Age-induced apoptosis in the male genital tract of the mouse

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Abstract

We have examined the effects of ageing on the increase in apoptotic cells numbers in the male genital tract of the house mouse (*Mus musculus*). We have found that not all organs have the same response. There is an induction of apoptosis in both the epididymis and ventral prostate. However, seminal vesicles and other prostatic lobes remain unaffected. Apoptosis was assessed by several methods: TUNEL, detection of the active fragment of caspase-3 and the pattern of DNA fragmentation on agarose gels. This increase in apoptosis is related to the fall in testosterone levels, although there is only a partial decrease in androgen receptor (AR). AR is still present in all tissues and only moderately reduced in the epididymis and ventral prostate. A more intense increase of lipofuscin granules, which may be indicative of oxidative stress, occurred in these tissues. Finally, testosterone supplementation reverses the changes (both in apoptosis and lipofuscin content in the tissue), suggesting a role of androgens in these processes.

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Introduction

Ageing has different effects on the reproductive tract. In the testes, spermatogenesis and steroidogenesis decrease with old age, as described in ageing laboratory rats (Levy *et al.* 1999, Zirkin & Chen 2000). In addition, in the epididymal epithelium, some striking segment-specific changes occur at the histological and biochemical levels prior to the major loss of spermatogenesis. These changes include some ultrastructural features which are characteristic of ageing, such as accumulation of lipofuscin granules, a dramatic increase in the thickness of the basement membrane and the number of halo cells (Serre & Robaire 1998), and changes in the junctional complexes between epithelial cells (Levy & Robaire 1999). In addition, changes in the expression of genes related to oxidative stress in the epididymis due to age have also been described (Jervis & Robaire 2002). Changes in gene expression related to ageing include the decrease of several mRNAs, such as the 5 α -reductase isozymes and proenkephalin, that occurs in the epididymis between 6 and 12 months of age (Viger & Robaire 1995). In fact, this decrease is the earliest marker for ageing in the male reproductive tract of the Brown Norway rat. Male accessory sex glands also experience changes. The secretory activity of the ventral prostate decreases and in the prostatic cells, supra- and paranuclear pleomorphic lysosomes can be observed (Zirkin & Strandberg 1984).

Apoptosis is an important physiological process that has been associated with ageing (Warner 1999, Higami & Shimokawa 2000). It is defined by a set of morphological and biochemical changes at different cellular levels (Wyllie *et al.* 1980), and the result is a physiological elimination of unwanted cells, leaving the surrounding tissue untouched. In relation to ageing, apoptosis has a primary negative effect by destroying essential and often irreplaceable cells, but it also acts to eliminate dysfunctional cells and protect the organs against cancer or hypertrophy (Warner *et al.* 1997).

In the testes, apoptosis increases with age, producing an accelerated germ cell loss (Wang *et al.* 1999, Kimura *et al.* 2003). This might be related to the fall in androgen levels (Steiner *et al.* 1984) and/or an increase in oxidative stress in the tissue (Samanta *et al.* 1999). On the other hand, these changes also occur in the epididymis and other reproductive organs, so it is not unreasonable to think of an increase in apoptotic indices in the ageing male tract. For this reason, we have examined the induction of apoptosis in the epididymis and male accessory sex glands in the senile mouse. We have estimated apoptotic indices and looked for a role of oxidative stress in the process. In addition, we studied the effects of testosterone administration in reversing age-induced apoptosis.

Materials and Methods

Animals

All male mice used in the experiments were CD-1 males bred in our colony. We had three groups. Group 1 was formed by six young (3 months old) mice and group 2, by 21 old (2 years old) males. In addition, six old mice received daily intramuscular injections of testosterone (5 µg/ml) for 2 weeks before death. They were assigned to group 3. The animal protocol used was covered by Law 223/88 on Animal Protection of Spain, and the European Union Agreement about Vertebrate Animal Protection (3/18/1986), and it has been approved by the CSIC ethics committee.

