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Decrease in Cellular Replicative Potential in "Giant" Mice Transfected With the Bovine Growth Hormone Gene Correlates to Shortened Life Span

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Adult mice, (C57BL/6 × SJl)F1 hybrids, transfected with the bovine growth hormone gene (bGH) grow to twice normal size, but have a mean life span less than 50% that of control siblings without the transgene. The replicative potentials of cells from six different tissue sites (tail skin and ear skin dermal fibroblasts, tail subdermal connective tissue fibroblasts, kidney medulla epithelial cells, bone marrow myofibroblasts, and spleen myofibroblasts) were assayed in vitro using clone size distribution analysis. Cells from all of the above bGH+ tissues produced a smaller fraction of large clones, relative to age-matched controls, in all of these cell types. The loss of replicative potential did not appear to be the result of negative conditioning of the cloning media by the bGH+ cells, and was tightly correlated to the period of accelerated growth in these animals (3–12 weeks), a time when additional GH receptors are expressed.

Loss of proliferative potential is one of the phenotypic expressions of aging in mammals and perhaps all vertebrates (see reviews Norwood and Smith, 1985; Stanulis-Praeger, 1987). The replicative potential measured as the maximum population doublings a cell population will undergo in vitro has been shown to be inversely proportional to the age of the donor in humans and other species (Martin, 1977), and is reduced in cells from patients with several progeroid diseases which shorten life span, such as Hutchinson-Gilford syndrome and Werner's syndrome (Martin, 1977; Brown, 1990). The maximum doublings that a particular cell lineage can undergo can be influenced by a number of extrinsic factors such as serum type or concentration (Schneider et al., 1978), media composition (Litwin, 1972), presence of specific mitogens (Wistrom and Villeponteau, 1990; Kill and Shall, 1990; Amtmann et al., 1990), or temporary arrest in late G1 (Pendergrass et al., 1989), but under standard conditions is reproducible (Norwood and Smith, 1985). The mechanism of loss of replicative potential with age is unknown. Hypotheses have been presented implicating both genetic factors (Peacocke and Campisi, 1991; Norwood et al., 1990) and stochastic damage (Rosenberger et al., 1991).

Another method of determining proliferative potential is by clone size distribution (CSD) analysis. CSDs display the fraction of clones capable of attaining a particular size (undergoing a given number of doublings) in a given period of time. When cell populations lose proliferative potential, the percentage of relatively large clones in the CSDs decreases. Originally developed by Smith et al. (1978), it has since been used by several others as a sensitive measure of proliferative potential that better represents the original heterogeneity of a cell population than does life span in doublings achieved by mass cultures (Angello et al., 1989; Angello and Prothero, 1989), and is also less influenced by transformation events that produce immortalization of some rodent cell populations (Stanulis-Praeger, 1987).

If proliferative potential of cells in various tissues and organs causally affects longevity, then interventions which modulate replicative potentials in an animal should also modulate its longevity. We have recently reported that caloric restriction, which lengthens life span 30–50% in rodents (Weindruch and Walford, 1988), slows the normal age-related loss of replicative potential in five different normal murine cell types examined (Li et al., 1991, unpublished data). In this report we examine replicative potential in a short-lived transgenic mouse ectopically overexpressing the bovine growth hormone gene (bGH). This transgenic mouse was developed by Palmiter et al. (1982) as a model for gigantism. The bGH transgene is driven by a metallothionine promoter, and is inserted as a tandem array in a single autosomal chromosome of affected mice, resulting in an autosomal dominant scheme of inheritance (Hammer et al., 1985). The

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transfected gene increases the serum content of GH 100–1,000 times, as well as raises the serum insulin-like growth factor-1 (IGF-1) level two to four times (Mathews et al., 1988). In addition to stimulating accelerated growth and producing adults of nearly twice normal body weight, the expression of the transgene causes early loss of fertility, early development of a variety of degenerative diseases, and a significant shortening of the life span to less than 50% of normal (Quaife et al., 1989; Doi et al., 1988; Brem et al., 1989). Here, we report that loss of proliferative potential is accelerated in this very short-lived transgenic mouse in cells from a variety of tissues, and that the loss of replicative potential is tightly correlated to the period of bGH+-sensitive acceleration of growth which occurs between 3–12 weeks of age.

