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The Effect of Aging on the Seminiferous Epithelium of the Brown Norway Rat

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ABSTRACT: Aging of the mammalian testis is often accompanied by loss of germ cells and, as a result, decreased daily sperm production. It is currently unknown whether cell loss is the result of aging-related changes in germ cells or whether there are also aging-related changes in the Sertoli cells that normally support germ development and differentiation. To begin to compare the effects of age on germ cells and on Sertoli cells, we examined brown Norway rats of 6, 12, 18, 21, and 24 months of age for the frequency of seminiferous tubule regression and total testis contents of transcripts for three Sertoli cell products: SGP-2, transferrin, and cyclic protein-2 (CP-2)/cathepsin L. Histological analysis revealed no changes in the seminiferous epithelium from 6 to 12 months of age. However, from 12 to 24 months of age, the percentage of normal tubules gradually decreased from 95% to 15% of the total while the percentage of fully regressed tubules (containing no germ cells per tubule cross section) increased from 0% to 78%. In our analysis of specific Sertoli cell transcripts, we noted no change in total testis content of SGP-2 mRNA from 6 to 24 months. However, total testis content of transferrin mRNA was unchanged from 6 to 18 months, but increased by 24 months to 368% of the content of a 6-month-old testis. In contrast, total testis content of CP-2/cathepsin L mRNA was unchanged from 6 to 12 months, but decreased by 24 months to 58% of the content of a 6-month-old testis. We conclude that aging-related loss of spermatogenic cells in the brown Norway rat is accompanied by specific changes in expression of particular Sertoli cell transcripts.

Key words: Aging, Sertoli cells, rat, gene expression, spermatogenesis.

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In many mammals, aging of the male leads to a decrease in the number of spermatogenic cells and, thus, decreased daily sperm production. In both humans and rodents, aging-related atrophy of the seminiferous tubules begins focally; atrophic tubules are often observed adjacent to tubules exhibiting normal spermatogenesis (vom Saal and Finch, 1988; Paniagua et al, 1991). This focal atrophy suggests that aging-related changes in the seminiferous epithelium are intrinsic to the testis and are not due to a decline in extrinsic factors that regulate testis function, such as serum levels of gonadotropic hormones. In rodents, the atrophy of the seminiferous epithelium is primarily due to loss of spermatogenic cells, as aged rats develop Sertoli cell-enriched tubules (Humphreys, 1977). In aged humans, atrophy results from a decrease both in numbers of Sertoli cells and in numbers of compacted spermatids per Sertoli cell (Johnson et al, 1983, 1984, 1990; Paniagua et al, 1987a,b, 1991). The specific loss of germ cells in both humans and rodents could be due to aging-related changes intrinsic to germ cells. Alternatively, the loss of germ cells could result in part or completely from a decreased ability of Sertoli cells to support germ cell survival and differentiation. Such aging-related changes in Sertoli cell function might be reflected in specific alterations in the steady state levels of particular Sertoli cell transcripts.

This paper compares the loss of germ cells and changes in Sertoli cell function as male brown Norway rats progress from 6 to 24 months of age. We studied the brown Norway rat because aged rats of this strain are remarkably disease free and they exhibit marked aging-related changes in testis structure and function (Zirkin et al, 1993). Thus, aging-related changes in testis structure and function of brown Norway rats are most likely due to aging per se and are not secondary to the effects of disease. To assess the effects of age on loss of spermatogenic cells, we determined, for each age, the percentage of tubules that were morphologically normal, partially regressed (contained fewer spermatogenic cells than a normal tubule), and fully regressed (contained no spermatogenic cells). To assess Sertoli cell function, we measured testis content of transcripts for three Sertoli cell products: cyclic protein-2 (CP-2)/cathepsin L, transferrin, and SGP-2. Three different transcripts were analyzed in order to determine whether aging leads to an overall decline in Sertoli cell function or whether aging has distinct effects on specific functions of this cell.

We now report that from 12 to 24 months of age, the

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male brown Norway rat experiences a progressive increase in frequency of fully regressed seminiferous tubules. We further report that while steady state levels of SGP-2 mRNA are unchanged from 12 to 24 months of age, levels of transferrin mRNA are increased and levels of CP-2/cathepsin L mRNA are decreased during this same period.

