Connecting serum IGF-1, body size, and age in the domestic dog

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Abstract Many investigations in recent years have targeted understanding the genetic and biochemical basis of aging. Collectively, genetic factors and biological mechanisms appear to influence longevity in general and specifically; reduction of the insulin/IGF-1 signaling cascade has extended life span in diverse species. Genetic alteration of mammals for life extension indicates correlation to serum IGF-1 levels in mice, and IGF-1 levels have been demonstrated as a physiological predictor of frailty with aging in man. Longevity and aging data in the dog offer a close measure of the natural multifactorial longevity interactions of genetic influence, IGF-1 signaling, and environmental

factors such as exposure, exercise, and lifestyle. The absence of genetic alteration more closely represents the human longevity status, and the unique species structure of the canine facilitates analyses not possible in other species. These investigations aimed to measure serum IGF-1 in numerous purebred and mixed-breed dogs of variable size and longevity in comparison to age, gender, and spay/neuter differences. The primary objective of this investigation was to determine plasma IGF-1 levels in the adult dog, including a wide range of breeds and adult body weight. The sample set includes animals ranging from just a few months of age through 204 months and ranging in size from 5 to 160 lb. Four groups were evaluated for serum IGF-1 levels, including intact and neutered males, and intact and spayed females. IGF-1 loss over time, as a function of age, decreases in all groups with significant differences between males and females. The relationship between IGF-1 and weight differs depending upon spay/neuter status, but there is an overall increase in IGF-1 levels with increasing weight. The data, currently being interrogated further for delineation of IGF-1 receptor variants and sex differences, are being collected longitudinally and explored for longevity associations previously unavailable in non-genetically modified mammals.

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Introduction

In recent years, many studies have been directed toward understanding the genetic and biochemical basis of aging. With escalating medical costs and a growing population of elderly in the global community, it is important to better understand the aging process to prevent age-related diseases such as cancer, cataracts, arthritis, and diabetes. Many investigations have approached this understanding by studying aging across species, and the common theme presented is that genetic factors and biological mechanisms heavily impact longevity. In vertebrates and invertebrates, aging processes are influenced by stress resistance, insulin signaling, caloric restriction, and the ability to repair cellular and macromolecular damage (Warner 2004). Specifically, reduction of the insulin/insulin-like growth factor-1 (IGF-1) or a homologous signaling cascade has significantly extended life span in diverse species, including yeast, worms, fruit flies, and mammals (Tatar 2003).

In *Caenorhabditis elegans*, the insulin/IGF-1 receptor homologous pathway is required for reproductive growth and metabolism, as well as for normal life span. Longevity in *C. elegans* is increased by reducing the activity of insulin signaling proteins and by reducing the effects of others. The insulin-like receptor, Daf-2, and its downstream effectors such as Age-1, phosphatidylinositol-3 kinase, phosphoinositide-dependent kinase 1, and the protein kinases, Akt-1 and Akt-2 demonstrate increased longevity for *C. elegans* when down-regulated (reviewed in Antebi 2007). In contrast, proteins that extend longevity by antagonizing Daf-2 and/or Age-1 signaling include: Daf-18, PTEN, and Daf-16, a forkhead transcription factor (Kimura et al. 1997; Morris et al. 1996; Paradis et al. 1999; Paradis and Ruvkun 1998; Mihaylova et al. 1999; Ogg et al. 1997). Studies in *Drosophila melanogaster* have revealed similar relationships (Garofalo 2002), as have studies designed to decrease insulin/IGF-1 signaling in mice (reviewed in Bartke 2005).

A multitude of information has been generated pertaining to insulin and IGF-1 signaling alterations in mice, including extensive information from genetically altered mice. Very low circulating levels of IGF-1 and circulating growth hormone (GH) are characteristic of the long-lived Snell dwarf mouse (Pit-1^{dw}; Hsieh et al. 2002), homozygous for a loss-