MATERIALS AND METHODS

Animals

Pathogen-free transgenic mice ectopically expressed the bGH transgene under the control of the metallothionine promoter [background strain (C57BL/6 × Sjl)F1] designated as Tg (Mt-1,GH) Bri 227 were obtained from the laboratory of R.L. Brinster (Hammer et al., 1985). The mice were continuously bred on site and maintained on an ad libitum diet (Tekalb Premier Wayne Autoclavable Rodent Blocks #8656, "Teklad/Wayne," Bartonville, IL). Specific pathogen-free (SPF) barrier conditions were used including the use of clean and dirty-sealed corridors, separate air flow, autoclaved food, water, and cages, and use of sterile gloves and gowns during handling. Sentinel mice were regularly tested and were found free of all testable mouse pathogens (McAlister et al., 1990). Sacrifices were by cervical dislocations.

Dot blot analysis for bGH gene

In order to determine which of the 2-week-old mice contained the bGH gene, animals were coded following sacrifice for cell culture work, and the liver was saved for dot blot analysis as previously described (Brinster et al., 1985). The dot blot analysis was done by the laboratory of R.D. Palmiter.

Cell isolation from nonhematopoietic tissues

Following sacrifice, tissue samples of the medullary portion of the kidney, ear skin, tail skin, and tail subdermal connective tissue were surgically removed from areas sterilized with 70% EtOH. The tissues were washed in phosphate-buffered saline (PBS) and divided into 2 mm pieces. Tissue pieces were washed in Dulbecco's modified Eagle's medium buffered with 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (DMEM + HEPES) then resuspended in a digestion solution of DMEM + HEPES containing 0.1% collagenase/dispase (Boehringer Mannheim, Indianapolis, IN) and 10% fetal bovine serum (FBS) (to reduce cell damage by the proteolytic enzymes). The digestion mixture was immediately transferred to a sterile plastic bag, then taped to the outside of a 1 liter roller bottle with 500 ml of water inside and run for the optimum time at 40 revolutions per hr at 37°C. The optimum time of maceration was 45 min for ear and tail skin; 60 min for subdermal tail tissue; and 30 min for back skin. Kidney pieces were macerated 15 min without enzymes. Digested material was filtered through a 20-5 m nylon screen mesh and washed with versene buffer to inactivate the proteases. Cells from all of these tissues, except kidney epithelial cells, were then plated into 25 cm^2 flasks with 7 ml of cloning medium (F-12 medium supplemented with 10% FBS, 50 units/ml penicillin, 50 μg/ml streptomycin, and 1.25 μg/ml fungizone). After 2 days, the cells were trypsinized (0.05% trypsin) and cloned into 25 cm^2 flasks with the cloning medium at 300–400 cells per flask. The plating efficiency of the cells from all tissues was 20–40%. Kidney epithelial cells, being more sensitive to passaging, were cloned directly following maceration for 15 min as above, but without any proteolytic enzymes. The freed cells were filtered through 20-5 m nylon mesh and directly cloned at 500 cells/25 cm^2 dish in the cloning medium supplemented with 10% FBS and 20 ng/ml epidermal growth factor (UBI, Lake Placid, New York). After allowing clones to grow for 7 days, they were fixed with 95% ethanol and stained with crystal violet and the number of cells per clone enumerated by eye with a dissecting microscope.

Bone marrow and spleen myofibroblast isolation

After sacrifice of mice, the femurs and spleens were taken by sterile technique. Femur medullary content was flushed repeatedly with DMEM-α, using needles of decreasing calibers. Spleens were minced finely with scissors in the same medium, passaged repeatedly through 20 and 22 gauge needles, and the cells then passed through a 100 mesh per square inch stainless steel screen. The single cell suspensions from either organ were counted with a ZBI Coulter cell counter. For both organs cultures were carried out in DMEM-α plus 15% heat-inactivated FBS (MEM-F), 10^{-4} M 2-mercaptoethanol, 100 units/ml penicillin, 50 μg/ml streptomycin, and 1.25 μg/ml fungizone at 37°C in humidified 7% CO_2, O_2, and 88% N_2. Myofibroblast clones (Penn et al., 1993) were obtained by direct plating of BDF1 spleen or marrow cells or by an improved method in BC3 spleen or marrow cells in which a 24-hr adherence step, followed by recovery of cells with 0.05% trypsin, 0.5 mM EDTA in PBS, 10 min, 37°C and final suspension in MEM-F was used. Myofibroblasts were identified by shape and size and confirmed by alkaline phosphatase staining and positivity for HHF-35 an anti-α-actin antibody, and for anti-α-sm-1 monoclonal antibody (obtained from Dr. Allen Gown, Department of Pathology, University of Washington) (note that fibroblasts from

