

Sexual Autophagic Differences in the Androgen-Dependent Flank Organ of Syrian Hamsters

ANA COTO-MONTES,* CRISTINA TOMÁS-ZAPICO,† JORGE MARTÍNEZ-FRAGA,* IGNACIO VEGANAREDO,* VERÓNICA SIERRA,*‡ BEATRIZ CABALLERO,* COVADONGA HUIDOBRO-FERNÁNDEZ,* CLARA SORIA-VALLES,* DELIO TOLIVIA,* AND MARIA JOSEFA RODRÍGUEZ-COLUNGA*

From the *Departamento de Morfología y Biología Celular, Facultad de Medicina, Universidad de Oviedo, Oviedo, Spain; the †Centro de Biología Molecular “Severo Ochoa” CSIC/UAM Campus de la Universidad Autónoma de Madrid, Cantoblanco, Madrid, Spain; and the ‡Servicio Regional de Investigación y Desarrollo Agroalimentario (SERIDA), Villaviciosa, Spain.

ABSTRACT: The flank organ of the Syrian hamster shows a biodynamic response to androgenic stimulation and is, therefore, a suitable model for the study of androgenic effects on hair and sebaceous glands. This organ is susceptible to programmed cell death (PCD), a prominent feature associated with sexual organ adjustment. In the present report, the type of PCD (apoptosis or autophagy) exhibited by this organ was evaluated. Caspase-3 activity, indicative of apoptosis, was not detectable in flank organ homogenates. Furthermore, cytokeratins, which are normally degraded during apoptosis, remained intact. On the other hand, Western blotting of Beclin 1 and light chain 3-II, both important

autophagy markers, revealed autophagic processes in the flank organ in both sexes, especially in females. Cathepsin D activity, higher in males than in females, and procathepsin D expression were also consistent with autophagy and not apoptosis. Taken together, these data indicate that macroautophagy, and not apoptosis, is the main mechanism by which the flank organ responds to androgen. This is the first direct evidence establishing the relationship between autophagy and morphological changes in androgen-dependent organs.

Key words: Beclin 1, cathepsin D, LC3.
J Androl 2009;30:113–121

The hamster flank organs, also known as the costovertebral spots or costovertebral organs, are 2 sebaceous glands located on each flank of the animal. In an adult male hamster, these organs have a diameter of approximately 8 mm and are heavily pigmented and covered with coarse, dark hairs. In female hamsters, they are undeveloped, with a diameter of approximately 2 mm. They are slightly pigmented, have no dark hairs, and are sometimes difficult to see (Voigt and Hsia,

1973). The flank organ has been widely used as a model of human sebaceous activity because androgens promote the growth of the sebaceous glands and hair follicles of this organ (Hamilton and Montagna, 1950; Mezick et al, 1999). Early studies demonstrated that the active androgens, testosterone and dihydrotestosterone, stimulate the sebaceous gland and that estrogens exert some inhibitory action (Ebling, 1948, 1963). Because there is growing evidence that the flank organ changes its histological features in response to androgens and their seasonal variations, it is likely that this organ has mechanisms to achieve these changes.

Programmed cell death (PCD) gives rise to 2 histological patterns as revealed by electron microscopy. Apoptosis, or type I PCD, is characterized by condensation of the cytoplasm and preservation of organelles, essentially with no autophagic degradation. In contrast, in autophagic cell death, or type II PCD, there is extensive autophagic degradation of the Golgi apparatus, polyribosomes, and endoplasmic reticulum preceding nuclear destruction (Bursch et al, 2000; Gozuacik and Kimchi, 2004). The mechanisms and molecules involved differ in the 2 types of cell death. The caspases, a family of cysteine proteases, play an essential role in inducing and carrying out apoptosis. When activated, caspases acquire the ability to cleave key intracellular

A.C.-M. is a contractual professor from the I3 Program application awarded by Gobierno del Principado de Asturias, Spain; C.T.-Z. is a postdoctoral fellow from Secretaría de Estado de Universidades e Investigación (Ministerio de Educación y Ciencia); I.V.-N. is an FPU predoctoral fellow from Secretaría de Estado de Universidades e Investigación (Ministerio de Educación y Ciencia), Spain; V.S. is a predoctoral fellow from INIA, Spain; and B.C. is an FICYT predoctoral fellow from Gobierno del Principado de Asturias, Spain.

Supported in part by grants FISS-06-RD06/0013/0011 from the Instituto de Salud Carlos III (Ministerio de Sanidad y Consumo) and FEDER fund (European Union).

Correspondence to: Dr Ana Coto-Montes, Departamento de Morfología y Biología Celular, Facultad de Medicina, C/ Julián Clavería s/n, 33006 Oviedo, Spain (e-mail: acoto@uniovi.es).

Received for publication March 13, 2008; accepted for publication September 23, 2008.

DOI: 10.2164/jandrol.108.005355

substrates, giving rise to the biochemical and morphological changes associated with apoptosis (Roberg et al, 2002).

In the search for biochemical differences between the 2 PCD processes, recent investigations have suggested that the cytoskeleton has distinct fates during autophagic and apoptotic cell death. The preparatory process that apoptotic cells undergo includes depolymerization or cleavage of actin, cytokeratins, lamins, and other cytoskeletal proteins (Hengartner, 2000; Bursch, 2001). In contrast, in cells undergoing autophagic death, the cytoskeleton, although redistributed, is largely preserved (Bursch et al, 2000; Bursch, 2001). Furthermore, Kabeya et al (2000) have suggested that the microtubule-associated protein light chain 3 (LC3) is an ideal marker for the autophagic process. LC3 is converted into LC3-I, and part of this modified protein is subsequently converted into LC3-II, which is localized in autophagosomal membranes. The conversion of LC3-I to LC3-II is induced under starvation conditions, which are well known to induce autophagy.