Materials and Methods

Animals

Brown Norway rats at 6, 12, 18, 21, and 24 months of age were purchased through the National Institute on Aging. Animals were housed for 2–3 weeks in pairs in our vivarium in microisolator cages with a light cycle of 14 hours light:10 hours dark. Animals used as the source of perfusion-fixed testes were killed by deep phenobarbital anesthesia and exsanguination. Animals used for quantitative analysis of seminiferous tubule atrophy and for analysis of specific Sertoli cell transcripts were killed by decapitation.

Analysis of Aging-Related Changes in the Cellular Composition of the Seminiferous Epithelium

To qualitatively describe aging-related changes in the seminiferous epithelium, testes of brown Norway rats at 6, 18, and 24 months of age (n = 3/age) were fixed with 5% glutaraldehyde in cacodylate buffer by whole body perfusion. Tissue was postfixed in osmium tetroxide–potassium ferricyanide and embedded in Epon; then 5-μm sections were cut and tissue was stained with toluidine blue.

To quantitatively describe aging-related changes in the seminiferous epithelium, testes from the animals also used for RNA analysis were weighed and cut in half with a razor blade. One-half of each testis was fixed overnight in Bouin's solution and stored in 70% ethanol. The fixed halves of each testis were then embedded in Paraplast and 5-μm sections were cut and stained with PAS–hematoxylin (Leblond and Clermont, 1952). At least 100 tubules from each testis were examined and scored as normal, partially regressed (having fewer germ cells per tubule cross section than a normal tubule), or fully regressed (having no germ cells per tubule cross section). In enumerating regressed tubules, we also noted whether the regressed tubule was undergoing fibrosis, which would constitute evidence of death of Sertoli cells.

Isolation of Testis RNA and Quantitation of Total Testis Content of Transcripts for Cyclic Protein-2/Cathepsin L, Transferrin, and SGP-2

Animals that were used for analysis of aging-related changes in the seminiferous epithelium were also used as the source of tissue for analysis of total testis content of mRNAs for CP-2/cathepsin L, transferrin, and SGP-2. Thus, for each animal, the halves of both testes that were not fixed in Bouin's solution were pooled, weighed, decapsulated, and homogenized with a Polytron (Brinkman Instruments, Westbury, New York) in 10 volumes of 4.2 M guanidinium isothiocyanate, 25 mM sodium citrate, and 0.7% β-mercaptoethanol. Homogenates were stored frozen at −70°C.

RNA was isolated from the homogenates by centrifugation at 150,000 × g through 5.7 M CsCl–0.1 M Na_2EDTA for 18 hours as previously described (Erickson-Lawrence et al, 1991) with the following exception: prior to centrifugation, 10,000 cpm of ^{35}S-labeled control RNA was added to each sample, and this control ^{35}S-RNA was used to determine percent recovery of total RNA, which was approximately 35% for all samples. This ^{35}S-RNA was synthesized using the control DNA template purchased from Promega, T7 polymerase, and ^{35}S-CTP. Based on mass of RNA recovered (measured by absorbance at 260 nm) from a specific volume of homogenate, the volume of the total testis homogenate, and the percent recovery of the ^{35}S-RNA standard, we calculated total testis RNA content. We then analyzed equal percentages of total testis RNA from each rat for each of the three transcripts. Approximately 4%, 2%, and 6% of total testis RNA from each rat was analyzed for CP-2/cathepsin L, SGP-2, and transferrin mRNAs, respectively. Assuming no loss of Sertoli cells during aging, analysis of equal percentages of total testis RNA allowed us to determine relative amounts of each transcript from equal numbers of Sertoli cells.

