

Role of Insulin-like Growth Factor Binding Protein-3 in Human Umbilical Vein Endothelial Cell Senescence

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ABSTRACT

Whereas insulin-like growth factor binding protein-3 (IGFBP-3) is frequently upregulated in senescent replicatively exhausted human umbilical vein endothelial cells (HUVEC), a systematic analysis of four different HUVEC donors revealed that IGFBP-3 is not consistently upregulated in all isolates at senescence. Lentiviral overexpression of IGFBP-3 inhibited cell proliferation induced apoptosis and senescence in young HUVEC. Knockdown of IGFBP-3 in senescent HUVEC by lentivirally expressed shRNA did not revert but rather enforced senescence-associated β -galactosidase staining and apoptosis. Together the data suggest that, although IGFBP-3 acts as an anti-proliferative and premature senescence-inducing protein, the role of IGFBP-3 on senescence depends on the genetic background of the donor, and additional factors might be important to maintain the senescent phenotype.

INTRODUCTION

INSULIN-LIKE GROWTH FACTORS (IGFs) are established as survival and proliferation factors in many cell culture systems.¹ These effects are primarily mediated by IGF-I dependent activation of the type 1 IGF receptor. The insulin/IGF signaling pathway is highly conserved through evolution and mutations in this pathway increase lifespan in *Caenorhabditis elegans*, *Drosophila melanogaster*, and mice.^{2,3} However the role of IGF-I signaling in human aging is not well understood. The bioactivity of IGF is regulated by IGF binding proteins (IGFBPs) which mainly act via the modulation of IGF bioavailability to the IGF-I receptor. There are six IGFBPs of which IGFBP-3 is particularly well studied.⁴ IGFBP-3 is upregulated in senescent fibroblasts where it may confer resistance to IGF mediated mitotic signaling.^{5,6} Our group

and others have reported an upregulation of IGFBP-3 in senescent endothelial cells suggesting a similar mode of action like in fibroblasts.^{7,8} While this study was in preparation, Kim et al. (2007) reported that ectopic expression of IGFBP-3 in young HUVEC induces a senescence response, suggesting that IGFBP-3 may be a rate-limiting regulator of endothelial cell senescence. In the present study, we have addressed the role of IGFBP-3 in HUVEC senescence using HUVEC isolates derived from four different donors to minimize variations that are due to genetic differences between individual cell strains.

METHODS AND RESULTS

To address regulation of IGFBP-3 in endothelial cell senescence in a systematic way, we