Beclin 1 is a 60-kDa protein that has been implicated as an important regulator of macroautophagy. It was originally discovered during the course of a yeast 2-hybrid screen of a mouse brain cDNA library using human Bcl-2 as the bait (Liang et al, 1998). Coimmunoprecipitation studies suggest that the major physiological partner for Beclin 1 is the mammalian class III phosphatidylinositol 3-kinase Vps34 (Zeng et al, 2006). Expression of Beclin 1 in MCF7 mammary carcinoma cells increases their autophagic response to nutrient deprivation (Liang et al, 1999). Consistent with this observation, several studies have implicated *Beclin* as an essential gene for cell survival under adverse nutritional conditions. In addition to its specific role in adaptation to nutrient deprivation, accumulating evidence suggests that Beclin may play a more general role in cell survival during embryonic development.

Isahara and collaborators (1999) have described a pathway for cell death in which cathepsin D has a relevant role. These authors postulate that cathepsin D acts as a death factor in this pathway. This enzyme translocates from lysosomal compartments to the cytosol, and seems to be related to PCD (Ishisaka et al, 2001). Cathepsin D, the main lysosomal aspartic protease, is synthesized and translocated into the endoplasmic reticulum as a 52-kDa inactive proenzyme (procathepsin D). It is converted in the endosomal compartment into a slightly active 48-kDa single-chain form (procathepsin D; Demoz et al, 2006). In humans and most other species, this single-chain form undergoes further proteolytic processing in late endosomes and lysosomes, yielding the mature form. Mature cathepsin D is composed of a light (14 kDa) chain and a heavy

(32 kDa) chain held together by disulfide bonds (Diment et al, 1989). However, extensive vacuolization resulting from autophagy interferes with trafficking of this enzyme through the endosome, increasing procathepsin D levels (Zeng et al, 2006). Therefore, indirect measures of autophagy may show a higher ratio of procathepsin D (52 and/or 46 kDa) to mature cathepsin D (33 kDa).

An evaluation of selected biomarkers of apoptosis (eg, caspase-3 activity) and autophagy (eg, Beclin 1 and LC3-II expressions), as well as ultrastructural studies, were performed in the present study to demonstrate the hypothesized primary role of autophagy in androgen-dependent PCD, as observed in the flank organs of male and female Syrian hamsters. The data collected in this study are important because they allow us to show that autophagy can act as an androgen-dependent mechanism, and this role is supported by the fact that autophagy acts differently in female vs male flank organs. This information can be used for improving knowledge about hair growth mechanisms, because it is known that the flank organ responds to androgen and antiandrogen treatments (Chen et al, 1996).

Materials and Methods

Animals

Thirty 2-month-old Syrian hamsters (*Mesocricetus auratus*; 15 male, 15 female) were obtained from Harlan Interfauna Ibérica (Barcelona, Spain). Before harvest of the flank organs, the hamsters were housed for 2 months (5 per cage) at a temperature of $22 \pm 2^\circ\text{C}$ and subjected to a 14:10-hour light:dark cycle. Food and water were provided ad libitum.

The Oviedo University Local Animal Care and Use Committee approved the experimental protocol. All experiments were carried out according to the Spanish Government Guide and the European Community Guide for Animal Care.

The animals were anesthetized and decapitated and their flank organs rapidly procured. For the biochemical studies, the flank organs were homogenized in a Polytron homogenizer at 4°C in 1 mL of lysis buffer (20 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, and 5 mM dithiothreitol) supplemented with protease inhibitors (1 mM Na_3VO_4 , 1 mM phenylmethylsulfonyl fluoride, and 1 $\mu\text{g}/\text{mL}$ aprotinin). Tissue homogenates were then centrifuged at $3000 \times g$ for 6 minutes at 4°C . Supernatants were collected and centrifuged again under the same conditions. The protein contents of the supernatants were determined by the Bradford (1976) method.

Caspase-3 Activity

Caspase-3 activity was determined using a colorimetric assay according to the manufacturer's instructions (CASP-3-C; Sigma, St Quentin-Fallavier, France). In this assay system,

the colorimetric substrate (acetyl-Asp-Glu-Val-Asp-p-nitroanilide) is hydrolyzed by caspase-3, and p-nitroaniline (pNA) is released from the substrate. To account for nonspecific hydrolysis of the substrate, the control reaction mixture contained tissue homogenates, substrate, and the specific caspase-3 inhibitor Ac-DEVD-CHO in assay buffer. Both mixtures were monitored for 5 hours at 37°C by a plate reader (ELx800 UV; Bio-Tek Instruments, Winooski, Vermont). Data obtained using the caspase-3 inhibitor were subtracted from data obtained without the caspase-3 inhibitor to correct for nonspecific hydrolysis. Blanks were incorporated and caspase-3 protein was used as a positive control. Data were normalized by protein content of each sample. The caspase-3 activity was expressed as nmol of pNA released per minute per milligram of protein (Tomas-Zapico et al, 2005).

Cathepsin D Activity

The aspartate-protease cathepsin D (EC 3.4.23.5) was assayed spectrophotometrically (Uvikon 930; Kontron Instruments, Madrid, Spain) at 280 nm according to the method described by Takahashi and Tang (1981) with minor modifications (Schreurs et al, 1995), using hemoglobin as the substrate. Two hundred microliters of tissue homogenate (see above) was pipetted with 500 μ L of substrate solution (3% hemoglobin in 200 mM acetic acid) in microcentrifuge tubes, followed by incubation for 30 minutes at 37°C. The reaction was stopped by adding 500 μ L of 15% trichloroacetic acid, and the tubes were kept at 4°C for 30 minutes and then centrifuged at 12 000 \times g for 5 minutes. The optical densities of the supernatants were used to calculate cathepsin levels, expressed as enzyme units per milligram of protein.

Detection of Autophagic Markers by Western Blotting

Tissue samples were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% skimmed milk in phosphate-buffered saline containing 0.05% Tween-20 solution and incubated with primary antibodies against anti-mouse cytokeratins (Sigma), anti-goat LC3 (sc-16756, Santa Cruz, Santa Cruz, California), anti-goat Cathepsin D (sc-6486, Santa Cruz), anti-goat Beclin 1 (sc-10086, Santa Cruz), and β -actin (sc-1615, Santa Cruz), and with the corresponding horseradish peroxidase-conjugated secondary antibodies (Sigma and Santa Cruz, for cytokeratin, LC-3, Beclin1, and β -actin). The membranes were developed using Western Blotting Luminol Reagent (Santa Cruz).