To quantify the levels of each transcript in each animal, RNA was fractionated in denaturing agarose gels along with four different amounts of a standard pool of total testis RNA isolated from 6-month-old rats. RNA was transferred overnight to nitrocellulose by capillary action and then hybridized to a radiolabeled cDNA probe for CP-2/cathepsin L mRNA (Erickson-Lawrence et al, 1991) or cRNA probes for transferrin or SGP-2 mRNAs (Collard and Griswold, 1987; Huggenvik et al, 1987; Morales et al, 1987). Blots were exposed to X-ray film and intensities of each band were determined by scanning densitometry using the Image Pro II (Media Cybernetics, Inc., Silver Spring, MD) system. Relative amounts of CP-2/cathepsin L, transferrin, and SGP-2 mRNAs in each sample were extrapolated from standard curves generated by titration of total testis RNA isolated from 6-month-old rats. Data were expressed as percentage of the content of each of the three transcripts in a standard 6-month-old rat testis.

Statistical Analysis

Data were analyzed by analysis of variance; differences between individual means were demonstrated by Duncan's multiple-range test (Snedecor and Cochran, 1967). Data on analysis of testis content of specific transcripts were subjected to log transformation prior to the statistical analysis. Significant differences were defined as $P \leq 0.05$.

Results

Aging-Related Changes in the Morphology of the Seminiferous Epithelium of the Brown Norway Rat

Figure 1A–C, which are low power light micrographs of testes, qualitatively illustrate the general aging-related changes in the seminiferous epithelium of the brown Norway rat. At 6 months of age, all seminiferous tubules were morphologically normal (Fig. 1A). By 18 months of age, both normal and regressed tubules were observed (not