of-function mutation in the Pit1 gene (Flurkey et al. 2001). These hormonal changes lead to a reduction in young adult body weight and 40% life span extension (Flurkey et al. 2001). Ames dwarf mice (Prop-1^{df}) and Snell dwarf mice not only have low GH levels, but also demonstrate prolactin and TSH deficiency. They live more than 40% longer than their wild-type counterparts (Brown-Borg et al. 1996; Bartke 2005). Growth hormone receptor knockout (GHRKO) mice have very low circulating IGF-1 levels and also a 40% life extension (Barbieri et al. 2003; Brown-Borg et al. 1996; Coschigano et al. 2000). The IGF-1 receptor knockout mice (Igf1r^{+/-}) provide strong evidence for the role of the IGF-1 pathway in longevity. The fat-specific insulin receptor knockout mice have a 60% reduction in body fat with weight reduction despite normal food intake and live significantly longer than their non-mutated littermates (Blüher et al. 2003). Characteristic of these knockouts is the lower circulating IGF-1 levels and higher stress resistance. Human studies have investigated IGF-1 levels in association with frailty prediction, and the circulating IGF-1 concentrations are associated with the frail condition (Espinoza and Walston 2005; Walston 2004); therefore, collective results obtained in different species identify insulin/IGF1 signaling as one of the major targets to be investigated in gerontology and age-related research.

Mutant and knockout animals with smaller body size and higher stress resistance have lower circulating levels of serum IGF-1 (Bartke, 2005). Because this trend holds true for several mouse mutants and because IGF-1 levels have been demonstrated as a physiological predictor of frailty with aging in man (Walston 2004; Kapatkin et al. 2007), the level of serum IGF-1 in various dog breeds has been determined. These studies, together with longevity and aging data in the dog, provide a close measure to the multifactorial interactions of genetic influence, IGF-1 signaling, and environmental factors such as environmental exposure, exercise, and lifestyle of human aging. The dog is the one animal in the USA living a life most similar to the human, not only in medical care but also in lifestyle. Dogs share their owners' homes, neighborhoods, and, often, their exercise habits. From birth, dogs are weighed, measured, and evaluated frequently. In the early developmental years, they receive vaccinations, as do humans. Canine quality of care is very high with dogs often

receiving extensive care from veterinary specialists, exactly as humans who are referred to medical specialists. Throughout life, dogs are medically followed closely, and in the USA, the amount of money spent on medical procedures and exams for dogs is second only to that spent on human medical care (U.S. Pet Ownership & Demographics Sourcebook, AVMA 2007). Therefore, these investigations aimed to measure serum IGF-1 in numerous purebred and mixed-breed dogs of variable size and longevity in comparison to various ages, the genders, and spay/neuter differences as a means of laying a definitive foundation for the investigation of the IGF-1 pathway factors potentially influencing aging and longevity.

Materials and methods

Animals

Height, weight, gender, birth date, and breed data were collected on more than 140 dogs within 43 independent American Kennel Club recognized breeds. Additionally, mixed-breed dogs were included and identical information recorded. Animal protocols as approved by Indiana University East Animal Care and Use committee were strictly followed. Study participants were recruited through independent veterinary practices, and each participating dog contributed a 3-ml EDTA-coated vial of whole blood. Exclusion criterion were utilized in an attempt to eliminate extraneous factors that would influence or skew analyses. Any animals with known or suspected metabolic, endocrine, or systemic disorders were excluded from the study. Individual data on participating animals were entered into a dedicated computer database for tracking, sorting, and analysis and exported to NCSS 2007 (described below). There were more than 20 neutered males evaluated by weight and age, more than 30 intact males evaluated by weight and age, and more than 30 and 20 females, both spayed and intact, respectively, evaluated by identical parameters such a weight parameter. The group totals of animals are presented on the figures.

Enzyme-linked immunoassays

Whole blood was separated by centrifugation and plasma recovered. The plasma was immediately

frozen at -80°C and stored until sufficient numbers of samples were collected to complete replicate analysis on an enzyme-linked immunoassay (ELISA) plate. ELISAs were performed using the human IGF-1 assay plate (Immunodiagnostic System Inc., Fountain Hills, AZ) according to the manufacturer's protocol. Although previously validated by the manufacturer for use with canine samples, preliminary experiments were performed to determine the plasma volume to be used in the assay.

Statistical analysis

Data files generated during the experiments were imported to Excel spreadsheets and assessed for integrity and distributional characteristics. Regression analyses were carried out using NCSS 2007.