Testosterone measurements

Blood was obtained by cardiac puncture from the animals previously anesthetized by an intraperitoneal injection of a mixture of ketamine (50 mg Ketalar, Parke-Davis, Barcelona, Spain) and Rompún (Bayer, Leverkusen, Germany). Typically, 0.8 ml of Ketalar was mixed with 0.15 ml of Rompún and 9 ml of PBS, and 0.8 ml of the mixture was injected into the mouse. Clots were left for 1–2 h at 4°C, and then supernatants were centrifuged at 16 000 *g*. Serum was stored frozen at –20°C until use. Testosterone values were measured in triplicate by radioimmunoassay using the Testosterone/Dihydrotestosterone Biotrak Assay kit manufactured by Amersham (Arlington Heights, IL, USA). Sensitivity of the method was of 6.25 pg per tube (coefficient of intra-assay variation was 4% and coefficient of inter-assay variation was less than 12%).

Detection of DNA fragmentation

After the animals were bled to death, the male genital tract was isolated and dissected free of fat. For fixation, pieces of the male ducts (caput, corpus and cauda epididymidis) and the various accessory sex glands (seminal vesicles, coagulating glands, and dorsolateral and ventral lobes of the prostate) were immersed in 4% paraformaldehyde in PBS, and kept for 1–2 h at room temperature, and then overnight at 4°C. In the caput epididymidis, segments II and III described by Abe *et al.* (1983) for the mouse, were analyzed. Samples were subsequently washed and embedded in paraffin-wax following standard procedures. Thin sections (5 µm) were obtained using a Leica microtome, mounted on slides, cleared with xylol, hydrated, and stained with Weigert's iron hematoxylin solution (Clark 1981). The stained sections were subsequently washed in distilled water, counterstained with saturated picric acid to increase contrast, dehydrated, mounted with Entellan (Merck, Whitehouse Station, NJ, USA) and scored under bright-field optics for the presence of apoptotic cells. Apoptosis was defined according to the presence of condensed chromatin granules in the nucleus.

Several series of sections were used for detection of DNA fragmentation *in situ* using the TUNEL (Terminal transferase dUTP nick-end labeling) detection kit supplied by Roche (Basel, Switzerland), following the manufacturer's instructions. Nuclei were counterstained with a solution 10 µM of Hoechst 33342 (Sigma, St Louis, MO, USA). Results were assessed under epifluorescence using the appropriate filters.

The percentages of apoptotic cells were determined by counting randomly selected areas with at least 3000 cells. There were three animals per groups; thus, a minimum of 9000 cells per group was scored.

In addition, samples from the different tissues were freshly isolated, cut into small pieces and digested with collagenase (10 mg/ml in RPMI 1640 medium) for 30 min at 37°C. The tissue fragments were pelleted by centrifugation at 1500 *g* for 5 min, and fresh collagenase solution was added. After a new 30-min digestion, the cells were washed 2–3 times, and the pellet was subjected to extraction of extrachromosomal DNA, as described by Fabregat *et al.* (1996). Total DNA extracts isolated from young and old tissues were labeled with ³²P-dCTP using terminal transferase (Roche, Basel, Switzerland) and cleaned using the QIAquick Nucleotide Removal Kit (Qiagen, Valencia, CA, USA). The labeled DNA was resolved by electrophoresis through 1.5% agarose gel at 40–45 V for 5 h. Then, it was dried and exposed to an X-OMAT-AR (Kodak, Rochester, NY, USA) to visualize the DNA ladder.

Statistical analyses

All data are expressed as means ± S.E.M. Statistical differences among groups were examined by one-way ANOVA followed by Student's *t*-test (*P* < 0.05).

Caspase-3 detection

Samples of the tissues were snap frozen in liquid nitrogen and sectioned using a cryostat. The sections (5–7 µm) were fixed for 10 min in an ice-cold mixture of acetone and methanol (1:1) and then air dried and stored frozen until use. The non-specific binding sites were blocked by

Figure 1 Serum testosterone values in the different groups (Group 1: young (3 months old). Group 2: aged (2 years old). Group 3: aged plus testosterone. For group 1, *n* = 6; for 2, *n* = 15; and for 3, *n* = 6. Values are expressed as means ± S.E.M. Different letters indicate statistically significant differences (*P* < 0.05).

