Reductions in serum IGF-1 during aging impair health span

Zhenwei Gong,1* Oran Kennedy,2* Hui Sun,3* Yingjie Wu,3,9* Garry A Williams,3 Laura Klein,1 Luis Cardoso,4 Ronald W. Matheny Jr,5,6,10 Gene B. Hubbard,7 Yuji Ikeno,7 Roger P. Farrar,8 Mitchell B Schaffler,4 Martin L Adamo,3,6† Radhika H Muzumdar11 and Shoshana Yakar312

1Department of Pediatrics, Albert Einstein College of Medicine, Bronx, New York 10461, USA
2Department of Orthopaedic Surgery, New York University Hospital for Joint Diseases, NY, NY 10003, USA
3David B. Kriser Dental Center, Department of Basic Science and Craniofacial Biology, New York University College of Dentistry, New York, NY 10010, USA
4Department of Biomedical Engineering, The City College of New York, New York, NY 10031, USA
5Department of Biochemistry, University of Texas Health Science Center, San Antonio, TX 78229, USA
6Sam and Ann Barshop Institute for Longevity and Aging Studies, University of Texas Health Science Center, San Antonio, TX 78229, USA
7Department of Pathology, University of Texas Health Science Center, San Antonio, TX 78229, USA
8Department of Kinesiology and Health Education, University of Texas at Austin, Austin, TX 78712, USA

Summary

In lower or simple species, such as worms and flies, disruption of the insulin-like growth factor (IGF)-1 and the insulin signaling pathways has been shown to increase lifespan. In rodents, however, growth hormone (GH) regulates IGF-1 levels in serum and tissues and can modulate lifespan via/or independent of IGF-1. Rodent models, where the GH/IGF-1 axis was ablated congenitally, show increased lifespan. However, in contrast to rodents where serum IGF-1 levels are high throughout life, in humans, serum IGF-1 peaks during puberty and declines thereafter during aging. Thus, animal models with congenital disruption of the GH/IGF-1 axis are unable to clearly distinguish between developmental and age-related effects of GH/IGF-1 on health. To overcome this caveat, we developed an inducible liver IGF-1-deficient (iLID) mouse that allows temporal control of serum IGF-1. Deletion of liver Igf1 gene at one year of age reduced serum IGF-1 by 70% and dramatically impaired health span of the iLID mice. Reductions in serum IGF-1 were coupled with increased GH levels and increased basal STAT5B phosphorylation in livers of iLID mice. These changes were associated with increased liver weight, increased liver inflammation, increased oxidative stress in liver and muscle, and increased incidence of hepatic tumors. Lastly, despite elevations in serum GH, low levels of serum IGF-1 from 1 year of age compromised skeletal integrity and accelerated bone loss. We conclude that an intact GH/IGF-1 axis is essential to maintain health span and that elevated GH, even late in life, associates with increased pathology.

Key words: aging; bone; growth hormone; IGF; insulin-sensitivity; LID; lifespan; liver; tumor.

Introduction

The role of growth hormone (GH)/insulin-like growth factor-1 (IGF-1) axis in lifespan has been studied in detail along the evolutionary lineage. Disruption of insulin/IGF-like signaling in yeast, worms, and flies has been conclusively shown to increase lifespan. In these lower order species, insulin and IGF-1 axis exists as a single axis. However, in rodents and other mammalian species, the insulin and IGF-1 axes are complex and distinct even though they share homology. In mammals, GH regulates serum IGF-1 levels, and while the insulin axis predominantly influences metabolism, the GH/IGF-1 axis is concerned with cell survival and growth.

The evidence for the role of IGF-1 in health/lifespan in primates is even less conclusive (Colman et al., 2009; Mattison et al., 2012; Stein et al., 2012). In humans, serum GH concentrations and GH secretion rates fall in adulthood, accompanied by a gradual fall in plasma IGF-1 concentrations, a state often termed the somatopause. In humans, reductions in IGF-1 also associate with higher incidence of diabetes (Barzilai et al., 2012), osteoporosis (Lombardi et al., 2005), dementia, and Alzheimer disease (O’Neill et al., 2012). Decline in GH/IGF-1 axis has been also shown to be associated with decreased incidence of tumors (Bartke et al., 2013). Indeed, human subjects with mutations in the GHR (Laron dwarfs) who show congenital IGF-1 deficiency do not exhibit increases in lifespan, but show protection from development of malignancies and diabetes when compared to their healthy siblings (Laron, 2005; Guevara-Aguirre et al., 2011).

Rodent models where global IGF-1 is decreased, such as caloric restriction (Wolf et al., 2000; Ward et al., 2005), and GH receptor deletion in mice (Hauck & Bartke, 2000; Westbrook et al., 2009) show increased lifespan. On the other hand, mice overexpressing GH throughout their lifespan live shorter (Bartke et al., 2002; Sonntag et al., 2012). Although the aforementioned studies point to a simple cause and effect relationship between the GH/IGF-1 axis and lifespan, experiments in many rodent models point to more complex interactions. The latter may depend on species, genetic background, gender, or even husbandry conditions. For example, female mice with igf1 receptor (igfr) haploinsufficiency lived longer than control females in one study (Holzenberger et al., 2003), but when repeated, the effect was smaller (Bokov et al., 2011). In addition to these studies, a brain-specific knockout of the igf1r was associated with increased median lifespan. However, these animals also show reduced GH levels, and thus, the effects of the igf1r deficiency cannot be distinguished from those of reduced GH levels (Kappeler et al., 2008). Furthermore, animal models in which components of the IGF-1 receptor signaling pathway were

Correspondence
Shoshana Yakar, PhD, Associate Professor, New York University College of Dentistry, David B. Kriser Dental Center, Department of Basic Science and Craniofacial Biology, 345 East 24th Street, New York, NY 10010-4086, USA. Tel.: +1 212 998 9721 Fax: +1 212 995 4087; e-mail: sy1007@nyu.edu All authors concur with the submission. The material submitted for publication has not been previously reported and is not under consideration for publication elsewhere.
*Equal contribution. †Shared senior PI.
*Present address: College of Integrative Medicine and Institute of Integrative Medicine, Dalian Medical University, Dalian, China
†Present address: US Army Research Institute of Environmental Medicine, Military Performance Division, Natick, MA 01760, USA

Accepted for publication 14 November 2013

© 2013 The Authors. Aging Cell published by the Anatomical Society and John Wiley & Sons Ltd. This is an open-access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.
ablated, exhibit no change, or even reduced lifespan (Taguchi et al., 2007; Selman et al., 2008) as compared to controls.