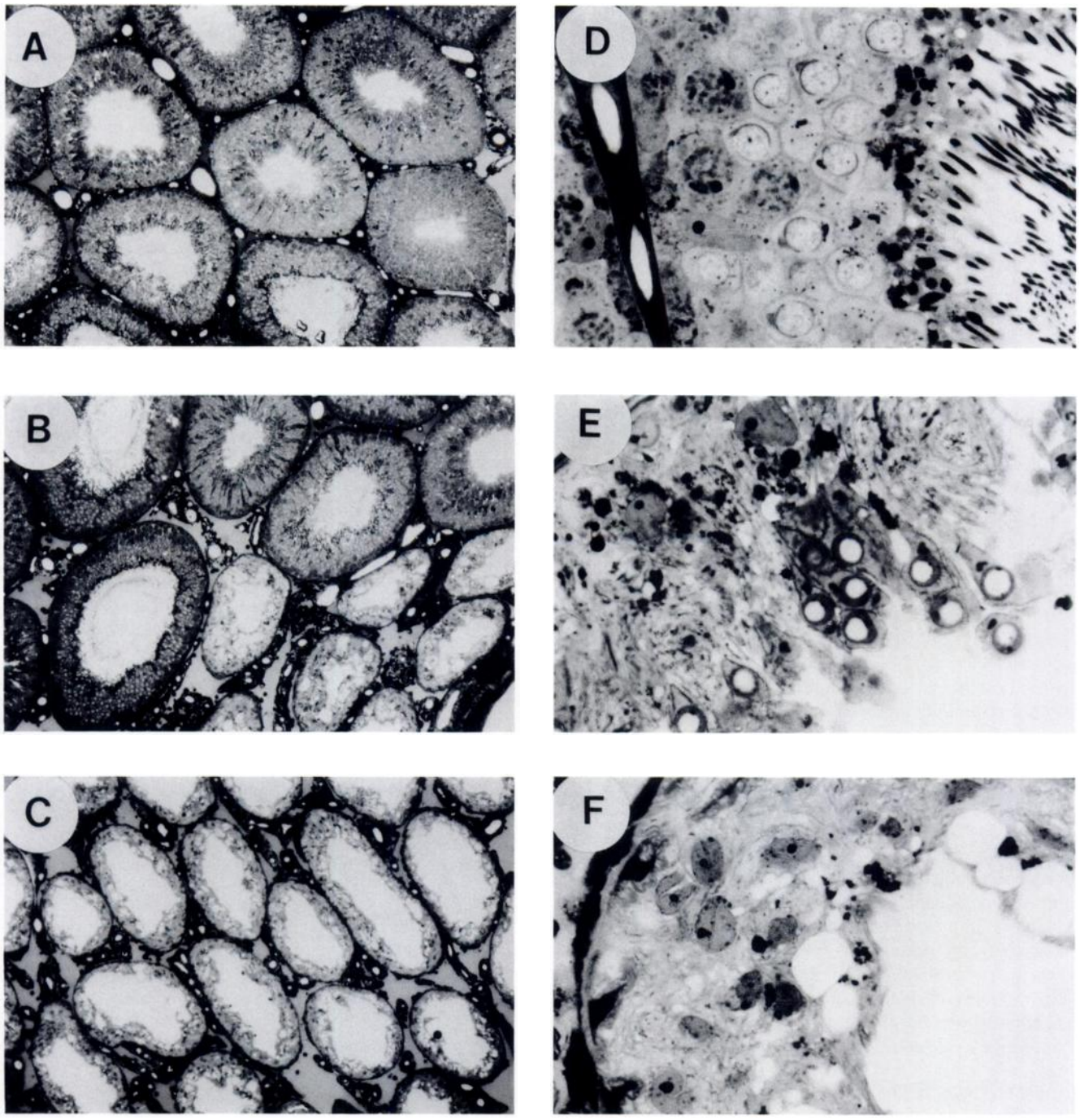

FIG. 1. The effects of aging on the morphology of the seminiferous epithelium of the brown Norway rat. **(A)** Normal seminiferous tubules in a 6-month-old rat. **(B)** The presence of adjacent normal and regressed seminiferous tubules in a testis of a 24-month-old rat. **(C)** Fully regressed tubules in a testis of a 24-month-old rat. **(D)** The appearance of a normal seminiferous tubule of a 6-month-old rat. **(E)** The appearance of a partially regressed seminiferous tubule of an 18-month-old rat. Sertoli cells and round spermatids are seen in this tubule; note the abnormal nuclear morphology of the round spermatids. **(F)** The appearance of a regressed seminiferous tubule of a 24-month-old rat. Note the complete absence of germ cells and the random distribution of the Sertoli cells in this tubule. A–C, ×80; D–F, ×640.

shown). At 24 months, testes contained either a mix of normal and regressed tubules (Fig. 1B) or contained only regressed tubules (Fig. 1C). We noted no substantial infiltration of lymphocytes or macrophages around the regressed tubules. Figure 1D–E provide a qualitative description of the process of regression. Figure 1D shows a normal stage VII tubule from a 6-month-old rat; morphologically normal Sertoli cells, spermatogonia, spermatocytes, and round and compacted spermatids are present in this tissue. Partially regressed tubules were observed

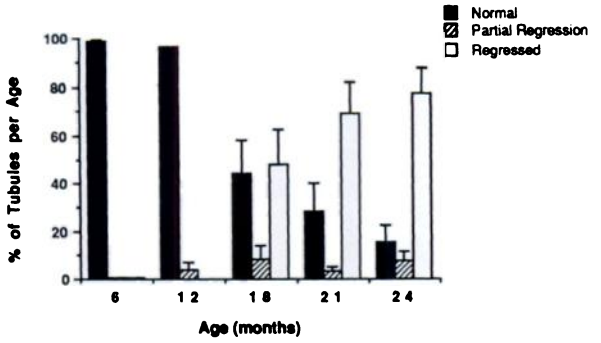

FIG. 2. The percentages of normal, partially regressed, and regressed tubules in rats at 6, 12, 18, 21, and 24 months of age. At least 100 tubules from six rats/age were examined. Data are expressed as means ± SEM.

both at 18 and 24 months and contained a few germ cells, which were often morphologically abnormal. For example, the tubule shown in Figure 1E contains no spermatocytes, but does contain round spermatids with abnormal nuclear morphology. In fully regressed tubules, which were abundant at 24 months, many Sertoli cells but few germ cells were observed (Fig. 1F). It was noteworthy that in normal and partially regressed tubules, all Sertoli cells exhibited normal polarized morphology, with their nuclei present in the basal portion of the seminiferous epithelium (Fig. 1D,E). In contrast, in fully regressed tubules, Sertoli cell nuclei were observed throughout the epithelium, indicating a loss of polarity of some of these cells. It should be noted, however, that we never observed pycnotic Sertoli cell nuclei in regressed tubules, suggesting that few Sertoli cells died during aging of the brown Norway rat.