Digital images were analyzed with a Chemidoc XRS system (Biorad, Hercules, California), which permits quantification of band intensity. Routinely, protein load was monitored by the Bradford method and phenol red staining of the blot membrane.

Ultrastructural Studies

Flank organs for morphological studies were lightly fixed by immersion in a solution containing 1.5% glutaraldehyde and 2.5% paraformaldehyde in phosphate buffer 0.1 M (pH 7.4).

Fixation was prolonged overnight using fresh fixative at 4°C. The tissue was then postfixed in 1% OsO₄ for 2 hours. After dehydration in a graded acetone series, the tissue fragments were embedded in Taab 812 (TAAB Laboratories Equipment Ltd, Aldermaston, United Kingdom) and semithin sections (1 μ m) were stained with toluidine blue. Ultrathin sections were collected on cooper grids, stained with uranyl acetate-lead citrate, and examined using a Zeiss EM-109 transmission electron microscope (Zeiss, Oberkochen, Germany) operating at 80 kV. Negatives were scanned by HP Scanjet 3970 (Palo Alto, California) and imported by Adobe Photoshop 7.0.1 (San Jose, California), and the images were incorporated into the figures using Corel Draw X3 (Ottawa, Canada).

Statistical Analysis

Data are presented as $\bar{x} \pm$ SD calculated for at least 3 separate experiments, each performed in triplicate. The normality of the data was established by the Kolmogorov-Smirnov test. Statistical comparisons between sexes were performed using Student's *t* test for data showing a normal distribution and the nonparametric Mann-Whitney test for nonnormally distributed data. The level of significance was set at $P < .05$.

Results

Study of Apoptosis

Caspase-3 Activity—Caspase-3 activity was determined colorimetrically during a 48-hour period in every sample from flank organs of both sexes. No caspase-3 activity was detected (negative data not shown), despite the use of several tissue concentrations (80–600 μ g/mL). Levels of excess pure caspase-3 (up to 5 μ g/mL), used as a positive control, were determined in parallel and found to attain values of 654 nmol pNA released per minute per milligram of protein. Therefore, the inactivity of caspase-3 in all assayed tissue concentrations was confirmed. However, the existence of other apoptotic processes not mediated by caspases is possible.

Ultrastructural Studies—Electron microscopy revealed that the studied cells of the flank organ in both sexes of Syrian hamsters do not undergo apoptosis because we were not able to observe, in any case, apoptotic nuclei in cells from flank organs of both sexes. This is a qualitative study, so the presence of undetected apoptotic nuclei could be possible.

Study of Autophagy

Cathepsin D Activity—Cathepsin D activity was assayed using its corresponding substrate in flank organs procured from male and female Syrian hamsters. The results showed statistical differences ($P \leq .05$) between male and female flank organs in relation to cathepsin D activity, because this protease activity was always 4 times higher in males than in females (Figure 1).

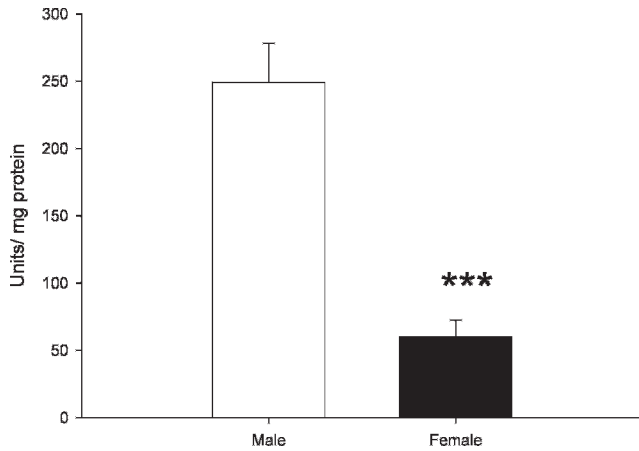


Figure 1. Cathepsin D activity determined using hemoglobin as substrate; □, male flank organ; ■, female flank organ. Values are $\bar{x} \pm SD$. *** $P \leq .005$.

Detection of Autophagic Markers by Western Blotting—Western blot analysis of flank organ extracts was performed using antibodies against cytokeratins, LC3, cathepsin D, Beclin 1, and β -actin.

The cytokeratin pattern was analyzed in untreated male and female flank organs. The study was developed using a wide range of anticytokeratin antibodies that recognize various cytokeratins, including the type II neutral-to-basic cytokeratin subfamily and the type I acidic subfamily. Male and female Syrian hamsters displayed a similar cytokeratin pattern (Figure 2A). The main cytokeratin detected was cytokeratin 8 (CK8). Additional bands were detected below the expected molecular weight for cytokeratins (33 and 22 kDa). In all cases, female hamsters expressed higher amounts of these proteins than males. Quantification of Western blot bands supported these data. Immunoblot analysis for β -actin demonstrated that there was no degradation of this protein, which is characteristic of autophagy (Figure 2B).

Microtubule-associated protein LC3 is an autophagosomal ortholog of yeast Atg8. LC3 modification is essential for the autophagic process, because the protein LC3-II is localized in preautophagosomes and autophagosomes, and it is considered an autophagosomal marker. Western blots showed 2 bands corresponding to LC3-I and LC3-II (18 and 16 kDa, respectively) in female flank organs, whereas in male organs, only 1 band of 18 kDa corresponding to LC3-I was observed (Figure 3).