While studies in rodents provide significant insights into the GH/IGF system and its role in aging, interpretations of the observations are complicated due to (i) developmental effects secondary to loss of the GH/IGF axis during crucial phases of growth and development and (ii) difficulties in distinguishing effects of serum IGF-1 from tissue IGF-1, and those arising from secondary increases in GH.

To better address how decline in serum IGF-1 affects aging, we used two mouse models: (i) congenital liver IGF-1-deficient (LID) mice, where serum IGF-1 is low throughout lifespan, and (ii) an inducible liver Igf1 gene-deleted (iLID) mouse model (Wu et al., 2010; Courtland et al., 2011), which allows temporal control of IGF-1. The iLID mice express the Cre recombinase under the liver-specific ET (tamoxifen-inducible-)α1-antitrypsin promoter, and induction of the Cre recombinase was achieved by a single intraperitoneal injection of tamoxifen.

Using the congenital LID cohort, we performed survival studies and end-of-life pathology. Using the iLID model, we depleted serum IGF-1 levels when mice reached 1 year of age and followed them up to 2 years of age. Unfortunately, we did not perform survival studies in the iLID cohort, but at 2 years, we studied inflammation, oxidative stress resistance, tumor incidence, and bone loss.

Results

Congenital decreases in serum IGF-1 impair lifespan in male mice

To understand how reductions in serum IGF-1 levels affect overall lifespan, we used the LID mouse model, where the Cre recombinase was expressed under the albumin promoter, specifically in the liver (Yakar et al., 1999). LID mice show early postnatal gene recombination (1 week old) and exhibit reductions in serum IGF-1 levels throughout life with concomitant increases in serum GH levels. Male and female (control and LID) mice were aged in the Nathan Shock Center at Texas Health Science Center, San Antonio (UTHSCSA). We observed robust longevity in both control male (median lifespan of 34.7 ± 1.3 months) and control female (median lifespan of 34.8 ± 1.0 months) mice. The overall survival, as analyzed by the log-rank test, was similar in female LID (with median lifespan of 36.2 ± 1.2 months). In contrast, overall survival of male LID mice was 27.9 ± 1.7 months, which was statistically significantly lower (P = 0.006) than the other groups (Fig. 1A, Table S1). Male LID mice showed decreased body weight throughout life (Fig. 1B) with similar food intake (Fig. 1C) when compared to controls.

Tissues dissected from a subcohort of male LID and control mice at 19-20 months of age (Table S2) revealed significant increases in absolute and relative liver weight in LID mice. Additionally, we found that absolute and relative kidney weight and quadriceps weight were significantly reduced in LID mice when compared to controls.

The probable causes of death for the LID and control groups are shown in Table 1. Approximately 22% of male control and 55% of female control mice died from neoplastic diseases. The major fatal neoplastic diseases observed in these control mice were hepatocellular carcinoma (HCC), pulmonary adenocarcinoma, and pituitary adenoma (female mice only). In addition, the neoplastic diseases were usually associated with metastasis to the other organs or other pathological lesions, for example pleural effusion, ascites, hemorrhage in the pleural and/or abdominal cavities, and severe congestion and edema in the lung. Male LID mice showed a higher incidence of fatal neoplasms (approximately 69%) than their control mice, although the changes in incidence were not statistically significant. The incidence of fatal HCC was significantly higher in male LID mice (approximately 69%) than the control group (5.6%; P < 0.05). The female LID mice showed a similar incidence of fatal neoplasms compared with their control mice. Although the incidence of fatal HCC was slightly higher in female LID
mice (approximately 24%) than in the control group (4.5%), the difference was not statistically significant. The major fatal non-neoplastic diseases observed in these mice were glomerulonephritis, thrombus in heart, hydropspherosis, and acidophilic macrophage pneumonia. These lesions were usually associated with other pathological lesions, for example pleural effusion, ascites, and/or severe congestion and edema in the lung. The incidence of fatal glomerulonephritis was slightly lower in both male and female LID mice than in the control group; however, the difference was not statistically significant.

The described studies with the LID model indicated that lifelong reductions in serum IGF-1 levels impaired health span mainly in male mice and coincided with hepatomegaly, decreased muscle and kidney weight, and increased HCC. However, one limitation of the LID mouse model is lifelong reductions in serum IGF-1; thus, the true impact of serum IGF-1 reductions after sexual maturation, during adulthood, remained unclear. To understand how reductions in serum IGF-1 during adulthood affect aging, we developed an inducible LID (iLID) model.

**Generation of a mouse model with age-dependent reductions in serum IGF-1 levels**

Generation of the iLID mice, described earlier (Wu et al., 2010; Courtland et al., 2011), was achieved by crossing mice homozygous for the floxed IGF-1 allele with mice expressing the Cre recombinase under the tamoxifen-inducible antitrypsin 1-α promoter specifically in the liver. Control mice were homozygous for the floxed IGF-1 allele but did not carry the Cre transgene. Ablation of hepatic IGF-1 gene was induced in iLID mice by a single intraperitoneal injection of 0.3 mg of tamoxifen at one year of age (Fig. 2A). Control mice were homozygous for the floxed Igf-1 allele but did not carry the Cre transgene. Ablation of hepatic Igf-1 gene was induced in iLID mice, we assessed Igf-1 gene expression in livers of control and iLID mice at sacrifice. Indeed, liver Igf-1 gene expression was significantly reduced in iLID mice (Fig. 2B), leading to marked reductions in serum IGF-1 levels (Fig. 2C) and a concomitant increase in serum GH levels (Fig. 2D).