Abbreviations

bGH	bovine growth hormone gene
CSD	clone size distribution
DMEM	Dulbecco's modified Eagle's medium
EDTA	ethylenediamine tetraacetic acid
FBS	fetal bovine serum
HDF	human diploid fibroblasts
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IGF-1	insulin-like growth factor-1
MEM-F	Dulbecco's modified Eagle's medium alpha + 15% heat-inactivated fetal bovine serum
PBS	phosphate-buffered saline
SPF	specific pathogen-free

the dermis also stain positive for α-actin antibodies in liquid culture) (Penn et al., in press; Boyd et al., 1988; Charbord et al., 1985; Peled et al., 1991). Myofibroblast colonies were read at 9 days.

CSD

The relative replicative potential was analyzed using clone size analysis as described by Smith et al. (1978), Schneider et al. (1979), and Angello et al. (1989). Cumulative CSDs consisting of a plot of the fraction of clones able to grow past a certain size were prepared for bGH+ transgenics and controls at similar ages (see Fig. 2). The statistical comparisons between bGH+ mice and controls at each age were made by comparing the percentage of large clones (over 16 cells for nonhematopoietic clones and over 32 cells for spleen and bone marrow-derived myofibroblasts) (see Fig. 2). The kidney cells formed compact epithelial-like colonies and the other tissues formed bipolar fibroblast-like colonies. Clones of inappropriate morphology were not counted (<10% for all tissues). The number of colonies per flask did not change from day 3 through day 10, indicating that the migration of cells or reseeding of clones did not alter CSDs.

Statistical analysis

The frequency of large clones (>16 cells) produced by cells from each tissue and age group were compared using Student's two-tailed t-test. Each tissue sample was cloned in triplicate and the clone size distributions were averaged for all three flasks. Means differing at the probability $P < .05$ level are marked with one asterisk (*P) and for $P < .01$ by a double asterisk (**P) (see Figs. 3–5).

RESULTS

Relative growth of bGH+ animals and control mice

About 3 weeks after birth the bGH+ mice, background strain random bred (C57BL/6 × SJL)F1 begin to show enhanced growth of body size and weight, as well as of many organs and tissues (Mathews et al., 1988). Prior to this time, bGH+ animals are indistinguishable from control siblings by size or weight. This increase in growth rate coincides with expression of GH receptors in target organs and a rise in IGF-1 levels (Mathews et al., 1988). By 3 months of age, bGH+ mice in our study weighed an average of 40 g while controls averaged 25 g. For studies on mice over 3 months old, the identification of genotype was made by increased body weight. On studies with younger mice, tissues were tested for the presence of the bGH gene by dot blot analysis, as previously described (Brinster et al., 1985).

Survival curves of bGH+ and bGH− mice

Survival curves for the bGH+ and bGH− animals are depicted in Figure 1. bGH+ animals had mean life spans less than half as long as control littermates raised under the same barrier conditions (see Materials and Methods). Each point represents one animal death. bGH+ males and females had median life spans of 11 and 12 months, respectively. This compares to medians of 24 and 27 for control males and females, respectively. Animals used for this analysis were set aside from several aging cohorts as they became available, and housed 4 or 5 to a cage. Large transgenic animals were housed separately from the small littermates to avoid fighting-related injuries to the smaller animals. Because of the relatively small sample sizes in the survival studies and the fact that they represent several generations, these curves should be considered preliminary; however, the bGH+ animals clearly have a greatly shortened life span.