Sections of paraffin-embedded testes from six rats/age-group were used to determine the frequency of normal, partially regressed, and fully regressed tubules at 6, 12, 18, 21, and 24 months of age (Fig. 2). At 6 and 12 months, 99% and 96%, respectively, of the tubules were normal, and the few abnormal tubules were partially regressed. By 18 months, however, normal tubules had been reduced to 44% of total, and fully regressed tubules increased to 48% of total (Fig. 2). From 18 to 24 months, the percentage of normal tubules was reduced significantly to 15% of the total, and regressed tubules increased to 78% of the total. Very few of the regressed tubules at any age showed evidence of fibrosis; of the total of 3,000 regressed tubules that were examined in this study, only 6 were fibrotic.

Seminiferous tubule regression tended to start in one testis. In four of the six 18-month-old rats we examined, almost all of the regressed tubules were present in one testis per animal; two of the testes containing regressed tubules were from the right side of the animal, and two were from the left side. Thus, there was no left–right bias to this asymmetric testicular regression. However, in 24-month-old rats, regressed tubules were present in both testes of all animals.

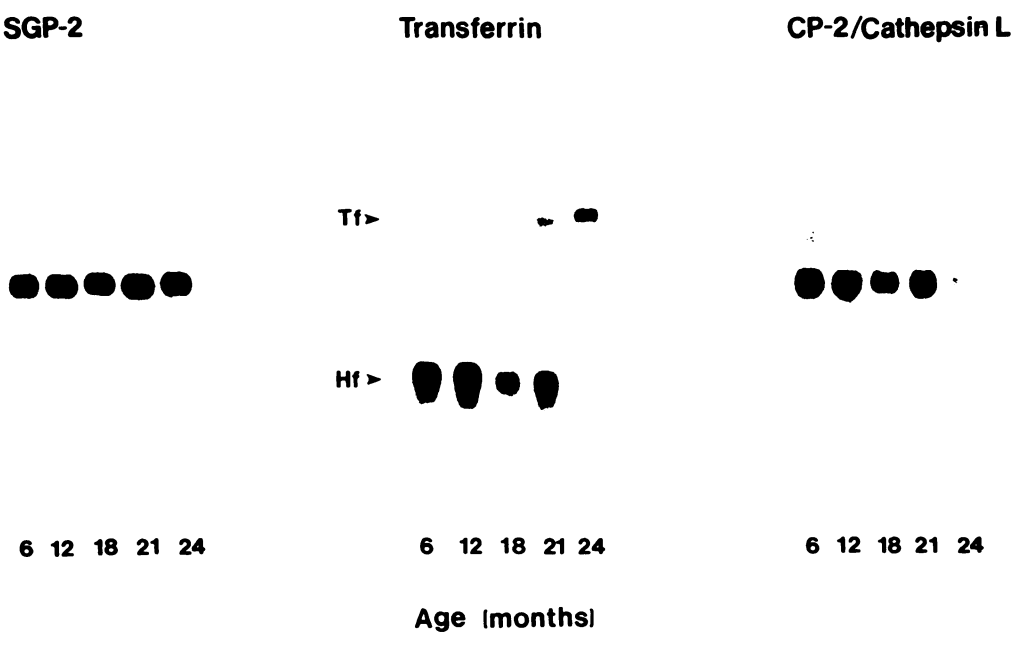

FIG. 3. Northern blot analysis of total testis content of transcripts for SGP-2, transferrin, and CP-2/cathepsin L in a single rat of 6, 12, 18, 21, and 24 months of age. Equal percentages of total testis RNA were analyzed for each transcript by agarose gel electrophoresis and Northern blot analysis. Shown here is a representative autoradiogram from our studies. Tf, transferrin mRNA, which is present in Sertoli cells; Hf, hemiferrin, which is present in germ cells.

