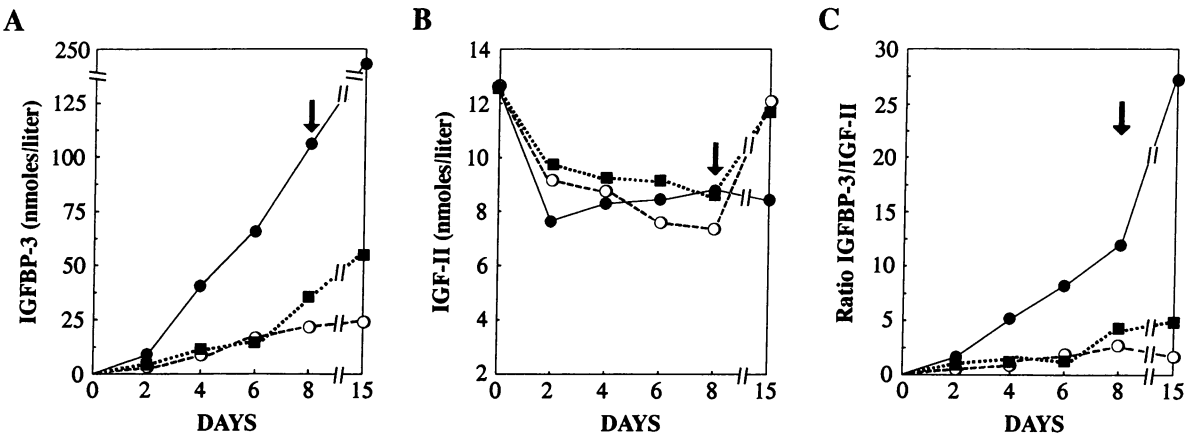

FIG. 4. Levels of IGFBP-3 and IGF-II and the IGFBP-3/IGF-II ratio in conditioned medium of young, old, and WS HDFs after subculture.

and Fig. 3 *C* and *D*) and IGFBP-3 protein production (Figs. 2*A* and 4*A*) that age- and density-dependent modulation occurs for IGFBP-3 mRNA translation, IGFBP-3 protein processing, and/or secretion, and this may be more specifically regarded as disregulation in the case of WS cells. WS is a genetically determined disorder of premature aging, which features genetic instability manifested as chromosomal rearrangements (32), high mutability (33), and an elevated rate of genetic recombination (ref. 34; see also ref. 35). These factors, by leading to one or more qualitative defects in transcription and/or quantitative and qualitative defects in translational and posttranslational steps, could thus contribute to the relative inability of WS cells, compared to senescent normal cells, to elicit an IGFBP-3 protein output commensurate with the generally high levels of cognate mRNA.

The peak values of IGFBP-3 attained in conditioned media of senescent normal cultures are considerably in excess of those observed in a previous report on HDFs (25). These cells, derived from neonatal foreskin and studied at early passage, were cultivated in Ham's F-12 medium supplemented with 0–10% FBS, a range shown to produce a concentration-dependent increase in IGFBP-3 production. Our cells, derived from skin of the adult arm, were cultivated in Eagle's minimal essential medium supplemented with 15% FBS, and it is likely that such variables involving donor age, tissue of origin and biopsy site, composition of basal medium, and quality and concentration of FBS might all contribute to the differences in IGFBP-3 production observed in the two reports. Other laboratories have also found that HDFs derived from various sources including skin and lung of embryos, newborns, and adults secrete a factor that meets the criteria of IGFBP-3 (36–38), but these determinations were semiquantitative. In any case, it is clear that HDF senescence is the major contributor to IGFBP-3 overexpression because the normal strain used here was compared at early and late passage. It is important to emphasize, therefore, that we have observed similarly augmented IGFBP-3 secretion at late passage in 11 additional strains of HDF, 3 derived from fetal lung and 8 from postnatal skin (S.G. and R.C.B., unpublished data).

Increasing molar ratios of IGFBP-3/IGF-II are evident during the 5-day interval of serum depletion and after serum repletion, as cells initiate cell growth, although these ratios do not exceed 1 until 48 hr postrepletion in senescent (old normal and WS) cells. However, in the longer range experiments examining cell proliferation from sparse to confluent and postconfluent cultures, molar ratios of IGFBP-3/IGF-II exceed 1 at day 4—i.e., during early stages of exponential or subconfluent growth in the medium of old cells—and climb rapidly at days 6, 8, and 15. In contrast, this ratio does not exceed 1 in the medium of WS and young cells until early stages of confluence, but it increases to higher levels in WS medium than in young HDF medium.