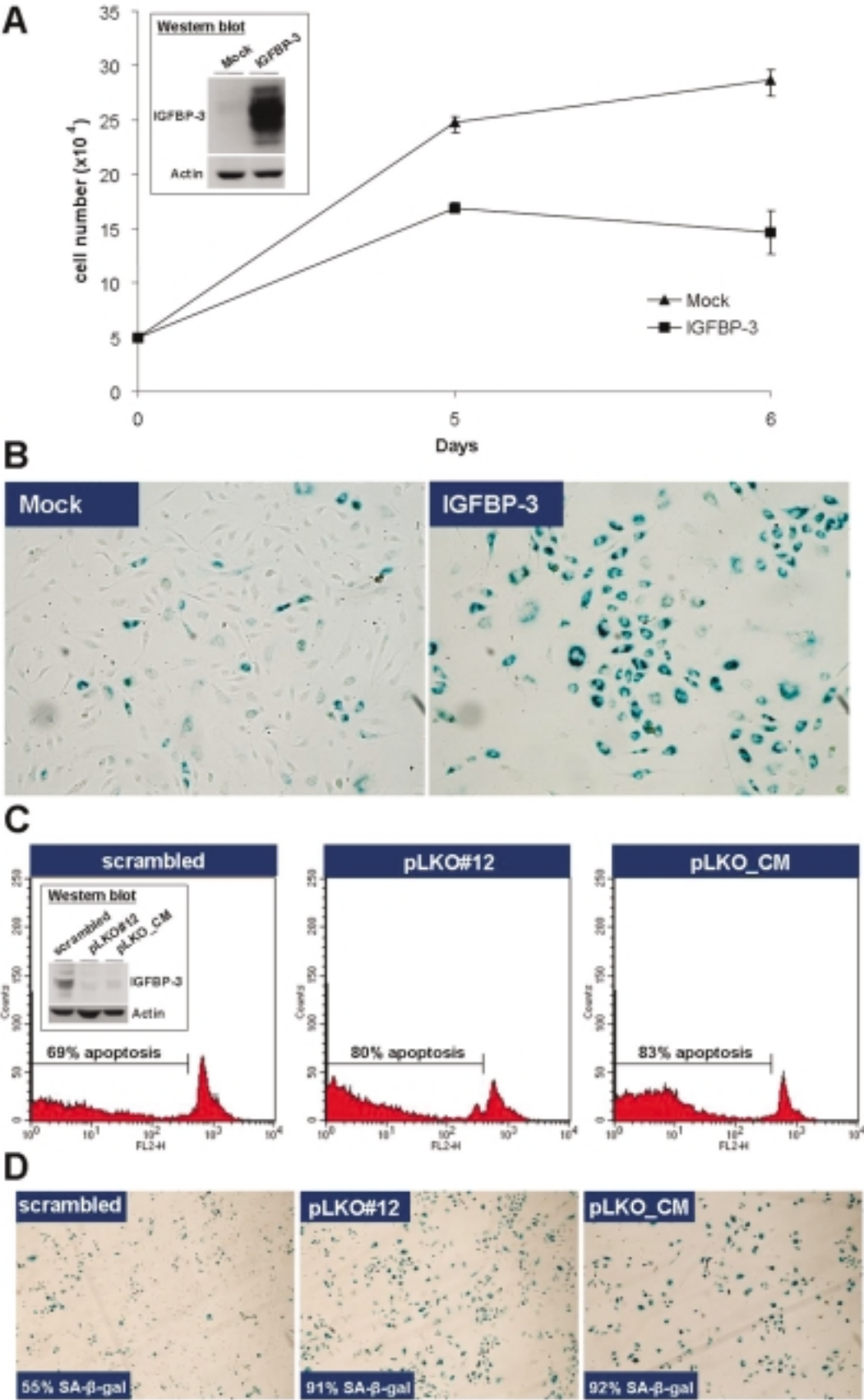
measured the relative amount of IGFBP-3 mRNA in young and senescent HUVEC of four different isolates by real-time quantitative PCR (RTQ-PCR). SYBR green I was used as a reporter and the housekeeper porphobilinogen deaminase as internal standard. The overall expression level of IGFBP-3 in senescent HUVEC was lower than observed for human fibroblasts, which have approximately 10- to 12-fold higher levels of IGFBP-3 mRNA and protein. IGFBP-3 mRNA in HUVEC derived from two donors (B and D) was significantly upregulated by roughly 10- and 4-fold in senescence, whereas IGFBP-3 mRNA levels were not regulated in HUVEC derived from two other donors (A and C, data not shown).

To determine a functional role of IGFBP-3 in endothelial cell senescence, we overexpressed IGFBP-3 by lentiviral transduction in young HUVEC (donor D). Lentiviral transduction of the cells with IGFBP-3 was effective as determined by Western blot after 7 days of overexpression (Fig. 1A). After 6 days of IGFBP-3 overexpression, significantly less viable cells were detected compared to empty vector transduced HUVEC (Fig. 1A). Cell proliferation was assayed by a BrdU-incorporation assay (Roche, Vienna, Austria) and a strong inhibition of cell proliferation was detected after 6 days of IGFBP-3 transduction. About 17% of empty vector transduced cells stained BrdU positive, whereas <1% of IGFBP-3 transduced cells stained positive for BrdU, suggesting that lentiviral overexpression of IGFBP-3 in young HUVEC strongly inhibits cell proliferation (data not shown). After transduction of HU-

VEC with IGFBP-3 we observed rapid induction of senescence within 7 days as monitored by senescence-associated β -galactosidase (SA β -Gal) staining (Fig. 1B). Since it is known that HUVEC senescence involves apoptotic cell death,⁹ IGFBP-3 transduced cells were stained and analyzed for nuclear fragments with DNA content <2N, which are characteristic of apoptotic cell death.⁹ Cell cycle distribution and apoptosis were monitored by propidium iodide (PI)-FACS. On day 5 after transduction, we found an increase of apoptosis in IGFBP-3 transduced HUVEC by 10% and on day 7 by over 40%. A slight reduction of G2 phase cells was observed on day 5 and cell cycle arrest in G1 was observed 7 days after IGFBP-3 transduction (data not shown).

The observation that IGFBP-3 can induce phenotypic features of senescence in young HUVEC raised the question if knockdown of IGFBP-3 in senescent HUVEC could reverse the senescent phenotype. Two lentiviral knockdown vectors targeting different sequences of the IGFBP-3 coding region were applied to address this question. As control, a lentiviral vector expressing a scrambled shRNA sequence was used. Efficient lentiviral knockdown of IGFBP-3 in senescent HUVEC was confirmed by Western blot (Fig. 1C). When we addressed phenotypical consequences of IGFBP-3 knockdown, we found that 7 days of lentiviral knockdown of IGFBP-3 by both short hairpin constructs increased the percentage of apoptotic cells by over 10% (Fig. 1C). We also observed an enforcement of SA β -Gal staining from 55% in control-transduced HUVEC com-