Figure 2 Detection of apoptosis in epithelia by hematoxylin staining (a–d) and TUNEL (e–h). (a) Caput epididymidis from an old mouse (group 2). The arrowheads indicate apoptotic bodies. Bar, 20 μ m. (b) Detail of a tubule of the caput epididymidis from an old mouse (group 2). Several apoptotic bodies, indicated by arrowheads, can be distinguished. Bar, 20 μ m. (c) Ventral prostate from a mouse from group 2. Apoptotic nuclei are indicated by arrowheads. Three of them (1, 2, 3) are magnified in the insets. Bar, 20 μ m. (d) Ventral prostate from an old mouse (group 2). Several apoptotic bodies (arrowheads) are seen. (e) Caput epididymidis of an old mouse stained by TUNEL. The positive reaction is visualized in green. Nuclei are counterstained with Hoechst 33342 and are observed in blue. Several apoptotic nuclei are indicated by arrows. Bar, 10 μ m. (f) Detail of an apoptotic cell (arrow) in the epididymal epithelium from an old mouse. Bar, 10 μ m. (g) Section of the ventral prostate from an animal from group 2 stained by TUNEL. Several apoptotic nuclei are indicated by arrows. Bar, 10 μ m. (h) Ventral prostate from an animal from group 3. No apoptotic nuclei can be seen. Bar, 10 μ m.

incubation for 1 h (RT) in 3% BSA in PBS. The primary antibody was an anti-ACTIVE Caspase polyclonal antibody (Promega, Madison, WI, USA) that specifically recognizes the cleaved active form of caspase-3 in human and mouse used at a concentration of 2 μ g/ml. An anti-rabbit IgG conjugated to FITC diluted 1:200 in PBS was used as the secondary antibody. The positive reaction was visualized in a Nikon fluorescence microscope. Images were recorded using a microscope equipped with a CCD system (200A, Polytronics, Emsworth, UK).

Androgen receptor expression

Androgen receptor expression was assessed by immunohistochemistry and Western blot, using as the primary antibody a polyclonal antibody, PG-21 (Upstate Biotechnology, Lake Placid, NY, USA). For Western blots, the tissues were homogenized and extracted with RIPA buffer (50 mM Tris, 150 mM NaCl, 1% NP40, 1% sodium deoxycholate, 1% SDS, 2 mM EDTA, pH 7.5) containing protease inhibitors (10 mM PMSF, leupeptin, antipain, chymostatin and pepstatin at 1 μ g/ml and benzamidine at

10 µg/ml) for 1 h in ice. The volume was 100 µl for every organ with the exception of the seminal vesicle, for which 200 µl were used. Protein concentration in the different samples was estimated using the BioRad Protein Assay kit (BioRad, Hercules, CA, USA). A total of 400 µg of proteins was loaded in 10% acrylamide gels and blotted onto 0.2 µm pore nitrocellulose filters. Nonspecific reaction was blocked by 1-h incubation in blocking reagent (Roche, Basel, Switzerland). Primary antibody was used at 1 µg/ml. Secondary antibody was an anti-rabbit IgG conjugated to alkaline phosphatase (KPL, Gaithersburg, MD, USA) diluted 1:1000 in PBS. The positive reaction was detected using a chemoluminescent substrate CDP-Star (Roche), following manufacturer's instructions.

For immunocytochemistry, samples of the frozen tissues were sectioned using a cryostat. The sections (5–7 µm) were fixed for 10 min in an ice-cold mixture of acetone and methanol (1:1) and then air dried and stored frozen until use. The non-specific binding sites were blocked by incubation for 1 h (RT) in 3% BSA in PBS. The primary antibody (PG-21) was used at a concentration of 2 µg/ml. An anti-rabbit IgG conjugated to FITC diluted 1:200 in PBS was used as the secondary antibody. The positive reaction was visualized in a Nikon fluorescence microscope. Images were captured using a microscope equipped with a CCD system (200A, Polytronics, Emsworth, UK).

Detection of lipofuscin

Lipofuscin was detected by the Popper's method (Kasten 1981). Briefly, samples from the tissues were snap frozen in liquid nitrogen and were used to obtain 10 µm cryosections that were directly scored under epifluorescence using a 580 nm filter to detect autofluorescent granules.

Results

Testosterone measurements

The testosterone values are indicated in Fig. 1. As can be observed, the old mice have a significantly lower concentration of testosterone in their blood than the young ones or those injected with testosterone.