Cathepsin D exhibited significant differences between the sexes with regard to the protein expression pattern (Figure 3). Both sexes showed 2 bands (52 and 46 kDa) corresponding to cathepsin D precursors, which are known as procathepsin D. Whereas the 52-kDa

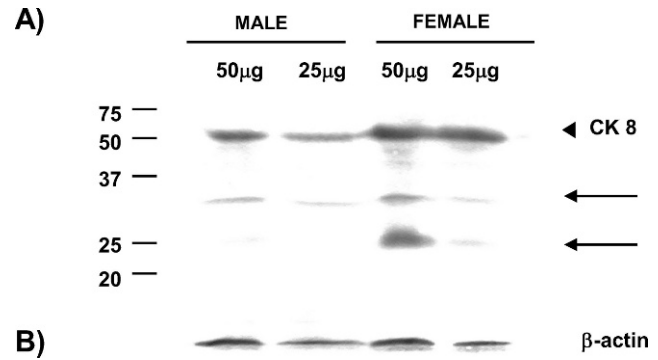


Figure 2. (A) Immunoblot analysis of cytokeratins in male and female flank organs. Total extracts of flank organ were subjected to Western blotting with an anti-Pan cytokeratin antibody. Male and female flank organs display the same cytokeratin pattern but show differences in protein expression. (B) β -actin immunoblot showed no protein degradation. These blots are representative of at least 3 different experiments.

procathepsin D showed high expression in females, the 46-kDa procathepsin D band was clearly more intense in males than in females. The expression of mature cathepsin D (33 kDa) is present in males but only slightly visible in females. Quantification of Western blot bands supports these data. As procathepsin D is processed, its proteolytic activity is increased, and therefore the data from Western blots are consistent with previous results from the cathepsin D activity study.

We also carried out an immunoblot analysis for Beclin 1. Both male and female flank organs showed a band of 60 kDa corresponding to Beclin 1. This band was clear in females but only slightly visible in males (Figure 3). Quantification of Western blot bands supports these data.

Ultrastructural Studies—Through electron microscopy, it was revealed that the cells of the flank organ in both sexes of Syrian hamsters undergo autophagy. Flank organ cells in males had a cytoplasm filled with ribosomes and rough endoplasmic reticulum surrounding abundant mitochondria with clear cristae. Along the cytoplasm, there were prominent Golgi complexes with several autophagosomes that contained cytoplasmic components with indistinguishable structures. In the same cell, individual membrane-bound autolysosomes could be observed (Figure 4). Hence, some cells showed diminished electrodensity as a result of the degradation of cytoplasmic components, which is a hallmark of autophagic cell death (Figure 5). Moreover, when both the nuclear envelope and the plasma membrane lost their integrity, the cells exhibited intense vacuolization of the cytoplasm (Figure 6).

The flank organ cells of female hamsters containing autophagic vacuoles were surrounded by whorls of

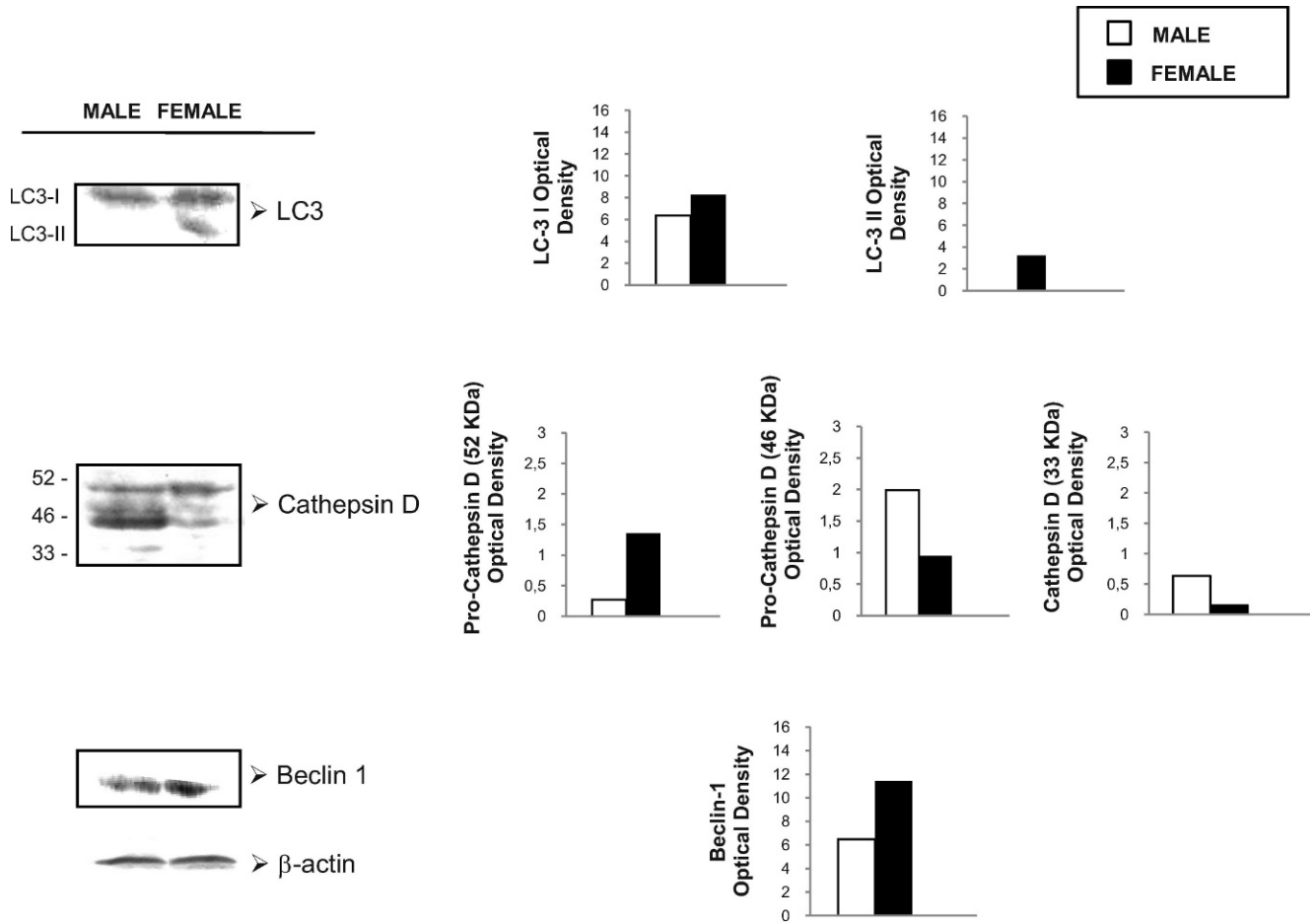


Figure 3. Immunoblot analysis of LC3, cathepsin D, Beclin 1, and β -actin in male and female flank organs. Female flank organs showed LC3-I and -II bands whereas male ones only showed the LC3-I band. Both sexes showed procathepsin D bands (52 and 46 kDa) although only the male flank organ showed a cathepsin D band (33 kDa). Both sexes showed a clear band of 60 kDa corresponding to Beclin 1; however, protein expression was very different. The Beclin 1 band was very strong in females, but in males it was tenuous. Bar chart shows optical densities of blot bands normalized in front of actin; \square , male flank organ; \blacksquare , female flank organ.