FIG. 1. (A) Western blot of IGFBP-3 transduced young HUVEC and the influence of IGFBP-3 on proliferation. HUVEC in passage 8 were lentivirally transduced using the pLenti6/Ubc/V5-DEST Gateway system (Invitrogen, Carlsbad, CA). The V5 Tag was removed by introduction of a stop codon after the coding sequence of IGFBP-3. For packaging of the lentivirus in 293FT cells (Invitrogen), we used psPAX2 and the envelope encoding plasmid pMD2.G from Addgene. 293FT cells were cultivated in T75 flasks to 90% confluence and transfected with a mixture of 3 μ g pLenti6/Ubc, 7.5 μ g psPAX2, and 2.5 μ g pMD2.G by Lipofectamine (Invitrogen). Supernatant was harvested 48 h and 72 h post-transfection. In U2OS cells the titer of lentiviral particles was approximately 5×10^5 TU/mL. For transduction of HUVEC, a multiplicity of infection of 5 was used together with 8 μ g/mL hexadimethrine bromide as transduction enhancer. (B) Increased SA β -Gal staining¹⁴ after 7 days of IGFBP-3 overexpression in young HUVEC of passage 8. Pictures were taken at 400 \times magnification. (C) Western blot and PI-FACS⁹ showing enforced apoptosis of lentiviral IGFBP-3 knockdown in senescent HUVEC of passage 25 after 7 days of lentiviral knockdown. For knockdown of IGFBP-3 in senescent HUVEC, we used the lentiviral pLKO.1-TRC short hairpin system from Addgene/Open Biosystems. We targeted sequences within the coding region of IGFBP-3: pLKO#12 (5'-GCCGTAGAGAAATGGAA-GACA-3'), pLKO_CM (5'-GCTACAAAGTTGACTACGA-3'). The shRNA for pLKO_CM was designed using the web-based program "siSearch."¹⁵ Lentiviral particles were produced as described in Fig. 1A. (D) Increased SA β -Gal staining after 7 days of IGFBP-3 knockdown in senescent HUVEC of passage 25. Photos were taken at 40 \times magnification.



pared to over 90% in IGFBP-3 knockdown (Fig. 1D).

DISCUSSION

Together these data suggest that high level overexpression of IGFBP-3 has the potential to induce phenotypical changes reminiscent of senescence. However, the observation that the senescent phenotype cannot be rescued and is even enhanced by knockdown of IGFBP-3 in senescent HUVEC suggests that IGFBP-3 is not essential for maintenance of the senescent phenotype in HUVEC.

It is well documented that manipulation of the IGF pathway has an impact on lifespan in many different species.² Due to its role as regulator of IGF-I bioactivity,⁴ IGFBP-3 is an attractive candidate as a modulator of lifespan. However IGFBP-3 has several IGF-independent functions in cell survival, proliferation, and apoptosis. The IGF-independent effects of IGFBP-3 are mediated through binding to matrix, cell surface, cytoplasmic, nuclear, and mitochondrial molecules.¹⁰ Furthermore IGFBP-3 modulates signaling via nuclear receptors, such as retinoid X receptor¹¹ and Nur77,¹² and regulates gene expression^{11–13} involved in regulation of apoptosis pathways.

For human aging, cell culture models are used and it was shown that IGFBP-3 is upregulated upon replicative senescence in fibroblasts.^{5,6} However the phenotypic consequences of IGFBP-3 knockdown in senescent fibroblasts are unknown. For human endothelial cells, we and others have reported an upregulation of IGFBP-3 upon replicative senescence.^{7,8} However the systematic analysis of four HUVEC donors in the current study showed that in just two of them IGFBP-3 was upregulated in senescence, suggesting that IGFBP-3 is not essential for the senescent phenotype in HUVEC senescence. Concerning the causal role of IGFBP-3 in HUVEC senescence, the data reported recently by Kim et al. (2007) and our own results reported here clearly indicate that high level overexpression of IGFBP-3 induces a premature senescence phenotype with induction of apoptosis, inhibition of cell proliferation, and increased SA β -Gal staining.

Downregulation of endogenous IGFBP-3 in senescent HUVEC did not revert the senescent phenotype; rather we noticed an increase in apoptosis and further decrease in cell number. This is in contrast to the report by Kim et al. (2007). These differences might be due to cell culture conditions and timing of IGFBP-3 knockdown. Moreover, genetic differences between individual donors may influence endothelial cell senescence *in vitro*.

In summary, our results suggest that high levels of IGFBP-3 are sufficient to induce senescence in human endothelial cells. However, IGFBP-3 overexpression is not always required for maintenance of the senescent phenotype. To further address this question, it will be most informative to analyze IGFBP-3 expression levels in human vascular biopsies from larger cohorts.

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