Aging-Related Changes in Steady State Levels of Transcripts for the Sertoli Cell Products Cyclic Protein-2, Transferrin, and SGP-2

Equal percentages of total testis RNA from each rat were fractionated on agarose gels and subjected to Northern analysis to determine relative amounts of transcripts for SGP-2, transferrin, and CP-2/cathepsin L. A representative analysis of each transcript in testes from a single animal at each age is shown in Figure 3. This analysis indicates that the amount of transcript for the Sertoli cell product SGP-2 was constant from 6 to 24 months, whereas the amount of testicular transferrin mRNA was substantially increased at 24 months. However, the germ cell transcript hemiferrin, which cross hybridizes with transferrin cRNA, was undetectable at 24 months. The lack of the hemiferrin transcript reflected the lack of germ cells in 24-month-old testes. In contrast to what was noted for transferrin mRNA, testis content of the transcript for a third Sertoli cell product, CP-2/cathepsin L, was substantially decreased at 24 months.

Quantitative analysis of testis content of these three transcripts in all rats were determined by scanning densitometry and by use of standard curves for all three transcripts. Titration of 2–10 μg testis RNA on agarose gels and hybridization of the RNA with cDNAs for CP-2/cathepsin L, transferrin, and SGP-2 generated linear dose responses (r = 0.95, 0.96, and 0.98 for the dose response of total testis RNA in assays for CP-2/cathepsin L, transferrin, and SGP-2 mRNAs, respectively; data not shown). Figure 4 shows the quantitative effects of age on testis content of all three Sertoli cell transcripts. There was an overall, significant effect of age on testis content of CP-2/cathepsin L mRNA. The amount of CP-2/cathepsin L mRNA per testis decreased gradually from 12 to 24 months. At 24 months, testis CP-2/cathepsin L mRNA content was 58% of the 6-month value, and at 24 months was significantly less than the content of this transcript in testes from 6–21-month-old rats. There was also an overall, significant effect of age on the amount of transferrin mRNA in a testis. In contrast to what we noted for CP-2/cathepsin L mRNA, transferrin mRNA content was unchanged from 6 to 18 months, but increased thereafter and at 24 months was 368% of the content of a 6-month-old testis. Testis transferrin mRNA content at 24 months was significantly greater than at all other ages we examined. Finally, quantitative analysis of SGP-2 transcripts demonstrated that total testis content of this transcript was constant from 6 to 24 months of age.

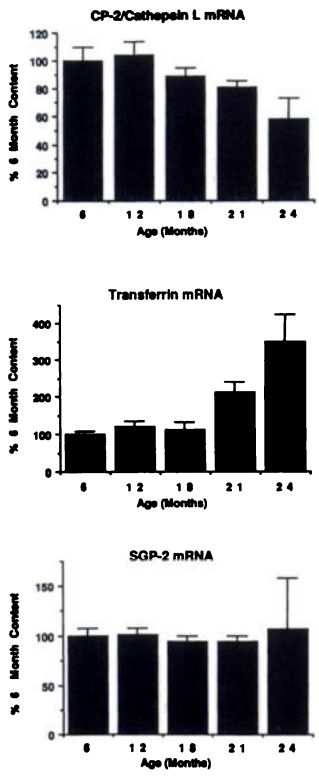

FIG. 4. Quantitative analysis of the effect of age on total testis content of CP-2/cathepsin L mRNA, transferrin mRNA, and SGP-2 mRNA. Data (means ± SEM) are presented as percentage of the content of each transcript in a standard 6-month-old testis. n = 6 for 6, 12, 18, and 24 months of age; n = 4 for 21 months of age.

Discussion

Our studies confirm and expand previous observations of aging of the rat testis by demonstrating that regression of the seminiferous epithelium in the brown Norway rat begins focally, but ultimately leads by 24 months to regression of most seminiferous tubules (Humphreys, 1977). We also noted that seminiferous tubule regression tended to begin in one testis and that regression was as likely to begin in the left testis as in the right testis. This conclusion, confirmed by ongoing studies in our laboratory (Wright, unpublished observations), is significant because the right testicular vein generally empties into the vena cava, whereas the left testicular vein generally empties into the left iliac vein (Goldstein et al, 1983). A left–right bias to the onset of seminiferous tubule regression would have implicated the venous drainage of the testis as a factor in seminiferous tubule regression. As there was no left–right bias to tubule regression, and as we have observed no significant vascular disease in any organ of aged brown Norway rats (Zirkin et al, 1993), we suggest that seminiferous tubule regression in the aged brown Norway rat does not result from vascular disease. Seminiferous tubule regression also does not appear to result from lack of hormonal support for the seminiferous epithelium. Zirkin et al (unpublished) have demonstrated that serum follicle-stimulating hormone (FSH) levels increase significantly from 6 to 24 months of age. They have also demonstrated