Results

To further define the relationships between insulin signaling, body size, longevity, and aging, we describe the quantitation and association of these parameters in dogs of widely variable size and expected longevity. Previous work demonstrated that a dog's weight is more predictive of life span than height, breed, or breed group (Greer et al. 2007). Studies in the mouse demonstrate that different dwarf mice, smaller than normal littermates by weight and characterized by decreased levels of IGF-1, live longer than littermates (Al-Regaiey et al. 2005). The current study evaluated serum IGF-1 levels in dogs of highly variable size on the basis of body weight. More than 140 dogs were successfully recruited for the study and comprised more than 40 distinct breed groups in addition to mixed breeds of equal size range. Data include a total sample set of animals ranging from 2 through 204 months (17 years). Breeds were inclusive of animals ranging from 5 to 160 lb at adult weight. The collective median age for the study animals is 84 months (7 years), while the median weight of all dogs in the study is 22 lb.

Regression analyses of IGF-1 levels as a function of age and weight were carried out separately for males, neutered males, females, and spayed females. For the comparison of IGF-1 levels to age, there were 35 intact male animals and 26 female intact animals. As a

function of age, and as depicted in Figs 1a and 2a, the analysis of data for intact males showed a decline of 0.39 ng/ml (± 0.21) of IGF-1 per month of age ($p < 0.06$), while data for intact females indicated a decline of 0.55 ng/ml (± 0.16) of IGF-1 per month of age. The comparison of IGF-1 loss between intact males and females was significant ($p < 0.003$). Alternatively, when comparing regression as a function of age in altered males ($N=24$; Fig. 1b) and females ($N=36$; Fig. 2b), higher variability was displayed with a trend toward more IGF-1 loss in altered males ($0.48 \pm 0.46 \text{ ng/ml}$, $p < 0.31$) when compared to the previously mentioned intact males ($0.39 \pm 0.21 \text{ ng/ml}$). Spayed females ($N=26$) displayed a decrease in IGF-1 loss over age of $0.19 \pm 0.18 \text{ ng/ml}$ ($p < 0.28$; Fig. 2b). Overall, all groups showed a decrease in IGF-1 levels with increasing age, and the heterogeneity in the rate of loss was not significant at the 0.05 level.

The relationship between IGF-1 and weight in groups separated by gender showed an increase in IGF-1 per

pound of body weight in both intact males ($N=34$) and females ($N=23$; $1.11 \pm 0.28 \text{ ng/ml}$, $p < 0.001$, and $0.84 \pm 0.34 \text{ ng/ml}$, $p < 0.023$, respectively; Figs. 1c and 2c). Spayed females ($N=36$) showed a similar trend between IGF-1 and weight, but the regression was not significant ($0.62 \pm 0.43 \text{ ng/ml}$, $p < 0.16$; Fig. 2d). Interestingly, castration in males ($N=22$) caused more than a twofold increase of IGF-1 when compared to intact males ($2.98 \pm 0.84 \text{ ng/ml}$, $p < 0.002$; Fig. 1c). Overall, groups showed an increase in IGF-1 levels with increasing weight; however, the rate of IGF-1 increase for the neutered males was considerably larger than the other groups which did not differ significantly from each other. Consequently, a multiple regression of age and weight upon IGF-1 levels was implemented for all groups except the neutered males. The resulting regression coefficients were: age = -0.34 ± 0.10 , $p < 0.002$; weight = 0.97 ± 0.19 , $p < 0.001$ (Fig. 3). The intercept was 90.37 ± 11.58 . The power, with alpha at 0.05, was > 0.89 for both coefficients.

Fig. 1 Simple linear regression: **a** IGF-1 and age in males, **b** IGF-1 and age in neutered males, **c** IGF-1 and weight in males, and **d** IGF-1 and weight in neutered males

Fig. 2 Simple linear regression: **a** IGF-1 and age in females, **b** IGF-1 and age in spayed females, **c** IGF-1 and weight in females, and **d** IGF-1 and weight in spayed females

Discussion

The primary objective of this investigation was to determine plasma IGF-1 levels in the adult dog, including a wide range of breeds and weights. A few previous studies analyzed IGF-1 levels in some breeds, but were primarily targeting other investigational goals. A priori data, therefore, including plasma IGF-1 measurements across numerous breeds and mixed-breed dogs, have not previously been available. This paucity of data led the investigators to evaluate IGF-1 levels on breeds of widely variable size and expected longevity because the clarity of these levels may provide valuable insight into the mechanisms of aging in a larger mammal. Studying these differences across dog breeds is especially interesting because the smaller breeds tend to live longer than the larger breeds, which is exactly the opposite of the general life span trend across mammalian populations (i.e., the mouse lives a much

shorter life than an elephant). The GH/IGF-1 axis has been proven important in life span extension for worms, flies, and mice, but its effects in larger mammals remain undetermined.