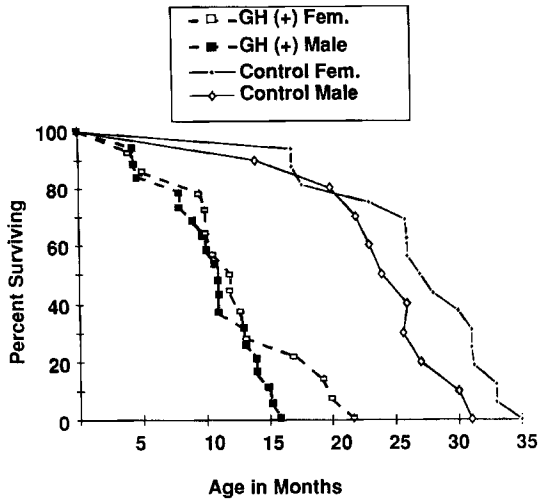

Fig. 1. Survival curves for bGH+ transgenic (GH+) and control animals. Each point represents one animal.

Proliferative potential of bGH+ and bGH− mice

Previous studies by this laboratory on female BC3 and male BDF1 mice (Jiang et al., 1992; Pendergrass et al., unpublished observation) indicate that the proliferative potential of the cells from a variety of tissues decreases significantly with the age of the donor, and that longer lived calorically restricted mice lose proliferative potential more slowly than ad libitum fed animals. In order to determine if the very short-lived bGH+ animals have an accelerated loss of proliferative potential, similar clone size analysis was carried out on tissues from female bGH+ and bGH− mice. Cells were isolated from various tissues and the clone sizes determined as described in Materials and Methods.

In Figure 2, the cumulative CSDs for cells from the six different tissue sites are shown. Cells from all six bGH+ tissues have less proliferative potential than from similarly aged bGH− mice. This is shown by a reduction in the fraction of large clones in each distribution. The tissues shown are tail skin fibroblasts, ear skin fibroblasts, tail subdermal (tendon) fibroblasts, kidney medulla epithelial cells, myofibroblasts from spleen, and myofibroblasts from femur bone marrow.

To quantitate the differences shown in Figure 2, we have compared the percentage of "large" (>16 cells) clones derived from each distribution. As depicted in Figure 3, all six bGH+-derived tissues produced significantly fewer large clones than the bGH− tissues. The P values (student two-tailed t-test) were <.01 for four of the tissues (ear skin, tail skin, tail subdermal fibroblasts, and kidney epithelial fibroblasts), and $P < .05$