**Table 1** End-of-life pathology of control and LID mice aged in the Nathan Shock Center at Texas Health Center, San Antonio (UTHSCSA)

|                     | Male   | Female  |
|---------------------|--------|---------|
|                     | Control| LID     | Control| LID     |
| Neoplasms           |        |         |        |         |
| Neoplasms           | 4 (22.2%) | 11 (68.8%) | 12 (54.5%) | 10 (58.8%) |
| Hepatocellular       | 1 (5.6%) | 11 (68.8%)* | 1 (4.5%) | 4 (23.5) |
| Adenocarcinoma       | 3       | 0       | 1       | 3       |
| Pituitary adenoma    | 0       | 0       | 2       | 1       |
| Others               | 0       | 0       | 8       | 2       |
| Non-neoplasms        | 14      | 5       | 10      | 7       |
| Glomerulonephritis   | 6       | 1       | 8       | 2       |
| Acidophilic mac.pn.  | 3       | 1       | 0       | 0       |
| Others               | 3       | 0       | 1       | 2       |
| Undetermined         | 4       | 1       | 1       | 3       |

*P < 0.05.

**Fig. 2** Validation of the iLID mouse model. (A) Schematic presentation of the iLID mouse model. (B) Hepatic Igf-1 gene expression at 2 years of age in iLID (n = 5) and control (n = 5) mice following a single intraperitoneal injection of 0.3 mg of tamoxifen at one year of age. (C) Serum IGF-1 levels at 2 years of age in iLID (n = 30) and control (n = 20) mice. (D) Serum GH levels at 2 years of age in iLID (n = 10) and control (n = 9) mice. Significance (*) was considered at P < 0.05.

**Age-dependent reductions in serum IGF-1 are associated with increased liver weight, inflammation, and tumors**

Depletion of liver Igf-1 at ‘middle age’ (~1 year old in a mouse lifespan) in male iLID mice resulted in minor, but significant reductions in body weight and gonadal fat pad weights at two years of age (Table S3). Although a direct comparison between the LID cohort and the iLID cohort cannot be made, we found that similar to the congenital LID mice, reductions in serum IGF-1 in the iLID mice (in mid-life) associated with significant reductions in kidney weight (Tables S2, S3). Additionally, we found that relative heart weight increased in iLID mice as compared to controls (while this was not significant in the LID cohort).

Interestingly, as seen in the LID cohort, we found that iLID mice had significantly increased relative liver weight (Fig. 3A). This was associated with enhanced basal STAT5b phosphorylation (pSTAT5b) in livers of iLID mice (P = 0.0015), possibly due to increased serum GH levels (Fig. 3B). Furthermore, in accordance with previous publications using GH transgenic mice (Miquet et al., 2013), we found that aged iLID hepatocytes manifested a higher mean nuclear area when compared to controls (Fig. 3C) with a shift to larger nuclear area in the nuclear size distribution histogram.

Histological examination of liver sections revealed no increase in hepatic lipid accumulation (data not shown). However, there was an
increased lymphoplasmacytic infiltrate in the portal regions in iLID mice, while no inflammatory infiltrates were found in control mice at 2 years of age (Fig. 3D). Additionally, the number of Iba1 (a marker of macrophages) positive Kupffer cells significantly increased in iLID livers (Fig. 3D). Likewise, F4/80 gene expression, a marker for macrophages, was significantly higher in iLID livers (Fig. 3E). Consistent with an increase in inflammatory infiltrate, gene expression of the cytokines IL-6 and TNFα was significantly increased in livers of iLID mice (Fig. 3E).

Moreover, the profile of cytokines in serum in mice showed marginally higher levels in the iLIDs compared with controls, although no significant differences were found (Fig. 3F, Table S4).

Macroscopic (PET scan) and histological examinations indicated an increase in the incidence of hepatic tumors in the iLID mice at two years of age (Fig. 3G). The incidence of hepatic tumors was 26% in control and 42% in iLID mice, but did not reach significance. Extrahepatic tumor incidence was 11% in control and 3% in iLID mice (Table S5).

Fig. 3 Inducible liver IGF-1-deficient mice show increased hepatic size, inflammation, and tumors. (A) Relative liver weight increased significantly in iLID mice (n = 36) as compared to controls (n = 27). (B) iLID mice (n = 10) show increased basal STAT5b-phosphorylation (pSTAT5b) as compared to controls (n = 10, P = 0.0015). Phosphorylated STAT5b levels were corrected to total STAT5b protein levels detected using the same membrane. (C) Nuclear size was evaluated in H&E-stained liver sections. Fifty nuclei per section were measured and calibrated to an image scale bar using ImageJ software. Distribution histogram shows nuclear size in control and iLID mice, and insert bar graph shows mean nuclear size (N = 5 mice per group). (D) The fraction (%) of Iba1-positive Kupffer cells counted in three sections per mouse from control (n = 4) and iLID livers (n = 5 per group). iLID mice showed significant (P = 0.008) increase in Iba1-positive cells in their livers. (E) Gene expression of markers of inflammation in iLID and control livers (n > 7). (F) Serum cytokine levels in control and iLID mice (graph represents overall cytokines measured in all mice, see also Table S4). (G) A representative PET scan image of iLID and control mice demonstrating increased uptake at the site of liver tumor.
of control and iLID mice, PET scans were taken to localize tumors. Figure 3G is a representative PET scan image of iLID and control mice demonstrating increased FDG uptake at the site of the tumor. The location of tumors as noted on PET scans was confirmed at the time of sacrifice.

**Age-dependent reductions in serum IGF-1 is associated with increased oxidative stress in muscle and liver**

We examined how reductions in serum IGF-1 levels during aging affect reactive oxygen species (ROS) generation by assaying lipid peroxidation (8-iso-prostaglandin F2-alpha (8-isoprostane)) in serum. This assay requires large amounts of sera, and therefore, we had to pool samples.

Surprisingly, we found that iLID and control mice had comparable levels of serum isoprostanes (Fig. 4A).

To better understand how hepatic Igf-1 gene deletion affected oxidative stress resistance in liver and muscle, we used the OxyBlot™ (OxyBlot Protein Oxidation Detection Kit S7150; Millipore, Billerica, MA, USA) Protein Oxidation Detection method. This assay detects carbonyl group-containing proteins that result from oxidative reactions with ozone, oxides of nitrogen, or by metal-catalyzed oxidation. We found that livers of iLID mice showed a 60% increase in oxidized proteins as compared to controls, whereas muscles of iLID mice had a twofold increase in oxidized proteins compared with controls (Fig. 4B).