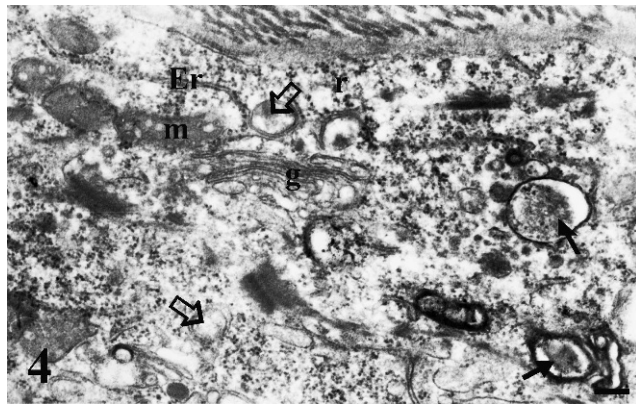


Figure 4. Ultrastructural features of autophagy in cells from the male flank organ. Portion of cytoplasm with abundant mitochondria (m), rough endoplasmic reticulum (Er), Golgi complexes (g), and ribosomes (r). Many autophagosomes (open arrows) and autolysosomes (arrows) could sometimes be seen. Bar = 0.3 μ m.

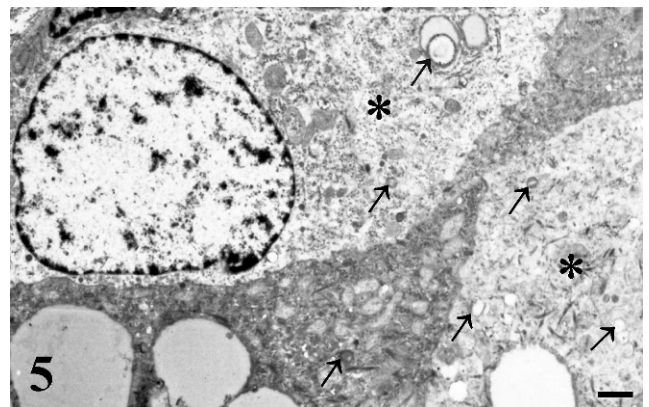


Figure 5. Electron-clear cells of the male flank organ (asterisks) and nucleus of an electron-clear cell showing a normal profile. Autophagic bodies (arrows) were distributed throughout the cytoplasm in both electron-clear and electron-dense cells. Bar = 2 μ m.

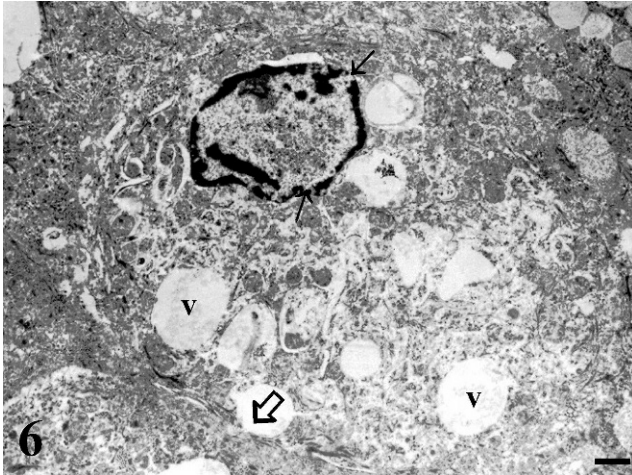


Figure 6. Ultrastructural appearance of disaggregated cells in the male flank organ. Note the vacuolized cytoplasm (v), disrupted nuclear envelope (arrows) and plasma membrane (open arrow). Bar = 2 μ m.

membranous material (Figure 7). These membranes seem to be made of smooth endoplasmic reticulum (Figure 8). Likewise, female flank cells showed numerous vesicles that abutted the nuclear membrane and appeared to produce nuclear indentations (Figure 9).

Discussion

The hamster flank organ has been widely used as a model of control of the peripheral androgenic actions

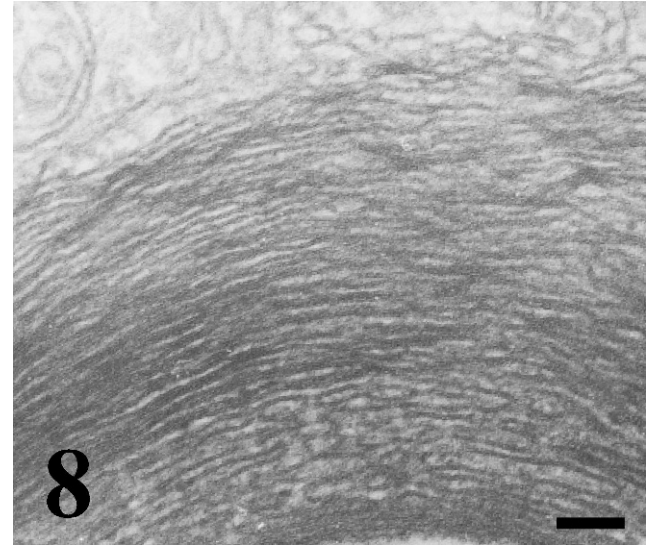


Figure 8. Detail of membranous material surrounding autophagic vesicles. Bar = 0.18 μ m.