Although IGF-II is nearly as potent as IGF-I in stimulating DNA synthesis of WI-38 (fetal lung) HDFs, with half-maximal effects occurring at 3.4 nM for IGF-II and 1.5 nM for IGF-I (39), it is 7-fold less potent than IGF-I in HDFs derived from adult skin (half-maximal effects of 8.5 nM and 1.2 nM for IGF-II and IGF-I, respectively) (40). However, in both fetal lung and adult skin HDFs, each IGF stimulates DNA synthesis to an equal extent at maximally effective concentrations, and stimulatory effects of both factors are apparently mediated by the IGF-I receptor (39, 40). We were unable to detect IGF-I in either naive or conditioned medium, although it is possible that levels below 15 ng/ml (2 nM) were present. The lack of sensitivity in IGF measurement resulted from dilution of samples during the HPLC fractionation, a method that rigorously removes all IGFBPs from medium (27), which might otherwise cause false-positive interference in the radioimmunoassay. Levels below 2 nM might be biologically significant in this study since we have found half-maximal values of ≈1 nM for IGF-I action on DNA synthesis for the young normal HDFs (strain J065) used here (E.J.M. and S.G., unpublished data). Nevertheless, even if ≤2 nM IGF-I levels were present, the molar excess of IGFBP-3 in senescent cells would have been greater for IGF-I than was observed for IGF-II and would still be rather high versus both IGFs combined.

Knowledge of IGFBP-3 physiology is at an early stage. Recent studies indicate a varied response of cultured cells to added IGFBP-3 depending on experimental conditions. Whereas preincubation of IGFBP-3 prior to the addition of IGF-I to HDFs and bovine fibroblasts potentiates IGF-I-mediated stimulation of DNA synthesis at molar ratios up to ≈3, coincubation of increasing amounts of IGFBP-3 with IGF-I progressively inhibits DNA synthesis beginning at <<equimolar ratios (17, 18). This is similar to the inhibition of IGF-I-mediated DNA synthesis by high concentrations of human IGFBP-3 in baby hamster kidney fibroblasts (41) and to the inhibition of serum-stimulated DNA synthesis in chicken embryo fibroblasts caused by IDF45, a murine IGFBP probably identical to IGFBP-3 (42). Comparable observations have been made *in vivo*, in wound healing models in which excess concentrations of IGFBP-3 inhibit IGF action on cellular proliferation and DNA synthesis (43). The conditions used here are best reconciled with the coincubation experiments because cells are exposed to IGFs in serum-containing medium and simultaneously begin to secrete IGFBP-3. Nonetheless, it would appear that precise spatiotemporal and molar relationships between IGFBP-3 and IGFs vis-à-vis their effects on cell replication and metabolism still need to be determined. We can envisage that if

growth inhibition is due to elevated ambient levels of IGFBP-3, such inhibition could be reversed by reducing IGFBP-3 levels, by increasing IGF levels, or both. Accordingly, refeeding with high serum-containing medium would be followed by the renewed proliferation evident in cells that had been serum depleted for 5 days (Fig. 1) and in cells undergoing density-dependent inhibition (days 8–15; Fig. 3). Conversely, addition of supraphysiologic concentrations of IGFs in the face of high IGFBP-3 levels could even lead to enhanced cell replication, possibly accounting for recent observations in the residual cycling fraction of senescent HDFs (12).

In conclusion, the concentrations of IGFBP-3 attained in the medium of senescent normal HDFs are in vast excess of those previously shown to be inhibitory to IGF-I-mediated stimulation of DNA synthesis. It is also noteworthy that in all three cell types, including young HDFs IGFBP-3 accumulates in the medium as cultures approach the quiescent state, due to either serum deprivation or high-density growth inhibition. We have no data bearing directly on the role of IGFBP-3 in the replicative arrest associated with senescence or quiescence. It is plausible that increased IGFBP-3 expression represents an adaptation by noncycling cells attempting to compensate for the reduced IGF-mediated stimulation of replication and metabolism. Alternatively, it is tempting to speculate that high levels of IGFBP-3 secreted into the medium by HDFs, and perhaps other cells *in vitro*, sequester IGFs and limit their bioavailability, thus playing a central role in both senescent and quiescent replicative arrest via an autocrine/paracrine mechanism. Indeed, this mechanism could explain several *in vivo* phenomena, including the somatic stunting in subjects with WS, poor wound healing in WS and in many aging normal persons, as well as normal maintenance of the steady-state cell mass of various organs during adulthood. Additional studies should help to distinguish between these adaptive and causal alternatives of growth regulation *in vitro* and *in vivo*.

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