Antioxidant defense includes induction of enzymatic machinery that converts superoxide to hydrogen peroxide and molecular oxygen. The

---

Fig. 4 Inducible liver IGF-1-deficient mice show increased markers of oxidative stress. (A) Serum isoprostanes in control (n = 4) and iLID (n = 4) mice. (B) Carbonyl group-contained proteins, detected by OxyBlot™ Protein Oxidation Detection Kit, increased in both livers and muscles of iLID mice compared with controls (n > 4 per group). Relative density corresponds to whole lane. (C) Gene expression of markers of antioxidant defense enzymes in the livers and muscles of iLID (n = 5) and control (n = 5) mice. (D) Peroxide-induced PARP cleavage in primary skin fibroblasts isolated from 2-year-old control (n = 4) and iLID mice (n = 5). Cultures were grown to 80% confluence and treated with 400 μM peroxide for 8 and 24 h. PARP cleavage was detected by conventional Western immunoblotting. The levels of cleaved PARP were corrected to both total PARP and β-actin, detected on the same blot.
Nrf2 transcription factor is a master regulator of genes that are involved in detoxification of harmful compounds such as glutathione peroxidase (Gpx1) and catalase 2 enzymes. We therefore examined the expression levels of Nrf2, Gpx1, and catalase in liver and muscle of iLID and control mice at 2 years of age. We found that there were no differences in Nrf2 gene expression (assessed by 2 sets of primers) in liver and muscle from iLID mice as compared to controls (Fig. 4C). However, Gpx1 gene expression significantly decreased (60% reduction) in the livers (but not in muscle) of iLID mice, while no changes were detected in the expression levels of catalase and superoxide dismutase in the livers (Fig. 4C) or muscle between the groups.

Lastly, we assessed oxidative stress resistance of dermal fibroblasts isolated at time of dissection from 2-year-old mice. Primary fibroblast cultures were grown to 80% confluence and treated with peroxide (400uM) for 8 or 24 h. As seen in Fig. 4D, peroxide induced significantly more PARP cleavage in fibroblasts isolated from aged iLID mice as compared to controls. This was evident when the intensity of the cleaved PARP band was corrected to intact PARP or to the β-actin band intensities. All together, these data indicate that iLID fibroblasts were more sensitive to the cytotoxic effects of peroxide.

Reductions in serum IGF-1 during aging compromise skeletal integrity

Femurs dissected from 2-year-old control and iLID mice were analyzed by micro-CT (Table 2, Fig. 5A). We found that reductions in serum IGF-1 during aging compromise skeletal integrity. For example, reductions in serum IGF-1 during aging compromise skeletal integrity. For example, reductions in serum IGF-1 levels in iLID mice at one year resulted in significant loss of cortical bone at two years. This was evident by reduced total cross-sectional area (Tt.Ar), cortical bone area (Ct.Ar), cortical bone thickness (Ct.Th), marrow area (Ma.Ar), and relative cortical bone area (RCA; Ct.Ar/Tt.Ar). We found that iLID aged bones were mechanically inferior when compared to controls and an increase in trabecular thickness. However, overall bone volume per total volume (%BV/TV) and bone mineral density (BMD) at the distal femur were comparable between the groups (Table 2). Mechanical properties of bones, dissected at 2 years, followed the morphological properties obtained by micro-CT. Four-point bending testing showed that iLID aged bones were mechanically inferior when compared to controls (Table 2). As such, max load, stiffness, yield to fracture, and postyield displacement were all significantly lower in iLID bones.

Bone mineral composition was measured by inductively coupled plasma (ICP) assay (Table 2) in whole femurs dissected from 2-year-old mice. We found that iLID mice had significantly lower ratio of Ca/Pi when compared to controls, which was not reflected in tissue mineral density (TMD) of cortical bone or in trabecular BMD.

To understand whether reductions in serum IGF-1 affected the number of osteoprogenitors in the bone marrow, we established osteoblast-like primary cultures. The number of alkaline positive colonies after 14, 21, and 28 days in cultures was similar between controls and iLID mice (Fig. 5B). Osteoclast primary cultures did not differ between the groups (data not shown).

Serum osteocalcin, a marker of bone formation, did not differ between control (29.8 ± 6.9 ng mL$^{-1}$) and iLID mice (28.1 ± 11.5 ng mL$^{-1}$) at two years of age. Another important hormone, which regulates bone remodeling during aging is parathyroid hormone (PTH). Elevations in PTH often associate with reduced bone mass. We found that control and iLID mice show similar levels of PTH in serum (Fig. 5C).

Discussion

In this study, we have aged two animal cohorts of congenital liver IGF-1 deficiency (LID) and an inducible LID (iLID) and studied the role of the GH/IGF-1 axis during aging. Although a direct comparison between the 2 cohorts cannot be made, we have revealed that reductions in serum IGF-1 levels in both LID and iLID mice with concomitant increases in GH associated with hepatomegaly, decreased kidney size and increased incidence of hepatic tumors. In both models, we show that in face of low levels of serum IGF-1, high levels of GH (even during aging only) associate with increased pathology.

Survival studies with the LID mouse model have revealed that congenital decreases in serum IGF-1 levels (LID) associated with decreased lifespan in male mice and increased incidence of hepatic tumors, likely reflecting lifelong increases in GH secretion. In contrast to our results showing no change in female LID lifespan, Svensson et al. (2011) observed increased median lifespan of female LID mice with no significant difference in male LID lifespan compared with wild-type mice. In that study, LID mice also showed reduced body weight similar to what we observed. The reason(s) why our current results differ from Svensson et al. is unclear, but could be related to the different genetic backgrounds between the control and LID mice in our study. However, it is also worth noting that the control mice in our study had robust median lifespan of over 30 months, whereas the control mice in the Svensson et al. study had median lifespans of 22-23 months, which is shorter than expected for C57BL/6 strain. Thus, the LID female mice in the Svensson study may have been protected against some life-shortening stimulus. Likewise, in longevity studies (conducted at three different locations) with mice homozygous for an insertion in the Igf1
A very large cohort of patients with acromegaly, with high levels of T-cell activity and a decrease in B-cell activity have been demonstrated in controls (Sneppen et al., 2013). However, mean lifespan was increased in female IGF-1-deficient animals in only one site and early life mortality was noted in one cohort of IGF-1-deficient mice, indicating that reductions in IGF-1 alone are insufficient to increase both mean and maximal lifespan in mice.