(Baillie et al, 1966; Brooks et al, 1991). Several studies have described its biodynamic response to androgens and antiandrogens (Pochi and Strauss, 1969; Chen et al, 1996), which produces rapid increases and decreases in the labeling intensity of these sebaceous glands. Excessive androgenic activity gives rise to several common cosmetically significant diseases, such as acne, seborrhea, hirsutism, and alopecia. Thus, our finding on the susceptibility of the flank organ cells to autophagy could

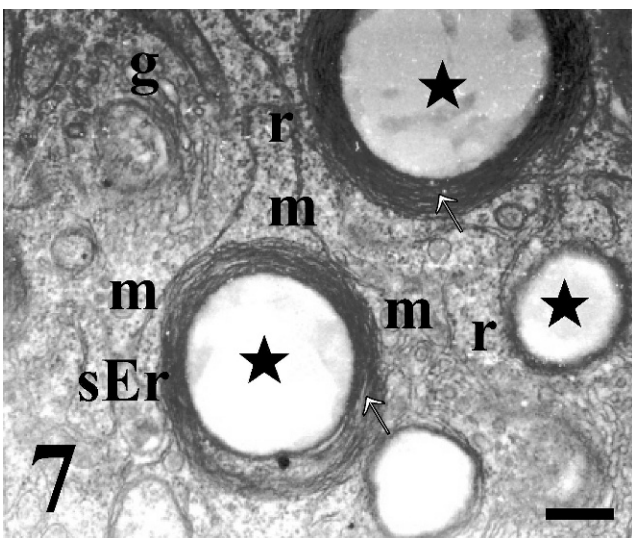


Figure 7. Ultrastructural appearance of a portion of cytoplasm with abundant mitochondria (m), Golgi saccules (g), free ribosomes (r), and smooth endoplasmic reticulum (sEr). Autophagic vesicles (stars) appear as whorls of membranous material (white arrows). Bar = 0.35 μ m.

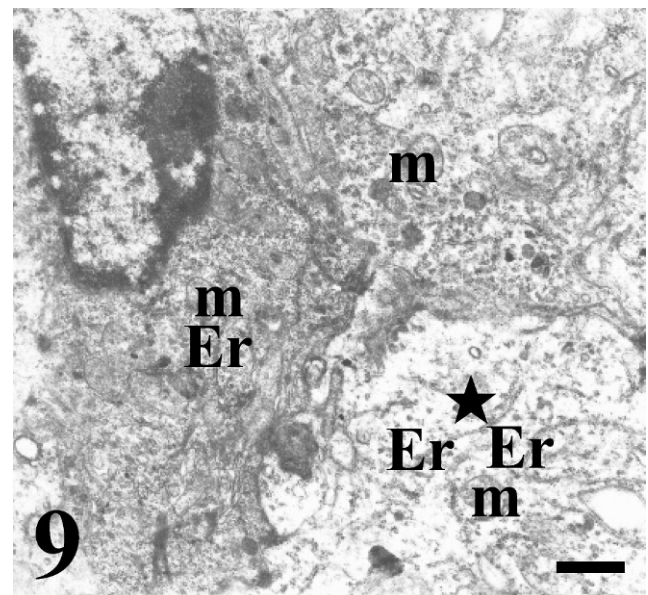


Figure 9. Electron-clear cell of female flank organ (star), close to an electron-dense cell, shows disaggregated cytoplasm with recognizable endoplasmic reticulum (Er) and some mitochondria (m). Bar = 0.6 μ m.

be useful for understanding the molecular mechanisms of hair growth control, which is presently poorly understood.

Caspases, a family of cysteine proteases, have been described to act as initiators of apoptosis, playing a central role in regulating this mechanism of PCD (Kitanaka and Kuchino, 1999). However, we observed that caspases were inactive in tissue from both sexes in the absence of external inhibition of caspase activities. Moreover, recent data suggest that different pathways of caspase-independent PCD can be activated, resulting mainly in a nonapoptotic form of cell death (Kitanaka and Kuchino, 1999; Bursch et al, 2000). The data shown in this article support the possibility that, in flank organs, a different type of PCD, such as autophagy, occurs. Our data do not exclude the possibility that autophagy and other kinds of apoptotic processes not mediated by caspases coexist (Joza et al, 2001).

Autophagy is a pivotal physiological process for survival during starvation, differentiation, and normal growth control, and may play a number of roles in other cellular functions via the turnover of cellular macromolecules and organelles (Kabeya et al, 2000). Autophagic vacuoles (autophagosomes) are initially formed from membranes of the endoplasmic reticulum that surround a region of cytoplasm. These structures, bounded by a double membrane, then develop into mature degradative vacuoles (autolysosomes) by progressive fusion with late endosomes and lysosomes. Accumulation of autophagosomes and autolysosomes is a morphological feature that is a marker of type II PCD, also referred to as autophagic cell death (Zeng et al, 2006).

LC3-II and Beclin 1 are classically described as autophagy markers (Zhu et al, 2007). Based on the results of this study, male flank organs show much lower levels of autophagy than do female organs, because they show reduced expression of Beclin 1 and an absence of LC3-II. However, using electron microscopy, we observed signs of extensive autophagy, including abundant autophagosomes derived from the rough endoplasmic reticulum. We saw normal nuclei in flank organ cells of both sexes. Because other parameters have pointed out clear sexual differences in autophagic processes of the flank organ, with higher development in females vs males, the combination of both biochemical and morphological data leads us to think that autophagy is more widespread in female flank organs than in male ones. This is in terms of affected area, and not in relation to the autophagic flux, which shows the same morphological characteristics in the flank organs of both sexes.

The autophagy process depends, to a large extent, on cytoskeletal proteins. The sequestration of parts of

cytoplasm requires intermediate filaments (cytokeratin and vimentin), and the movement and fusion of lysosomes with the late autophagosomes requires the microtubular system. So, autophagic death needs a preserved cytoskeleton, although the cytoskeleton could be redistributed (Bursch et al, 2000; Bursch, 2001). Thus, we have also studied the cytokeratin pattern in the flank organ, as described by Bursch et al (2000), who indicated that the fate of the cytoskeleton is different between apoptosis and autophagy.