that seminiferous tubule testosterone levels in 24-month-old rats are sufficient to maintain spermatogenesis in a normal adult animal. Finally, seminiferous tubule regression does not appear to be due to an acute autoimmune disease, because we observed no significant accumulation of lymphocytes around regressing tubules. Because seminiferous tubule regression does not appear to have a vascular origin, a hormonal origin, or an autoimmune origin, we suggest that aging of the seminiferous epithelium results from aging-related processes within the testis that we currently do not understand.

The demonstration of constant testis content of SGP-2 mRNA suggests that some Sertoli cell functions are unaffected by testicular aging. The lack of change in SGP-2 mRNA levels with age is consistent with observations by other investigators that testis content of this transcript is unaffected by changes in testicular hormone levels or by the loss of germ cells (Roberts et al, 1991, 1992). Additionally, because SGP-2 mRNA content in other tissues such as the rat prostate and corpora lutea increase during programmed cell death (Sensibar et al, 1991; Keynard et al, 1992), the constant testicular content of this transcript is consistent with the hypothesis that there is no significant death of Sertoli cells during aging of the male brown Norway rat. This hypothesis is further supported by the facts that we have never observed a Sertoli cell with a pycnotic nucleus and that of the 3,000 regressed tubules we examined in this study, only 6 were fibrotic. Thus, it seems likely that in comparison to what has been reported for the human male (Johnson et al, 1984), there is no significant death of Sertoli cells in the aged brown Norway rat.

In contrast to what we observed for SGP-2 mRNA, testicular transferrin mRNA content began to increase between 18 and 21 months of age, and by 24 months was 368% of the content measured in testes of 6-month-old rats. The response of CP-2/cathepsin L mRNA to aging was the opposite to what we noted for transferrin mRNA. Testis CP-2/cathepsin L mRNA content began to decrease after 12 months, and at 24 months was 58% of the content measured in testes of 6-month-old rats. Because we have no evidence for significant death of Sertoli cells during aging of the brown Norway rat and because we analyzed equal fractions of total testis RNA per rat, which, therefore, contain transcripts from an equal number of Sertoli cells, the changes we noted in transferrin and CP-2/cathepsin L mRNA levels must reflect aging-related changes in the function of the average Sertoli cell. Thus, whereas CP-2/cathepsin L mRNA content of the average, aged Sertoli cell is decreased, transferrin mRNA content is increased.

Although significant changes in CP-2/cathepsin L and transferrin mRNAs were detected at 24 months, significant numbers of regressed seminiferous tubules were noted by 18 months. One possible explanation for this lag between the loss of germ cells and changes in Sertoli cell gene expression is that the changes in Sertoli cell gene expression resulted not from an intrinsic aging of Sertoli cells but from the loss of germ cells. It should be noted, however, that our observation that transferrin mRNA increases with aging is inconsistent with this suggestion, because *in vivo* and *in vitro* experiments document that germ cells stimulate transferrin mRNA expression by normal rat Sertoli cells. For example, Sertoli cells from 60-day-old rats with normal testes contain significantly higher levels of transferrin mRNA than Sertoli cells from 60-day-old rats that had been irradiated *in utero* and thus had few germ cells (Stallard and Griswold, 1990). Additionally, *in vitro*, germ cells have been shown to stimulate both transferrin mRNA levels in Sertoli cells and the synthesis and secretion of transferrin by these cells (Djakiew and Dym, 1988; La Magueresse and Jegou, 1988; Castellon et al, 1989; Stallard and Griswold, 1990). Thus, if germ cell loss was the primary cause of age-related changes in transferrin mRNA levels, all published data indicate that total testis transferrin mRNA levels should have decreased, not increased as we observed. Thus, we suggest that aging-related increases in transferrin mRNA levels of Sertoli cells reflect an intrinsic aging-related change in Sertoli cell function. One potential cause of this change is an increased responsiveness of Sertoli cells to hormones such as FSH. It should be noted, however, that aged human Sertoli cells have a decreased responsiveness to FSH (Tenover et al, 1988). Alternatively, aged Sertoli cells may have an increased basal level of transcription of the transferrin gene or a decreased basal rate of turnover of the transcript.