Using the iLID model, we induced IGF-1 deficiency after the critical phase of growth and development and followed the mice up to two years of age. We have found that in iLID mice reductions in serum IGF-1 and concomitant increases in GH levels during aging associated with increased liver weight, systemic and liver inflammation, increased oxidative stress, and increased incidence of hepatic tumors, although this did not reach significance. We postulate that this phenotype relates to the unopposed action of high GH levels on hepatocytes, as evidenced by the increased basal STAT5b phosphorylation in livers from iLID mice. Possible increase in hepatic GH action was also evident by increase in hepatocyte nuclear size in the iLID mice. These data are in accordance with previous observations using the GH transgenic mice (Miquet et al., 2013), which also manifested increased hepatocellular nuclear size. Using the iLID model, we show, for the first time, that elevations in GH late in life have detrimental effects on the liver.

Inducible liver IGF-1-deficient mice showed increased macrophage infiltration and other cellular infiltrate in the liver. GH has been implicated in recruitment of inflammatory cells (Francescchi et al., 2005), suggesting that the excess of GH action in the liver of the iLID mice triggered hepatic inflammation. In GH-deficient (GHD) patients, who exhibit major reductions in serum IGF-1, there is significantly impairment of natural killer (NK) cell activity, both unstimulated and stimulated by INFγ or IL-2. Similarly, NK cell concentration and the proportion of NK cells (CD16+) were reduced in GHD patients compared with controls (Sneppen et al., 2002). On the other hand, an increase in T-cell activity and a decrease in B-cell activity have been demonstrated in a very large cohort of patients with acromegaly, with high levels of serum GH and IGF-1 (Colao et al., 2002). However, despite this valuable information, these studies demonstrate conditions in which the changes in GH and IGF-1 paralleled, namely both decreased as seen in states of GHD, or increased as seen in acromegaly. Thus, the relative contribution of each hormone to the inflammatory phenotype is hard to be determined. In the iLID model, we show that in the face of low levels of serum IGF-1, high levels of GH in serum associate with increased inflammation. These results strongly suggest causality between GH elevations and hepatic inflammation.

The aforementioned increases in hepatic inflammation, oxidative stress, and GHR activation in livers of the iLID mice were associated with increased incidence of hepatic tumors, although this did not reach significance. In LID mice, however, where serum IGF-1 decreased throughout lifespan, we have shown a significant increase in liver tumors. We attribute the increase in hepatic tumor incidence to the high levels of GH in the LID and iLID mice. Our findings are in line with previous observations showing a link between increased GH signaling and hepatic tumors (Baik et al., 2011; Mueller et al., 2011; Miquet et al., 2013). Accordingly, Sonntag et al. (2005) has shown that reductions in both GH and IGF-1 during adulthood and aging, reduced neoplastic disease, nephropathy, and total disease burden. Unfortunately, we did not perform survival studies in the LID cohort, and thus, we cannot conclude that the findings above contributed to the overall mortality of the iLID mice.

Aged IID mice demonstrated increased oxidative stress in liver and muscle. This was evident by significant increases in tissue levels of oxidative proteins in both livers and muscles of iLID mice as compared to controls. Our findings are consistent with recent studies demonstrating increased cellular oxidative stress in aged animals with adult onset IGF-1 deficiency (Bailey-Downs et al., 2012). In that study, cultured aorta segments of IGF-1-deficient mice treated with a panel of oxidative stressors showed attenuation in Nrf2-driven genes, leading to endothelial dysfunction, increased oxidative stress, and apoptosis. In line with these data, it has been shown that IGF-1 can oppose oxidative stress-induced damage in the vasculature by up-regulating glutathione peroxidase (Higashi et al., 2013). Consistent with this evidence, we have found that fibroblasts isolated from aged iLID mice show increased sensitivity to peroxide, as reflected by increased levels of cleaved PARP. We postulate that the increased sensitivity to oxidative stress is due to increases in serum GH levels during aging. It has been previously shown that fibroblast cultures from aged GH-deficient Snell, Ames, and GHKO mice were resistant to the cytotoxic effects of peroxide and paraquat (Murakami et al., 2003; Salmon et al., 2005). In Snell dwarfs, resistance to peroxide and paraquat was associated with increased catalase activity (Page et al., 2009).

Lastly, we studied bone loss, another physiological hallmark of aging. In humans, the physiological decline in GH and IGF-1 levels is linked to osteoporosis. Using the iLID model, we were able to delineate the role of the GH/IGF-1 axis in skeletal aging, without the confounding effects of

© 2013 The Authors. Aging Cell published by the Anatomical Society and John Wiley & Sons Ltd.
this axis on the growing skeleton. In our previous studies characterizing the LID mice during development, we found that reduced serum levels of IGF-1 in LID mice were associated with the development of slender bones (Eliš et al., 2010a). This slender phenotype developed at an early pubertal age and persisted to 52 weeks, affecting mostly radial bone growth and minimally affecting linear growth. In a subsequent study, we performed skeletal characterization of the iLID mice, where depletion of serum IGF-1 was induced at 4 (prepuberty) and at 8 (postpuberty) weeks of age (Courtland et al., 2011). We showed that depletion of serum IGF-1 during advanced adulthood (iLID), when bone loss becomes more prevalent, and we showed that reductions in serum IGF-1 during aging impaired bone integrity. Taken together, data from both studies show that depletion of serum IGF-1 during growth affects skeletal development and peak bone accrual, while during aging, serum IGF-1 appears to play protective roles on the skeleton and its reductions affect mainly the cortical compartment.

In conclusion, animal and human studies have revealed major roles of the GH/IGF-1 axis in the regulation of growth, development, and organ function. As such, decline in these two pleiotropic hormones during aging was shown to associate with organ dysfunction. On the other hand, high normal levels of GH/IGF-1 during aging have been associated with the development of neoplastic disease in both human and animals, and complete lifelong suppression of GH signaling in rodents produces robust extension of longevity. Thus, the involvement of this axis in the aging phenotype remains controversial, and the relative contribution of each hormone (GH or IGF-1) to age-related pathology is still doubtful and may be tissue specific. We have shown here that low circulating levels of IGF-1 coupled with high levels of GH during aging, associate with hepatomegaly and increased hepatic pathology in both LID and iLID models and with reduced longevity of LID males. At the same time, we show that elevations in GH were insufficient to protect against bone loss during aging.