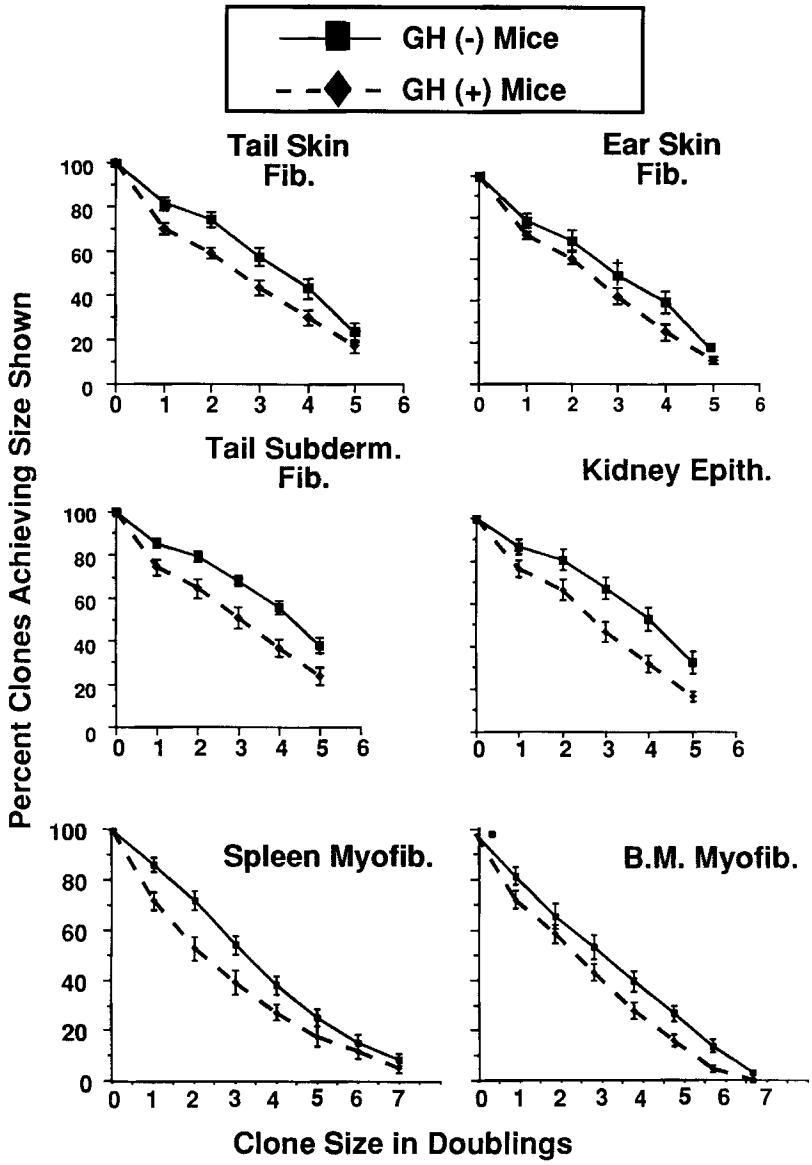

Fig. 2. Cumulative CSD of tissues from bGH+ transgenic (GH+) and control (GH−) animals. Cells from each tissue were isolated and cloned as described in Materials and Methods. The means from 7–15 animals aged 3–6 months were used to prepare each distribution. The bars represent the standard errors at each point. fib, fibroblast; epith, epithelium; myofib, myofibroblast.

for myofibroblasts from two hematopoietic stromas, spleen and bone marrow. This indicates that a significant reduction in replicative potential has occurred by 3–6 months in the bGH+ animals, which does not occur in normal wild-type mice until after 12 months of age (Pendergrass et al., unpublished observation; Jiang et al., 1992).

Effects of serum and conditioned media on proliferative potential of bGH+ cells

The reduced replicative potentials of cells from bGH+ tissues was maintained relative to bGH− controls with a variety of serum concentrations and conditioned media types. In Figure 4A, the proliferative advantage of tail skin fibroblasts from bGH− over bGH+ is shown to be maintained in a variety of FBS concentrations from 10–60%. In Figure 4B we examined whether the poorer growth of bGH+ cells was due to negative conditioning of the media, perhaps as a result of bGH secretion in vitro, or whether the loss of replicative potential occurred in vivo in the transgenic animals. The ratio of large clones produced by bGH+ cells to that produced by bGH− cells was similar in all media whether or not it was conditioned by previous growth of bGH+ or bGH− cells. Thus, it is unlikely that bGH or excess IGF-1 secreted into the cloning medium was responsible for the reduced replicative potential of the bGH+ cells in vitro. However, this experi-

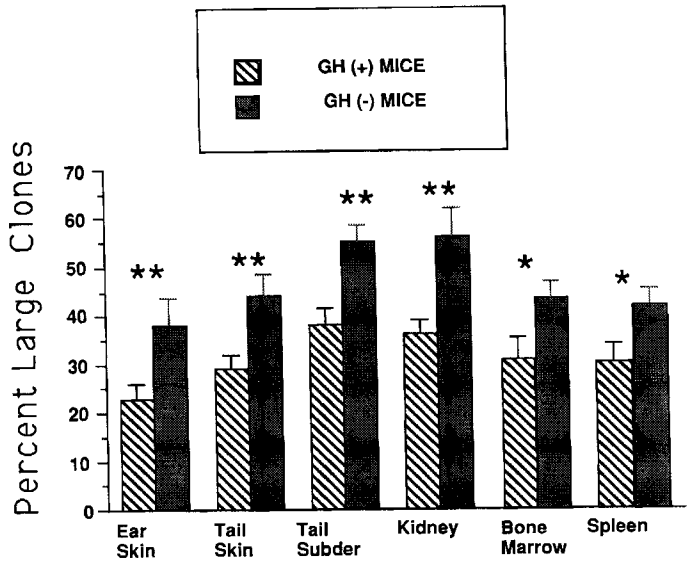

Fig. 3. Percentage of large clones (>16 cells) from six sites. The isolation of cells from each tissue is described in Materials and Methods. Ear skin, tail skin, and tail subdermal tissues produced fibroblast-like clones, kidney medulla produced epithelial clones, and bone marrow and spleen produced myofibroblasts. The percentage of large clones was calculated from the CSDs as shown in Figure 2. The mean percentage of large clones represents the average for 7–12 animals shown with standard error bars. The levels of significance between bGH− and bGH+ tissues were determined by Student's two-tailed t-test: $*P < .05$, $**P < .01$.