Early IGF-1 analyses completed by Eigenmann et al. (1988) indicated that adult poodles had high correlation of circulating IGF-1 levels and adult body size. The correlation held between dogs of similar breed origin and different adult body size: the toy ($N=4$), miniature ($N=4$), and standard poodle ($N=4$). Given the population structure of purebred dogs, it is possible that these breeds, derived from very similar or identical predecessors, are not representative of the wide range of variability within the species. This variability is extreme in adult body size, with the smallest breed averaging 5 lb at adult weight and the largest breed weighing approximately 225 lb as an adult. Additionally, the domestic dog has the widest life span variation among mammals between its independent breed groups. The species is also

Fig. 3 Simple linear regression in all groups except the neutered males: **a** IGF-1 and age; **b** IGF-1 and weight

intriguing in that with so much species variation, all members of the species are capable of mating regardless of highly variable physical characteristics. By utilizing these unique parameters of the species, analyses can advantageously investigate the natural occurrence of extreme variation within genetically isolated populations which are represented in canines by breed group structure and strictly maintained by national canine organizations. One or two other previous studies into canine IGF-1 concentrations compared dissimilar breeds, but targeted very young animals. Nap et al. (1994) determined that toy poodles and great Danes between the ages of 7 and 27 weeks maintain similar plasma IGF-1 levels. A later study confirmed this result by demonstrating that beagles ($N=6$) and great Danes ($N=6$) at the ages of 6 and 24 weeks have no significant differences in plasma

IGF-1 levels regardless of size and gender in the pups (Favier et al. 2001). Further analysis led these investigators to conclude that early circulating GH levels rather than circulating IGF-1 levels were significantly associated with the dogs' final adult size. The results reported here significantly expand upon data pertaining to correlations in adult body weight and size rather than early life growth rate or size. With increased sample size, the breeds analyzed are expanded to include breeds that are not closely genetically related in origin. This sample set includes animals ranging from just a few months of age through 204 months, or 17 years, and ranging in size at adulthood from 5 to 160 lb.

Regression analysis of IGF-1 levels is presented as a function of age and weight for four categories of animals, each inclusive of all the breeds and ages. For regression analysis comparing IGF-1 with age, the four groups (intact males, neutered males, intact females, and spayed females) are similar in that all groups have a noticeable, yet not significant, negative correlation of serum IGF-1 levels with age. These changes have been noted in other species as well, but an interesting point of the diminishing IGF-1 levels with age, uniquely facilitated by canine analysis, is the apparent drastic difference in levels between the intact females and the spayed females. Intact females decrease their serum IGF-1 levels at a rate of 55 ng/ml (± 0.16) per month of life, while the spayed females lose IGF-1 at a level of 0.19 ng/ml (± 0.18) per month of life. The dramatic decrease demonstrated by the intact females may be due to hormonal effects. Clearly, there is a difference in the biological maintenance of IGF-1 levels between the intact and altered females, and the dog provides a unique opportunity to more closely investigate hormonal effects on the GH/IGF-1 axis. Waters et al. (2009) have noted differences between ovariectomized dogs and dogs with 4 years or more of ovarian exposure, reporting that those maintaining greater ovarian exposure live longer. Therefore, more intense analyses of these effects are currently underway.

Another interesting difference in the data exists between the intact males and the neutered males. There is a significant difference in the serum IGF-1 levels between these two groups, with the neutered males losing IGF-1 at a level of 2.98 ng/ml per month of life while the intact males decrease their serum IGF-1 levels at a much more modest rate, 1.11 ng/ml

per month of life. Being the first report of such a difference in males, longitudinal data on the dogs contributing to this investigation will be vital for continued data analyses as the relationship between IGF-1 and life span has not previously been analyzed with consideration to spay/neuter situations. This parameter for the study population may offer insight unavailable in other organisms.