Cytoskeletal proteins appear to be preserved, at least in part, during autophagic-driven cell death. This pattern of change is clearly different from that ascribed to apoptosis (Bursch, 2001). However, all experiments focusing on preservation of the cytoskeleton in autophagy have been performed *in vitro* using specific PCD inducers, such as tamoxifen (Bursch et al, 2000). This type of study is difficult to perform in *in vivo* conditions because of a lack of controls. According to previous results, we obtained, in our model lacking of any form of induction, the same cytokeratin pattern in both sexes, although it was more intense in female organs than in male ones. Our Western blots indicate that most cytokeratins remained undegraded, such as the simple epithelial CK8, the most expressed cytokeratin. Moreover, the observed 23-kDa band was present only in females, supporting the hypothesis that autophagy takes up a bigger area of flank organs in females than in males.

Because our experiments were conducted using *in vivo* conditions, we cannot compare our results to a baseline control (at time 0) as with cell cultures. Nevertheless, the cytokeratins determined here confirm previous findings wherein CK8 appeared in autophagy. This cytokeratin has been observed in MCF-7 cells incubated with tamoxifen (Bursch et al, 2000) and more recently in the Syrian hamster Harderian gland (Tomas-Zapico et al, 2005). Our Western blots of total actin revealed no signs of proteolytic cleavage, which is also consistent with the data reported by Bursch et al (2000).

Cathepsin D is a lysosomal aspartic proteinase, initially synthesized as an inactive precursor, procathepsin D, which upon arrival at the lysosome is proteolytically cleaved to yield the mature active enzyme (Neurath, 1991, 1994). Beclin 1, as part of 2 distinct protein complexes that contain the PI 3-kinase Vps34 (Kihara et al, 2001), causes vacuolation of late endosomal compartments, impeding the traffic of procathepsin D from late endosomes to lysosomes (Zeng et al, 2006) and, therefore, impedes lysosome development. The main proteolytic activation event is presumed to depend on the action of lysosomal cysteine proteinases. In agreement with preceding hypotheses and previous biochemical data, our results show that

female cathepsin D activity is low in comparison to male cathepsin activity, that cathepsin D is present in males, and that there is higher expression of procathepsin D in both sexes. Therefore, the presence of either cathepsin D or procathepsin D could be used as indirect evidence of autophagy, and, once more, autophagic processes in flank organs are stronger in females than in males.

The flank organ contains highly developed clusters of sebaceous glands immersed in both sides of the back skin. The skin possesses all the enzymes required for transformation of the steroid precursors of adrenal origin into active androgens and estrogens, and these enzymes are expressed at a particularly high level in sebaceous glands as in the flank organ (Sansone-Bazzano and Reisner, 1974). Moreover, since 1972, it has been known that the flank organ contains a considerable amount of dihydrotestosterone compared with the prostate and seminal vesicle (Takayasu and Adachi, 1972). Likewise, the hamster flank organ is a scent gland that produces a high amount of pheromones that allow animals to distinguish males from females, and to identify the reproductive status of both sexes and whether an animal will be aggressive or submissive (Johnston and Rasmussen, 1984). The flank organ can also be considered a steroid-secreting organ because of its combination of sexual steroid transformation and steroidal pheromone production. This information is very important in relation to autophagy because this kind of PCD takes place in normal steroid-secreting cells at higher frequencies than in many other cells, probably as a mechanism to adapt to or even moderate the varying steroid-secreting level by changing the intensity of autophagic activity (Yi and Tang, 1995). In light of these findings, the autophagic processes that we have observed in the flank organ are very understandable. The preference, or even specificity, for destroying some of the mitochondria and smooth endoplasmic reticulum would be responsible for the turnover of steroid-producing organelles, and may consequently result in an influence upon the rate of steroid secretion. Likewise, our group has demonstrated autophagic processes in the Harderian gland, which is also present in Syrian hamsters and has a lot of similarities to the flank organ. This is also an organ that is a pheromone producer and sexual steroid transformer (Tomas-Zapico et al, 2005). This similarity between 2 organs that develop their functions in relation to sexual hormones seems to support the hypothesis that autophagy could be a common mechanism of detoxifying these kinds of organs.

However, sexual differences between female and male flank organs were unexpected. The decrease in flank organ size could be caused by orchidectomy, partially reversed by dehydroepiandrosterone and completely

reversed by 4-enedione, testosterone, and dihydrotestosterone implants (Chen et al, 1996). Androgen hormones seem to play an important role in flank organ formation and structural maintenance. Therefore, this article shows that flank organs in Syrian hamsters develop autophagy, as PCD, at different degrees dependent on sex. Because the flank organ is an androgen-dependent organ, this is the first time that variations in androgen levels have been shown to be the most important factor for the development of autophagy, and this information could be very useful for gaining knowledge into the control mechanisms for hair growth.

Acknowledgments

We thank Fernando Jáñez and Gloria Menéndez for their excellent technical assistance.

References

- Baillie AH, Thomson J, Milne JA. The distribution of hydroxysteroid dehydrogenase in human sebaceous glands. *Br J Dermatol.* 1966; 78:451–457.
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976;72:248–254.
- Brooks JR, Primka RL, Berman C, Krupa DA, Reynolds GF, Rasmussen GH. Topical anti-androgenicity of a new 4-azasteroid in the hamster. *Steroids.* 1991;56:428–433.
- Bursch W. The autophagosomal-lysosomal compartment in programmed cell death. *Cell Death Differ.* 2001;8:569–581.
- Bursch W, Hochegger K, Torok L, Marian B, Ellinger A, Hermann RS. Autophagic and apoptotic types of programmed cell death exhibit different fates of cytoskeletal filaments. *J Cell Sci.* 2000; 113(pt 7):1189–1198.
- Chen C, Belanger A, Labrie F. Adrenal steroid precursors exert potent androgenic action in the hamster sebaceous glands of flank organs and ears. *Endocrinology.* 1996;137:1752–1757.
- Demoz M, Castino R, Follo C, Hasilik A, Sloane BF, Isidoro C. High yield synthesis and characterization of phosphorylated recombinant human procathepsin D expressed in mammalian cells. *Protein Expr Purif.* 2006;45:157–167.
- Diment S, Martin KJ, Stahl PD. Cleavage of parathyroid hormone in macrophage endosomes illustrates a novel pathway for intracellular processing of proteins. *J Biol Chem.* 1989;264:13403–13406.
- Ebling FJ. Sebaceous glands I. The effect of sex hormones on the sebaceous glands of the female albino rat. *J Endocrinol.* 1948;5: 297–302.
- Ebling FJ. Hormonal control of the sebaceous gland in experimental animals. In: *Advances in Biology of Skin: The Sebaceous Glands.* Oxford, United Kingdom: Pergamon Press; 1963:200–219.
- Gozuacik D, Kimchi A. Autophagy as a cell death and tumor suppressor mechanism. *Oncogene.* 2004;23:2891–2906.
- Hamilton JB, Montagna W. The sebaceous glands of the hamster; morphological effects of androgens on integumentary structures. *Am J Anat.* 1950;86:191–233.
- Hengartner MO. The biochemistry of apoptosis. *Nature.* 2000;407: 770–776.
- Isahara K, Ohsawa Y, Kanamori S, Shibata M, Waguri S, Sato N, Gotow T, Watanabe T, Momoi T, Urase K, Kominami E, Uchiyama