ment does not speak to the possibility that very high concentrations of bGH or IGF-1 present in vivo might directly damage the proliferative potential of the cells in various tissues when assayed later in vitro (see Discussion).

Proliferative potential is lost in the bGH+ animals after the commencement of accelerated growth at 3 weeks of age

The transgenic animals begin an accelerated GH-dependent growth spurt about 3 weeks postpartum, which is roughly coincident with the expression of GH receptors in all young mice and an increase n IGF-1 levels in the transgenics (Mathews et al., 1988). If the loss of proliferative potential in the adult transgenic animals is a result of bGH and/or IGF-1 accelerated growth, it should not occur until after 3 weeks. To test this hypothesis, 2-week-old animals were sacrificed and cells taken from tail and kidney for the CSD assay. The genotypes of these animals were determined by dot blot analysis of their liver extracts (courtesy of Dr. Richard Palmiter, University of Washington, Seattle, WA, as described in Materials and Methods). In Figure 5, the percentage of large clones from 2-week-old transgenics and controls are compared. No difference in the percentage of large clones is seen among these young animals indicating that loss of proliferative potential did not occur until the bGH/IGF-1-induced accelerated growth phase was underway (Mathews et al., 1988), and suggests receptor activation must first occur, at least in these tissues.

DISCUSSION

We have shown by CSD analysis that the in vitro replicative potentials of cells from six different tissues (dermal fibroblasts from ear and tail, subdermal connective tissue fibroblasts from tail, kidney medullary epithelial cells, as well as bone marrow and spleen myofibroblasts) are significantly reduced in short-lived transgenic bGH+ mice. The major part of the proliferative loss in the transgenic mice occurs between 2 weeks and 3 months of age, which is also the period of rapid body and organ growth to mature size. By comparison, in other studies by the authors, cells from the same tissues in two normal mouse strains, (C57BL/6 × C3H)F1 and (C57BL/6 × DBA/2)F1, control (ad libitum fed) mice did not lose significant amounts of replicative potential until after 12 months of age (Li et al., 1991 and unpublished data). It was also noted that caloric restriction, which lengthened the life span of these normal mouse strains by one third, significantly delayed the age-related accrual of proliferative loss. Interestingly, caloric restriction reduces both the GH and IGF-1 levels in rodents significantly (Armario and Jolin, 1989; Merry and Holehan, 1985).

It is relevant that preliminary studies on CSDs of skin fibroblasts derived from dog breeds of widely different sizes also demonstrate a similar phenomenon (Wolf et al., unpublished data). Eigenmann et al. (1984a,b) have previously shown that "giant" dog breeds (100–180 pounds) have elevated serum GH and, especially, IGF-1 levels relative to small dog breeds (5–25 pounds) and thus are similar to large bGH+ mice