Methods

Animals

The iLID model (C57BL/6 background) has been described previously (Courtland et al., 2011). Male mice used in this study were given unrestricted access to water and food and housed to a maximum of 5 per cage under a 12 h light/dark cycle. Animal care and maintenance were provided through the UTHSCSA AAALAC Accredited Animal Facility. All procedures approved by the Institutional Animal Care and Use Committee of the NYU. See additional details in supplemental methods. Lifespan studies of LID mice on a mixed genetic background (C57BL/6, FVB/N, and 129svs) were conducted in the Nathan Shock Center at Texas Health Center, San Antonio (UTHSCSA). Food intake was also recorded. Pathological lesions were identified and graded for severity as previously described (Ikeno et al., 2005). See additional details in Methods S1.

Animal care and maintenance were provided through the UTHSCSA AAALAC Accredited Animal Facility. All procedures approved by the Institutional Animal Care and Use Committee of the UTHSCSA.

Hormone measurements

Mice were bled between 7 and 9 AM via the mandibular vein before sacrifice. Serum samples were collected in a fed state at 22-24 months of age. Serum IGF-1 levels were measured by RIA (American Laboratory Products Company, Inc., Salem, NH, USA). Serum GH levels were determined using commercial ELISA kit (Millipore, Temecula, CA, USA). Serum osteocalcin levels were measured by ELISA (Millipore Inc). Serum PTH levels were determined by mouse PTH ELISA (Immutopics Inc., San Clemente, CA, USA).

Serum isoprostanes

8-iso PGF2α levels were measured by GC/MS as described by Roberts and Morrow (Roberts & Morrow, 2000). The 8-iso PGF2α concentrations are computed using either peak area or peak height, that is, area of sample/area of standard x ng standard added = ng 8-iso PGF2α in the sample. Data were normalized to plasma volume.

Serum cytokine assay

Serum cytokine levels were assessed using mouse proinflammatory 7-plex ultra-sensitive kit (K15013C-1, Meso Scale Discovery) according to the manufacturer’s instructions. Plates were read on a SECTOR plate reader.

Gene expression

Total RNA was extracted from tissues using TRIzol (Invitrogen, Carlsbad, CA). RNA samples (1 µg) were reverse-transcribed using oligo-dT primers (Invitrogen), and quantitative real-time PCR was performed following the manufacturer’s instructions using the QuantiTectSYBR green PCR kit (Qiagen, Valencia, CA, USA) on an ABI PRISM 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA). Transcript levels were assayed 3 times in each sample, and the fold-change ratios between experimental and control samples were calculated. We used three housekeeping genes; β-actin, GAPDH, and 18S. As no differences were found when data corrected to each housekeeping gene, we present data obtained after correction to 18S. For primer sequences, refer to Table S6.

Tissue oxidative stress levels

Oxidative stress was measured using OxyBlot Protein Oxidation Detection Kit (S7150, Millipore). Proteins extracts obtained in RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS). 20 µg of protein extracts from liver and muscles of control and iLID mice were analyzed according to the manufacturer’s instructions.

Primary fibroblast cultures

One centimeter of mouse tail was rinsed in 70% ethanol three times and consequently with PBS to remove excess alcohol. Tails were minced with a sterile scalpel (#22 Blade, Fisher, 08-918-5C; Scalpel Handle, 08-917-5) and digested overnight with collagenase (400 U mL⁻¹, Gibco, 17101-015). Cells were then passed through a sterile nylon netting (40-µm nylon mesh, BD Falcon, 25-2340 with Falcon 50 mL culture tubes, 35-2098) to remove small pieces of skin and hair. Cells were counted and seeded at 0.25 x 10⁶ cells in 6 mL complete DMEM in a T-25 tissue culture flask. To evaluate oxidative stress resistance, cells were seeded in 6-well plates and treated with 400 µM peroxide for 8 or 24 h. At the end of treatment, cells were harvested in RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS). Twenty microgram of total protein extracts was run on 4-10% SDS-PAGE, and PARP (#9542, Cell Signaling, Danvers, MA, USA) cleavage was detected by immunoblot. The levels of cleaved PARP were
corrected to both total PARP and β-actin (Cell Signaling, #4970), detected on the same blot.

**Tissue protein extraction and detection**

Liver proteins were obtained from 50 mg liver tissue homogenized in RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS). Twenty microgram of total protein extracts of control and iLID mice were run on 4-20% SDS-PAGE. Membranes were blocked in 5% nonfat dry milk and subjected to immunoblot. Phospho-STAT5b (Cell Signaling, CAT # 9359) and total STAT5b (Cell Signaling, CAT# 9363) were diluted in TBS-T (1:1000) and detected according to manufacturer’s instructions. β-actin (Cell Signaling, CAT# 4970) was used as a loading control. Levels of phosphorylated STAT5b were corrected to total STAT5b, using a stripped membrane. Quantification was performed by FUJIFILM LAS3000, and 10 mice from each group were used for the quantification.

**Primary osteoblast-like cultures**

Bone marrow cells were flushed out from femurs using a 26-gauge needle and collected in αMEM culture medium and drawn through 18-gauge needle to achieve single-cell suspension. Cells were then washed in αMEM culture medium and cultured in αMEM supplemented with 10% heat-inactivated FBS with β-glycerophosphate (10 mM) and ascorbic acid (50 mM). Cellular alkaline phosphatase activity was determined using a para-nitrophenol phosphate-based colorimetric assay. Cultures were carried out for different time points as indicated. Alkaline phosphatase (ALP) staining was performed using an Alkaline Phosphatase kit according to the manufacturer’s instructions (Sigma-Aldrich, St. Louis, MO, USA).

**Histology**

Tissues were fixed in 10% buffered formalin phosphate (Fisher Scientific) and embedded in paraffin. Immunohistochemistry with rabbit anti-mouse Iba1 (Wako Chemicals, VA) was performed in 5 um sections of paraffin-embedded livers. Three sections were analyzed per mouse, where the percentage of Iba1-positive cells was calculated per each section. Nuclear size was evaluated in H&E-stained liver sections. We analyzed one section per mouse and 5 mice per group that were blinded to the observer. Fifty nuclei per section were measured and calibrated to an image scale bar using IMAGE J software (National Institutes of Health, Bethesda, MD, USA).