The regression analysis of IGF-1 as a function of weight revealed a significant relationship for all groups of animals except the spayed females. For both intact males and females, an increase in overall body weight was significantly associated with higher levels of IGF-1. While the trend remained the same for the spayed females, the correlation was not significant. The data, therefore, verify the previous work by Eigenmann et al. (1988) indicating that adult dogs have high correlation of circulating IGF-1 levels with adult body size as opposed to early body size. Apparently, the early life body size and IGF-1 levels were significant for association to GH, but do not correlate well with IGF-1 parameters. It will be interesting upon follow-up of these animals, therefore, to see if the final longitudinal parameters continue to correlate with adult body size and IGF-1 values as indicated in this study.

The adult body size appears to be the key factor for association with IGF-1 levels. This parameter sheds light on the studies by both Favier et al. (2001) and Nap et al. (1994) which indicated no correlation between IGF-1 and early life size up to 27 weeks of age. When these results are compared to previously published life span analyses (Greer et al. 2007; Eigenmann et al. 1988), the correlation between body size and longevity corresponds to those demonstrated here of larger body weight being in positive correlation to increased IGF-1 levels or diminished longevity. Although this study correlates IGF-1 levels with size in dogs without direct measurements of individual animals' life span, the findings could be indicative that the rate of growth is actually an important key factor for subsequent life span. Clearly, growth records indicate that the larger breeds experience a significantly more rapid and lengthier period of rapid growth in early life. An indication may exist then that an organism's extremely accelerated growth trajectory is mechanistically linked to final longevity. This idea, as suggested by Samaras et al. (2003), notes that the initial birth weight of small dogs is not significantly

different than that of large or giant dogs. These parameters were reiterated with Favier and colleague's publication (2001), potentially suggesting that a higher cell turnover during rapid growth is required to obtain adult body size. Rapid body growth also presumably lends to more rapid cellular senescence and, therefore, ultimately organismal aging. It seems possible that a rapid and prolonged cell turnover in several tissues increases the risk of cell mutations that are not repaired or eliminated, also resulting in more rapid cellular senescence and thus contributing to those diseases producing a shortened life span (Campisi 2008). There is evidence for these life span-related events in rapidly growing animals (Coile 2005; Galis et al. 2007; Samaras 2009; Deeb and Wolf 1994; Miller et al. 2002; Snibson et al. 1999).

Although the exact relationship between IGF-1 and life span may be indirect and proceed through additional biochemical pathways, the correlations between body size, serum IGF-1, and longevity have been demonstrated in shorter lived, much smaller mammals. Mice deficient in IGF-1 are not only smaller, but live longer and have significantly reduced prevalence of age-related physical complications such as cataracts, atherosclerosis, and cancer (Bartke et al. 2003; Deeb and Wolf 1994; Menezes Oliveira et al. 2006; Shechter et al. 2007; Shevah and Laron 2007). In fact, when longitudinal studies (as are ongoing in the dog) are completed in the mice, the most apparent correlations with longevity are overall body size and IGF-1 levels. The genetically manipulated mice, including those with mutations in the GH/IGF-axis, Snell and Ames dwarfs, are significantly smaller than their normal littermates and benefit from significantly increased life span (Brown-Borg et al. 1996). GHRKO mice, and those with a defect in the GH-releasing hormone receptor (GHRHR) signaling pathway, demonstrate similar results. In human studies, low IGF-1 levels have been associated with lack of pituitary and cardiovascular disease (Colao et al. 2008), and men with higher levels of circulating IGF-1 have significantly increased risk of developing prostate cancer (Cheng et al. 2006). Longitudinal studies have not been completed in humans because of the length of time involved, similar to longitudinal studies in the dogs; however, the current parameters for indicating increased or decreased longevity as proven in the mouse are present in the dogs analyzed.

Furthermore, the indications for human longevity such as a lack of cardiovascular disease are further correlated with diminished IGF-1 levels, which may prove to be associated to longevity in the dog. IGF-1 and its receptor alleles have been linked both directly and indirectly to body size and skeletal structure (Eigenmann et al. 1984; Sutter et al. 2007), as well as to life span in several mammals, including dogs (Suh et al. 2008; Taguchi and White 2008; Bartke et al. 2003; Samaras et al. 2003; Miller et al. 2002). The longitudinal data currently being collected from animals intermediate of mice and men will certainly prove beneficial in determining definitive correlation of longevity potential and circulating IGF-1. Additionally, these data can be explored for further delineation of sex differences in IGF-1 correlations, while the IGF-1 receptor variants, GH receptor variants, and GHRHR receptor variants are also being explored for polymorphisms and longevity associations in the study animals.

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