Detection of DNA fragmentation in situ

We observed apoptotic cells in the epididymides and ventral prostates of the old mice (Fig. 2a–d). The use of the TUNEL technique allowed us to assess whether these cells were indeed undergoing apoptosis (Fig. 2e–h). There were no significant numbers of apoptotic cells either in the dorsal and cranial lobes of the prostate or in the seminal vesicles. On the other hand, apoptosis was abolished in the epididymis and ventral prostate by testosterone treatment. These results were confirmed by caspase-3 detection. The active fragment of the enzyme was detected in epithelial cells from both the epididymis and ventral prostate (Fig. 3).

The apoptotic indices are indicated in Fig. 4 (a and b). The highest values were found in the caput epididymidis. Those found in the corpus and cauda were lower and similar to those found in the ventral prostate. Testosterone supplementation resulted in the indices falling to control values.

DNA fragmentation in agarose gels

Results are shown in Fig. 5. Both the epididymis and ventral prostate reveal an obvious DNA ladder indicative of DNA fragmentation. This was reversed by testosterone treatment. There is also a ladder in tissue from young

Figure 3 Detection of the active fragment of caspase-3 in the different tissues in aged animals (group 2). The positive reaction is visualized in green. Nuclei are counterstained with Hoechst 33342 and are observed in blue. (a) caput epididymidis, (b) cauda epididymidis, (c, d) ventral prostate. The bars represent 10 µm.

Figure 4 (a) Number of apoptotic cells in the different regions of the mouse epididymis of the different groups. Group 1 (open bars): young (3 months old). Group 2 (gray bars): aged (2 years old). Group 3 (black bars): aged plus testosterone. Values are expressed as means $\pm$ S.E.M. Different letters indicate statistically significant differences ($P < 0.05$). (b) Number of apoptotic cells in the different male accessory sex glands of the different groups. For the three groups, $n = 3$. Values are expressed as means $\pm$ S.E.M. Different letters indicate statistically significant differences ($P < 0.05$). VP, ventral lobe of the prostate; CG, coagulating glands; DP, dorsolateral lobe of the prostate; SV, seminal vesicles.

animals, but it is faint, representing the background levels of normal apoptosis on every organ. In both dorsal prostate and seminal vesicles, old animals show comparable levels of apoptosis to the young ones (Fig. 5c and d).

Androgen receptor detection

The androgen receptor was localized in all tissues analysed. The levels detected by Western blots were not very different among groups, although a lower amount occurred in the epididymides and ventral prostates of the senile mice. No differences were detected by immunocytochemistry among young and old animals. All the organs showed androgen receptors in a nuclear localization (data not shown).

Detection of lipofuscin granules

We detected lipofuscin granules in all organs, but they were more evident in those organs that presented apoptosis (that is, epididymis and ventral prostate), as can be

Figure 5 Analysis of DNA fragmentation in different tissues studied on agarose gels. Total extranuclear DNA obtained as described in the text from epididymis (a), ventral prostate (b), seminal vesicles (c) and dorso-lateral prostate (d) was resolved on a 1.5% agarose gel and exposed to X-OMAT AR Kodak film to visualize the DNA ladder. Numbers at the bottom of each lane indicate the group. Numbers on the right and left represent the molecular size in kb of the markers (Roche No XIV).

seen in Fig. 6. The content of lipofuscin granules was decreased in all cases by testosterone treatment.

Discussion

It is well known that ageing affects reproduction in the male. Old age brings about degenerative changes in the testes. Apoptosis has been shown to play a role in these changes (Wang *et al.* 1999, Kimura *et al.* 2003). Here we showed that not only the gonads but also the epididymis and the ventral prostate do undergo an increase in apoptotic rates. The present results after using TUNEL and the ladder procedure, the existence of apoptotic bodies, the isolated distribution of positive cells and the absence of inflammatory reaction, clearly indicates that positive cells are apoptotic. The changes in the epididymis are not surprising; since the epididymis depends on androgens and testicular factors, it may well be reflecting the changes in the testes. Moreover, there was also a regional response, and apoptotic indices were higher in the caput

Figure 6 Detection of lipofuscin granules in the caput epididymidis (A1–A3), the cauda epididymidis (B1–B3), the ventral prostate (C1–C3), and the seminal vesicles (D1–D3). Numbers indicate the different groups: 1) young, 2) aged, 3) aged plus testosterone. Each case is observed under phase contrast (at the left) and fluorescence (at the right). Lipofuscin granules are prominent in the epididymis and ventral prostate of old animals. Bars, 10 μ m.