**Micro-CT**

Femoral bone morphology at the mid-diaphysis, and trabecular bone volume fraction and micro-architecture in the distal femoral metaphysis were assessed as previously described (Elis et al., 2010a,b) and according to JBMR guidelines (Bouxein et al., 2010). See additional details in Methods S1.

**Micro-Positron Emission Tomography/Computed Tomography**

Positron emission tomography scan was performed in a Micro-Positron Emission Tomography/Computed Tomography (µ-PET/CT) (Siemens, TN, USA) using 18F-fluorodeoxyglucose (18F-FDG). The animals were fasted for 8-10 h before undergoing PET. They were anesthetized using 1 L min⁻¹ of 2% isoflurane in 100% oxygen and injected intravenously with 10 MBq 18F-FDG. After 1 h had been allowed for clearance of nonspecifically bound tracer, the animals received an intraperitoneal injection of 30 µL of a mixture of ketamine and xylazine (80 and 20 mg mL⁻¹, respectively) and subsequently imaged. Static acquisition was performed for 30 min.

**Mechanical testing by 4-point bending assay**

Mouse femora from 2-year-old control and iLID mice were tested to failure by 4-point bending using a servohydraulic materials testing system (Bose, Canton, MA, USA). This test measures whole-bone stiffness, maximum load, postyield deflection and work to failure. Femora were placed with the anterior surface down on two lower supports. The two lower and two upper supports were set apart by 6.35 and 2.2 mm, respectively. Loading was centered over the mid-shaft, at a displacement of 0.05 mm s⁻¹ until failure.

**Inductively coupled plasma**

Thermo Jarrell Ash Trace Scan Advantage was used to determine bone mineral compositions (Ca,P). 10 mg of bone mineral powder was dissolved in 17% HCl and made up to 100 mL in a volumetric flask with double distilled water. Appropriate standard solutions for Ca (0, 1 and 50 ppm) and P (0, 1 and 30 ppm) were prepared from 100 ppm standard solutions of the respective elements (Fisher Scientific). The specimen in solution and standard solutions was pumped through argon plasma excited by 2 kW 27.12 MHz radio frequency generator. The concentrations of each element were determined using their characteristic wavelengths (Ca: 317.9 Å, P: 213.6 Å).

**Statistical analysis**

For survival studies of the iLID mice and end-of-life pathology, total frequency and grade of lesions were compared between genotypes using a chi-square test. When the expected frequencies were too small for the chi-square test, the data were analyzed using Fisher’s exact test. The Kaplan–Meier survival curves for fatal neoplastic lesions and adenocarcinoma were analyzed using a log-rank test (Lawless 1990). All differences in mean serum hormone levels, organ weights, and growth between controls and iLID mice were assessed by T-test. Values are presented as the mean ± SEM, and P < 0.05 was considered statistically significant. All micro-CT measurements, mechanical tests, and ICP data are presented as the mean ± SD.

**Funding**

Financial support was received from funding agencies in the United States (NIH/NIAMS AR054919 & AR055141) to SY. Supported in part by UTHSCSA PREF Award, IGF1/Insulin Signaling in Aging – Project 1 (MLA). Supported in part by NIH Grants AG13319 (YI and GBH) and a grant from the Glenn Foundation for Medical Research (YI).

**Conflict of interest**

None of the authors have conflicting financial interests.

**References**

Baik M, Yu JH, Hennighausen L (2011) Growth hormone-STAT5 regulation of growth, hepatocellular carcinoma, and liver metabolism. Ann. N. Y. Acad. Sci. 1229, 29–37.
Bartke A, Courtland HW, Elis S, Wu Y, Rosen CJ, Jepsen KJ, Majeska RJ, Yakar S (2005) Somatopause: dismetabolic and bone effects. J. Endocrinol. Invest. 28, 1265–1270.

Bartke A, Sun LY, Longo V (2013) Consequences of growth hormone (GH) overexpression and GH resistance. Neuropeptides 36, 201–208.

Bartke A, Sun LY, Longo V (2013) Somatotropic signaling: trade-offs between growth, reproductive development, and longevity. Physiol. Rev. 93, 571–598.

Barzilai N, Huffman DM, Muzumdar RH, Bartke A (2012) The critical role of metabolic pathways in aging. Diabetes 61, 1315–1322.

Bokov AF, Garg N, Ikeno Y, Thakur S, Musi N, DeFronzo RA, Zhang N, Erickson RC, Gelfond J, Hubbard GB, Adamo ML, Richarson A, Wieland R, Baumler M, Kratky D, Sevi V, Henner L, Kozlov AV, Terracciano L, Zechner R, Schuetz G, Casanova E, Pospisilik J, Heim MH, Morjig R (2011) Impairment of hepatic growth hormone and glucocorticoid receptor signaling causes steatosis and hepato-cellular carcinoma in mice. Hepatology 54, 1398–1409.

Murakami S, Salmon A, Miller RA, Faseb J (2003) Multiplex stress resistance cells in from long-lived dwarf mice. Faseb J. 17, 1556–1566.

O’Neill C, Kiley AP, Coakley MF, Mannings S, Long-Smith CM (2012) Insulin and IGF-1 signalling: longevity, protein homeostasis and Alzheimer’s disease. Biochem. Soc. Trans. 40, 721–727.

Page MM, Salmon AB, Leser SF, Robb EL, Brown MF, Miller RA, Stuart JA (2009) Mechanisms of stress resistance in Snell dwarf mouse fibroblasts: enhanced antioxidant and DNA base excision repair capacity, but no differences in mitochondrial metabolism. Free Radic. Biol. Med. 46, 1109–1118.

Roberts LJ, Morrow JD (2000) Measurement of F2-isoprostanes as an index of oxidative stress in vivo. Free Radic. Biol. Med. 28, 505–513.

Salmon AB, Murakami S, Bartke A, Kopchick J, Yasumura K, Miller RA (2005) Fibroblast cell lines from young adult mice of long-lived mutant strains are resistant to multiple forms of stress. Am. J. Physiol. Endocrinol. Metab. 289, E139–E149.