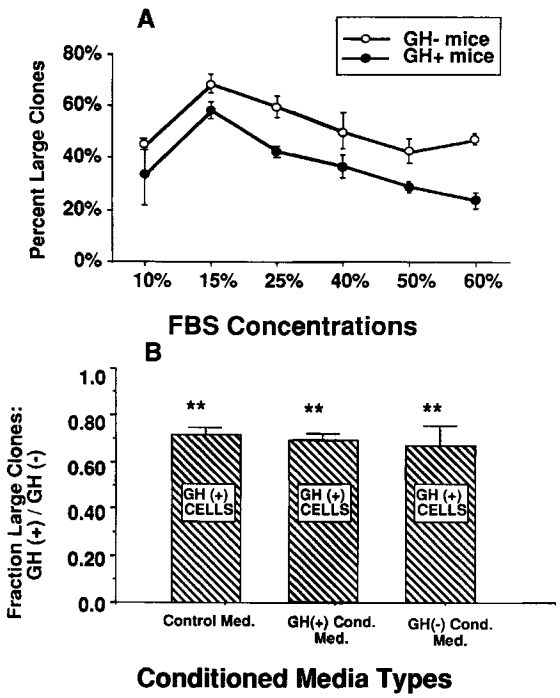

Fig. 4. Lack of effect of FBS concentration or of conditioned media on the relative percentage of large clones produced by tail skin fibroblasts from bGH+ and bGH− mice. **A:** Increasing amounts of FBS were added to the culture medium of triplicate cultures of skin fibroblasts from one bGH+ and one bGH− and the percentage of large clones (>16 cells) determined as described in Materials and Methods. More large clones were produced by cells from bGH− than bGH+ at all serum concentrations. The error bars represent one standard error of the mean. **B:** The fraction of large clones (>16 cells) produced by skin fibroblasts from bGH+ animals divided by the fraction of large clones produced by bGH− animals in various media. The results depict the mean and standard errors for five independently assayed animals. Bar 1, bGH+/bGH− cells grown in control media only (F-12 + 10% FBS); bar 2, bGH+/bGH− cells in media preconditioned by growth with bGH+ cells (mixed 1:1 with control medium); bar 3, bGH+/bGH− cells in media preconditioned by bGH− cells (mixed 1:1 with control medium). The conditioned media were prepared by cloning 500 fibroblasts/25 cm^2 flask from a bGH+ or a bGH− animal for one week in 7 ml standard F-12 cloning medium (with 10% FBS). The ratio of large bGH+ clones/large bGH− clones was significantly less than 1.0 (**$P < .01$) for all three media tested, but no differences were found due to conditioning of the media.

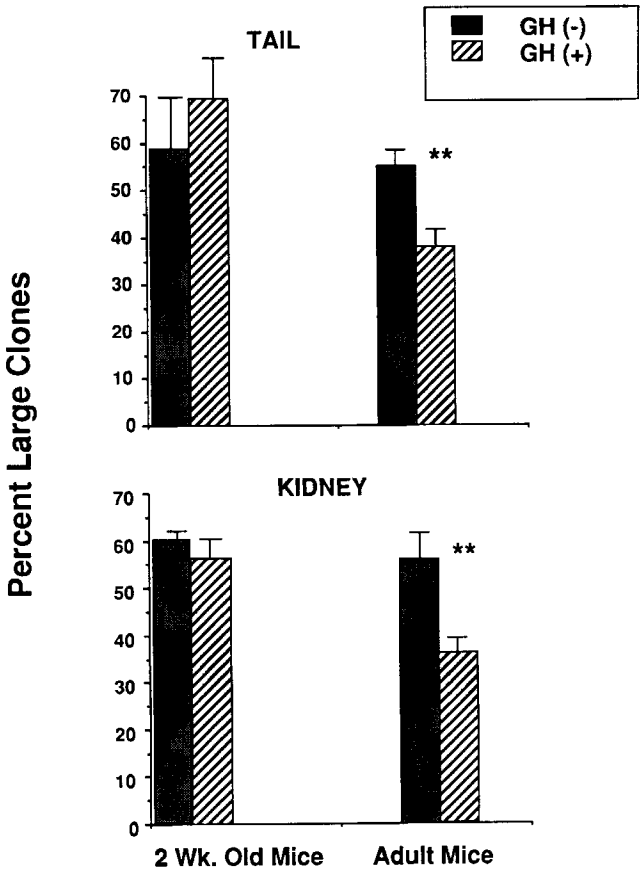

Fig. 5. Comparison of fraction of large clones produced by infant and adult bGH+ and bGH− mice. Cells were cloned from tail skin and kidney medulla from infant (2-week-old) and adult bGH+ mice (3–6 months) as described in Materials and Methods. Because the bGH+ transgenics are the same size as the control bGH− animals until 3–6 weeks of age, the identity of the 2-week-old bGH+ animals was determined by dot blot analysis (see Materials and Methods). Cells from 10 adult mice and five 2-week-old animals were analyzed, and the mean and standard error of the mean shown for each condition; *$P < .05$, **$P < .01$. Solid bars, control mice; hatched bars, bGH+ mice.