epididymis that in the corpus or cauda. This regional response is also evident in the epididymis of castrated rats (Adams 1984). This was explained as a reflection of the differences in androgen concentration in the luminal fluids along the epididymis (Fan & Robaire 1998). It may be the same situation in the old mice, since the old testes in the rat secrete much less testosterone (Turner *et al.* 1984), and actually our RIA analyses in the mice showed a considerable decrease in androgen content. Of all the accessory glands examined, only the ventral prostate showed signs of apoptosis. Interestingly, this is the only prostatic lobe that shows castration-induced cell death (Chen *et al.* 1994). However, castration induces apoptosis in the seminal vesicles (Tsuji *et al.* 1998), and we did not see any apoptosis in the tissues from old animals. We must consider, however, that, although the testosterone levels fall, they are still measurable, and when the androgen receptor levels were examined, there were significant amounts of them with normal nuclear localization. Thus, it is likely that only the most sensitive organ, the ventral prostate, can react to the changes. That the androgen withdrawal was ultimately responsible for the induction of apoptosis is supported by the fact that androgen supplementation abolished the age-induced apoptosis in all epididymal segments and the ventral prostate.

The lipofuscin detection deserves some special comment. Lipofuscin is also called age pigment (Brunk *et al.* 1992). Oxidative stress, as well as diminished activity of lysosomal proteolytic enzymes, is known to induce lipofuscin accumulation in a variety of cell types (Banerjee

et al. 1995). Lipofuscin cannot be degraded and accumulates with age, being at least partially responsible for the senescent phenotype in the cell (Terman & Brunk 1998). Interestingly, we saw larger lipofuscin deposits in the epididymis and the ventral prostate than in the other prostatic lobes and other accessory sex glands. Moreover, the lipofuscin contents decreased after testosterone treatment. It is possible that this decrease is due to a dilution effect caused by an increase in cell size or the presence of new cells generated by mitosis which occurs after testosterone treatment. Thus, lipofuscin contents match the apoptotic indices in the tissue. This could indicate that both phenomena are closely related. In fact, lipofuscin granules have been seen in the diaphragm muscles of the X chromosome-linked muscular dystrophic (mdx) mice in cells undergoing apoptosis (Sitte *et al.* 2000), a finding which supports this hypothesis. Moreover, in the dog prostate, apoptotic cells that appear after castration are loaded with lipofuscin granules (Nakae *et al.* 2001). Oxidative stress could be the link to both processes. Testosterone has been shown to be a protective agent against cellular damage induced by oxidative stress in cerebellar neurons (Ahlbom *et al.* 2001, Niu *et al.* 2001). On the other hand, oxidative stress influences the accumulation of lipofuscin granules (Ahlbom *et al.* 1999) and can induce apoptosis in a variety of cell types (Brunk & Terman 2002, Curtin *et al.* 2002), so it is possible that the fall in testosterone can induce a joint increase in oxidative stress and apoptosis.

One of the most surprising facts that deserves some comment is how testosterone supplementation was able to suppress the deleterious effects of old age in the male tract, at least regarding apoptosis. Testosterone is a well-known proliferative agent in the prostate both *in vitro* (Kagan *et al.* 2002) and *in vivo* (Silva *et al.* 2001). This suggests that testosterone treatment could be effective in restoring some of the reproductive organs. In fact, supplementation with low doses of testosterone has some beneficial effects on the male rat, such as preventing the loss of body and bone mass (Waltregny *et al.* 2001). Moreover, androgen supplementation in the old rat is partially able to restore sperm numbers in the epididymis (Vanderschueren *et al.* 2000), suggesting that the male tract in the old animal is able to respond to exogenous stimulation. In old men, the benefits of testosterone-replacement therapy are still under evaluation (Gooren 2003, Morley & Perry 2003). Anticipated risks lie principally with the prostate and the cardiovascular system. The risks with regard to prostate disease are often over-rated; nevertheless, there is an impending need of more data (Tenover 1999, Gooren 2003).

In summary, old age induces apoptosis in the epididymis and ventral prostate, which seems to be caused by the fall of testosterone levels and is probably related to an increase in the oxidative stress in the tissues. More detailed analyses are needed to elucidate the precise molecular mechanisms involved in this age-induced apoptosis.

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