We have previously demonstrated that in normal, mature rat testes, CP-2/cathepsin L mRNA is expressed at high levels only in stage VI–VIII seminiferous tubules, and we attributed this stage-specific expression to the stimulation of Sertoli cells by specific germ cells (Erickson-Lawrence et al, 1991). Quantitative analysis of the stage-specific synthesis of CP-2 protein showed trends identical to what was observed for levels of the transcript and demonstrated that 85% of all CP-2 synthesized in a testis was synthesized by stage VI–VIII tubules, which constitute 39% of the tubules in a testis (Parvinen, 1982; Wright, 1988). When we began the studies of aging of the testis of the brown Norway rat, we anticipated that loss of all germ cells would cause CP-2/cathepsin L mRNA levels per Sertoli cell to be reduced to basal levels. Based on the frequency of each stage of the cycle and assuming that rates of synthesis of the protein reflected the steady state levels of the transcript, we anticipated that CP-2/cathepsin L mRNA levels in aged, germ cell-depleted testes would be 24% of what we observed in normal 6-month-old rats. However, our data demonstrate that total testis levels of this transcript in 24-month-old rats, although significantly

reduced, were 58% of 6-month-old levels and thus, twice as high as we anticipated. Only a small part of the difference between observed and anticipated results is attributable to the fact that about 15% of all tubules in the 24-month-old rats were not regressed (see Fig. 2). Thus, we propose two possible explanations to explain our observations. First, stage-specific expression of this transcript may be partially retained by the Sertoli cells in the absence of germ cells. This suggestion is consistent with the observations of Boockfor and colleagues, who demonstrated that stage-specific secretion of transferrin is maintained by mature Sertoli cells cultured in the absence of germ cells (Garza et al, 1991). Second, although stage-specific expression of CP-2/cathepsin L mRNA may be lost during aging, there may be an increase in the constitutive expression of this gene by all Sertoli cells, regardless of the stage of the cycle or the presence or absence of adjacent germ cells. Exploration of these two possibilities requires analysis of transcript levels in individual Sertoli cells *in situ*.

Our observation that aging affects expression of specific Sertoli cell transcripts is consistent with what has been observed with aging of other cell types. For example, senescent fibroblasts fail to express c-fos and the histone H3 genes when stimulated by serum *in vitro* (Seshadri and Campisi, 1990). Additionally, *in vivo* studies demonstrate that during aging of the mouse, liver content of glucocorticoid and insulin receptor mRNAs increases while levels of cJun mRNA decrease and then increase (Mote et al, 1991; Spindler et al, 1991). These aging-related changes in gene expression can be cell-type specific. In aged rats, levels of GAPDH and β-actin are increased in spleen but not in liver or brain (Slagboom et al, 1990). These data are therefore consistent with a conclusion that aging has specific effects on expression of particular genes in Sertoli cells.

In summary, our data suggest that during testicular aging of the brown Norway rat, there is an initially focal but progressive loss of germ cells. There is no evidence that this loss has a vascular, immune, or hormonal basis, and we therefore suggest that loss of germ cells is due to aging of the testis. Loss of germ cells is followed by changes in levels of a subset of some Sertoli cell transcripts. Increased levels of transferrin mRNA in aged rats has led us to suggest that age-related changes in Sertoli cell gene expression may be a primary result of aging and not a secondary effect of loss of germ cells. However, whether loss of germ cells in the aged rat results in part from altered Sertoli cell function requires further investigation.

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