in this respect. In preliminary results skin fibroblasts from adult dogs of several giant breeds formed significantly fewer large clones than did adult dogs of small breeds of matched ages. Although formal life-span tables are not available for all the breeds, an examination of veterinary necropsy records (nonaccident-related cases) indicates that only 14% of these giant breeds survive past 9 years; whereas, 42% of the small and average sized breeds do so (Vet Med Data Base, School of Veterinary Medicine, Purdue). Importantly, the high levels of GH and IGF-1 present in the large dogs are the result of a normal endocrine mechanism and loss of proliferative potential in this system cannot be attributed to abnormal expression of GH as in the transgenics (Quaife et al., 1989). The parallel loss of replicative potential and shortened life span in two such widely different animal models strengthen the case for a causal connection. We also note that life span is reduced in acromegaly and gigantism, which involve overproduction of GH and IGF-1 via the normal endocrine route in humans (Wright et al., 1970). Finally, these findings are consistent with older reports that unknown pituitary factor(s) may accelerate aging in mice which have been previously presented by several authors (Everitt, 1980, 1982; Denckla, 1978; Harrison et al., 1982).

Out studies raise several questions about bGH+ transgenic mice: 1) Are the short life spans of these mice due to an accelerated form of normal aging? 2) Are the reduced replicative potentials of cells from bGH+ mice causally linked to their shortened life spans? and 3) What are the mechanism(s) by which high levels of GH and IGF-1, normally considered mitogenic for cells (Isaksson et al., 1991), reduce replicative potential in these mice? These questions are only partially answerable at this time. The direct cause of death in the short-lived transgenic animals has been attributed to the

formation of degenerative lesions, especially in the kidney (Quaife et al., 1989; Doi et al., 1988; Behringer et al., 1990). Although the severity and early occurrence of the lesions in the bGH+ mouse are specific to this model, many of these lesions including the probably fatal renal lesions also occur during normal aging in mice (Bronson and Lipman, 1991). Furthermore, we are confident that the ubiquitous loss of proliferative potential is not secondary to formation of pathological lesions since no lesions have been reported in four of the tissues studied (ear and tail skin, tail connective tissue, and bone marrow stroma) (Quaife et al., 1989; Doi et al., 1988; Brem et al., 1989).

The mechanism(s) that cause early loss of proliferative potential in the bGH+ mice are not known. An attractive hypothesis is that the extra doublings forced upon mouse or dog cells by the mitogenic activities of high levels or GH and/or IGF-1 needed to achieve and maintain a large stature have exhausted the proliferative potential of the tissues, reducing replicative reserves later in life (Hayflick, 1965). It is also possible that GH and IGF-1 levels may directly or indirectly induce some form of cell "damage" that results in an acceleration of cell "senescence." In fact, we have observed (unpublished data) that treatment of human diploid fibroblasts (HDF) with IGF-1 (50 ng/ml), in medium with low FBS for 1–2 weeks, results in substantial loss of replicative potential when treated cells are subsequently cloned. Under the low serum conditions used, the IGF-1 does not induce a significant increase in cell number; but rather, appears to increase cell size in the absence of cell division. Cells left in low serum medium alone have previously been observed to undergo similar "unbalanced growth" and loss of proliferative potential but at a slower rate than the cells exposed to IGF-1 (Angello et al., 1989).

Clearly, long-term exposure to high levels of GH and/or IGF-1 are associated with early loss of cellular replicative potential and early death. Studies by others on the effects of low levels of GH injected into normal animals are less clear. Rudman et al. (1990) have recently reported that weekly injections of low levels of GH into old men actually reversed some aspects of age-related deterioration. However, this study lasted only 6 months and long-term effects are not known. Also, Khansari and Gustad (1991) have reported that biweekly injections of low doses of human GH into mice actually lengthen life span. The latter report is difficult to interpret because human GH has been reported to raise a strong immunological response in rats which reduces or abolishes biological activity, and a similar immunological interference may occur in mice (Groeseck and Parlow, 1987; M.J. Cronin, personal communication). Finally, since long-lived calorically restricted rodents actually have reduced levels of GH and IGF-1, reduced levels of these hormones are at least consonant with life-span extension (Armario and Jolin, 1989; Merry and Holehan, 1985).

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