- Y. Regulation of a novel pathway for cell death by lysosomal aspartic and cysteine proteinases. *Neuroscience*. 1999;91:233–249.
- Ishisaka R, Kanno T, Akiyama J, Yoshioka T, Utsumi K, Utsumi T. Activation of caspase-3 by lysosomal cysteine proteases and its role in 2,2'-azobis-(2-amidinopropane)dihydrochloride (AAPH)-induced apoptosis in HL-60 cells. *J Biochem (Tokyo)*. 2001;129:35–41.
- Johnston RE, Rasmussen K. Individual recognition of female hamsters by males: role of chemical cues and of the olfactory and vomeronasal systems. *Physiol Behav*. 1984;33:95–104.
- Joza N, Susin SA, Dugas E, Stanford WL, Cho SK, Li CY, Sasaki T, Elia AJ, Cheng HY, Ravagnan L, Ferri KF, Zamzami N, Wakeham A, Hakem R, Yoshida H, Kong YY, Mak TW, Zuniga-Pflucker JC, Kroemer G, Penninger JM. Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. *Nature*. 2001;410:549–554.
- Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, Kominami E, Ohsumi Y, Yoshimori T. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosomal membranes after processing. *EMBO J*. 2000;19:5720–5728.
- Kihara A, Noda T, Ishihara N, Ohsumi Y. Two distinct Vps34 phosphatidylinositol 3-kinase complexes function in autophagy and carboxypeptidase Y sorting in *Saccharomyces cerevisiae*. *J Cell Biol*. 2001;152:519–530.
- Kitanaka C, Kuchino Y. Caspase-independent programmed cell death with necrotic morphology. *Cell Death Differ*. 1999;6:508–515.
- Liang XH, Jackson S, Seaman M, Brown K, Kempkes B, Hibshoosh H, Levine B. Induction of autophagy and inhibition of tumorigenesis by Beclin 1. *Nature*. 1999;402:672–676.
- Liang XH, Kleeman LK, Jiang HH, Gordon G, Goldman JE, Berry G, Herman B, Levine B. Protection against fatal Sindbis virus encephalitis by beclin, a novel Bcl-2-interacting protein. *J Virol*. 1998;72:8586–8596.
- Mezick JA, Gendimenico GJ, Liebel FT, Stenn KS. Androgen-induced delay of hair growth in the golden Syrian hamster. *Br J Dermatol*. 1999;140:1100–1104.
- Neurath H. Proteolytic processing and regulation. *Enzyme*. 1991;45:239–243.
- Neurath H. Proteolytic enzymes past and present: the second golden era. Recollections, special section in honor of Max Perutz. *Protein Sci*. 1994;3:1734–1739.
- Pochi PE, Strauss JS. Sebaceous gland response in man to the administration of testosterone, delta-4-androstenedione, and dehydroisoandrosterone. *J Invest Dermatol*. 1969;52:32–36.
- Roberg K, Kagedal K, Ollinger K. Microinjection of cathepsin D induces caspase-dependent apoptosis in fibroblasts. *Am J Pathol*. 2002;161:89–96.
- Sansone-Bazzano G, Reisner RM. Steroid pathways in sebaceous glands. *J Invest Dermatol*. 1974;62:211–216.
- Schreurs FJ, van der Heide D, Leenstra FR, de Wit W. Endogenous proteolytic enzymes in chicken muscles. Differences among strains with different growth rates and protein efficiencies. *Poult Sci*. 1995;74:523–537.
- Takahashi T, Tang J. Cathepsin D from porcine and bovine spleen. In: Loran L ed. *Proteolytic Enzymes, Part C Methods in Enzymology*. New York, NY: Academic Press; 1981:567–589.
- Takayasu S, Adachi K. The in vivo and in vitro conversion of testosterone to 17-hydroxy-5-androsten-3-one (dihydrotestosterone) by the sebaceous gland of hamsters. *Endocrinology*. 1972;90:73–80.
- Tomas-Zapico C, Caballero B, Sierra V, Vega-Naredo I, Alvarez-Garcia O, Tolivia D, Rodriguez-Colunga MJ, Coto-Montes A. Survival mechanisms in a physiological oxidative stress model. *FASEB J*. 2005;19:2066–2068.
- Voigt W, Hsia SL. The antiandrogenic action of 4-androsten-3-one-17 beta-carboxylic acid and its methyl ester on hamster flank organ. *Endocrinology*. 1973;92:1216–1222.
- Yi J, Tang XM. Functional implication of autophagy in steroid-secreting cells of the rat. *Anat Rec*. 1995;242:137–146.
- Zeng X, Overmeyer JH, Maltese WA. Functional specificity of the mammalian Beclin-Vps34 PI 3-kinase complex in macroautophagy versus endocytosis and lysosomal enzyme trafficking. *J Cell Sci*. 2006;119:259–270.
- Zhu JH, Horbinski C, Guo F, Watkins S, Uchiyama Y, Chu CT. Regulation of autophagy by extracellular signal-regulated protein kinases during 1-methyl-4-phenylpyridinium-induced cell death. *Am J Pathol*. 2007;170:75–86.