Selman C, Lingard S, Choudhury AI, Battersham RL, Claret M, Clements M, Ramadani F, Okkenhauk K, Schuster E, Blanc E, Piper MD, Al-Qassab H, Speakman JR, Carmignac D, Robinson IC, Thornton JM, Gems D, Partridge L, Withers DJ (2008) Evidence for lifespan extension and delayed age-related biomarkers in insulin receptor substrate 1 null mice. Faseb J. 22, 807–818.

Snapp SB, Mersebach H, Ullum H, Feldt-Rasmussen U (2002) Immune function during GH treatment in GH-deficient adults: an 18-month randomised, placebo-controlled, double-blinded trial. Clin. Endocrinol (Oxf.) 57, 787–792.

Sonntag WE, Carter CS, Ikeno Y, Ekenstedt K, Carlson CS, Loeser RF, Chakraborty S, Lee S, Bennett C, Ingram R, Moore T, Ramsey M (2005) Adult-onset growth hormone and insulin-like growth factor-I deficiency reduces neoplastic disease, modifies age-related pathology, and increases life span. Endocrinology 146, 2920–2932.

Sonntag WE, Csiszar A, deCabo R, Ferrucci L, Uyngvi Z (2012) Diverse roles of growth hormone and insulin-like growth factor-1 in mammalian aging: progress and controversies. J. Gerontol. A Biol. Sci. Med. Sci. 67, 587–598.

Stein PK, Soare A, Meyer TE, Cangemi R, Holloszy JO, Fontana L (2012) Caloric restriction may reverse age-related autonomic decline in humans. Aging Cell 11, 644–650.

Svensson J, Spogenous J, Faldt J, Andersson N, Isaksson O, Jansson JO, Ohlsson C (2011) Liver-derived IGF-1 regulates liver function and insulin-like growth factor-I deficiency reduces neoplastic disease, modifies age-related pathology, and increases life span. Endocrinology 146, 2920–2932.

Svensson J, Spogenous J, Faldt J, Andersson N, Iaksson O, Jansson JO, Ohlsson C (2011) Liver-derived IGF-1 regulates liver function and insulin-like growth factor-I deficiency reduces neoplastic disease, modifies age-related pathology, and increases life span. Endocrinology 146, 2920–2932.

Wolf NS, Leifer BL, Brown MF, Miller RA, Stuart JA (2009) Mechanisms of stress resistance in Snell dwarf mouse fibroblasts: enhanced antioxidant and DNA base excision repair capacity, but no differences in mitochondrial metabolism. Free Radic. Biol. Med. 46, 1109–1118.

Roberts LJ, Morrow JD (2000) Measurement of F2-isoprostanes as an index of oxidative stress in vivo. Free Radic. Biol. Med. 28, 505–513.

Salmon AB, Murakami S, Bartke A, Kopchick J, Yasumura K, Miller RA (2005) Fibroblast cell lines from young adult mice of long-lived mutant strains are resistant to multiple forms of stress. Am. J. Physiol. Endocrinol. Metab. 289, E139–E149.

Selman C, Lingard S, Choudhury AI, Battersham RL, Claret M, Clements M, Ramadani F, Okkenhauk K, Schuster E, Blanc E, Piper MD, Al-Qassab H, Speakman JR, Carmignac D, Robinson IC, Thornton JM, Gems D, Partridge L, Withers DJ (2008) Evidence for lifespan extension and delayed age-related biomarkers in insulin receptor substrate 1 null mice. Faseb J. 22, 807–818.

Snapp SB, Mersebach H, Ullum H, Feldt-Rasmussen U (2002) Immune function during GH treatment in GH-deficient adults: an 18-month randomised, placebo-controlled, double-blinded trial. Clin. Endocrinol (Oxf.) 57, 787–792.

Sonntag WE, Carter CS, Ikeno Y, Ekenstedt K, Carlson CS, Loeser RF, Chakraborty S, Lee S, Bennett C, Ingram R, Moore T, Ramsey M (2005) Adult-onset growth hormone and insulin-like growth factor-I deficiency reduces neoplastic disease, modifies age-related pathology, and increases life span. Endocrinology 146, 2920–2932.

Sonntag WE, Csiszar A, deCabo R, Ferrucci L, Uyngvi Z (2012) Diverse roles of growth hormone and insulin-like growth factor-1 in mammalian aging: progress and controversies. J. Gerontol. A Biol. Sci. Med. Sci. 67, 587–598.

Stein PK, Soare A, Meyer TE, Cangemi R, Holloszy JO, Fontana L (2012) Caloric restriction may reverse age-related autonomic decline in humans. Aging Cell 11, 644–650.

Svensson J, Spogenous J, Faldt J, Andersson N, Isaksson O, Jansson JO, Ohlsson C (2011) Liver-derived IGF-1 regulates liver function and insulin-like growth factor-I deficiency reduces neoplastic disease, modifies age-related pathology, and increases life span. Endocrinology 146, 2920–2932.

Sonntag WE, Csiszar A, deCabo R, Ferrucci L, Uyngvi Z (2012) Diverse roles of growth hormone and insulin-like growth factor-1 in mammalian aging: progress and controversies. J. Gerontol. A Biol. Sci. Med. Sci. 67, 587–598.

Stein PK, Soare A, Meyer TE, Cangemi R, Holloszy JO, Fontana L (2012) Caloric restriction may reverse age-related autonomic decline in humans. Aging Cell 11, 644–650.

Svensson J, Spogenous J, Faldt J, Andersson N, Iaksson O, Jansson JO, Ohlsson C (2011) Liver-derived IGF-1 regulates liver function and insulin-like growth factor-I deficiency reduces neoplastic disease, modifies age-related pathology, and increases life span. Endocrinology 146, 2920–2932.
Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site.

Methods S1 Reductions in serum IGF-1 during aging impair health span.

Table S1 Support information of survival studies with the congenital LID mouse cohort.

Table S2 Body and organ weights of a subcohort of control and LID mice dissected at 19-20 months of age.

Table S3 Body and organ weights of 2-year-old control and iLID mice.

Table S4 Cytokine levels in serum assessed by Mouse pro-inflammatory 7-plex ultra-sensitive kit.

Table S5 Tumor incidence in control and iLID mice.

Table S6 Primers used for gene expression studies using real-time PCR.