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Apoptotic cell death in canine hair follicle

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Summary. Apoptotic cell death is an essential homeostatic mechanism involved in the control of cellular turnover in a variety of adult tissues.

Cytoplasmic and nuclear condensation morphologically define this process whose biochemical hallmark is extensive DNA fragmentation into discrete oligonucleosomic units.

Hair follicle growth and regression has been shown to be correlated with apoptosis in humans, mice, rats and guinea pigs.

The present study was carried out to evaluate its implication in canine hair biology in order to define the spatio-temporal relationship between apoptosis and the hair cycle in dogs.

As assessed by terminal deoxy-nucleotidyl transferase-mediated d-UTP nick-end-labelling (TUNEL) and by basic histological and ultrastructural assays, apoptotic cells appeared both in the growing and in the regressing follicle epithelium showing the well characterized morphological features described in the previous relevant literature.

Key words. Hair follicle, Dog, Apoptosis, TUNEL, Ultrastructure

Introduction

The mammalian hair follicle is a highly dynamic and plastic skin appendage that repeatedly and perpetually undergoes tightly programmed cyclical transformations. Three distinct developmental stages have been defined for the mammalian cycle: anagen (growing phase); catagen (regressing phase) and telogen (resting phase) (Al-Bagdadi et al., 1979; Von Tscharner and Suter, 1994; Scott et al., 1995; Paus and Cotsarelis, 1999). Hair follicle cycles of growth and regression are characterized by dramatic changes in follicular microanatomy and in keratinocyte biology that involve a closely co-ordinated machinery of cell proliferation, differentiation, mesenchymal/epithelial interactions and apoptosis (Weedon and Strutton, 1981; Cotsarelis, 1997; Paus et al., 1999) .

Concerning the last aspect, no other mammalian organ displays such rhythmical, physiological apoptosis during the entire lifespan of the organism, offering one of the most attractive models for the study of programmed epithelial cell death in situ and its role in the morphogenesis and the regression of multicellular systems (Lindner et al., 1997).

Previous studies in murine hair follicles have largely characterized the sequence of morphological changes and the topographical distribution of apoptotic cells during the anagen-catagen-telogen transformation: because both spontaneous and induced hair follicle cycling in murine skin appears in waves, with all hair follicles in almost the same stage of the cycle, the synchronized and relatively short hair cycle in mice represents an excellent model to study the physiological remodelling associated with distinct phases of the hair cycle (Paus et al., 1994; Lindner et al., 1997; Matsuo et al., 1998; Müller-Rover et al., 2000) .

Lindner et al. (1997) and Soma et al. (1998) respectively reported that in murine and human hair follicles apoptosis takes place both in anagen (restricted to the area of terminal differentiation of the central inner root sheath and in the outer root sheath) and in catagen (in the outer root sheath, in the epithelial strand, in the secondary hair germ and in the bulb epithelial cells closely adjacent to the dermal papilla).

Besides these two main reports, other studies extensively documented apoptosis in terminal differentiating cells of the medulla and the outer root sheath of anagen follicles and in catagen hair follicles of guinea pigs (Kishimoto et al., 1997), rats (Iwamoto et al., 1998) and mice (Weedon and Strutton, 1981; Matsuo et al., 1998) suggesting the crucial role of apoptotic cell death in both the morphogenesis and the regression of this skin appendage.

Considering the lack of any similar evaluation in dogs, this study was aimed at detecting and morphologically characterizing apoptosis in spontaneously cycling canine hair follicles which normally display a mosaic growth pattern (Scott et al., 1995).

Except for rare congenital hair defects and

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inflammatory and "scarring" alopecias, hair loss in canine species often reflects aberrations of hair follicle cycling characterized by early and massive apoptosis-driven catagen induction (es. alopecia areata, telogen effluvium, post-clipping alopecia, hyperadrenocorticism, hypothyroidism, hyperestrogenism, growth hormone/ castration–responsive dermatosis, etc.) (Gross et al., 1992a,b; Dunstan and Credille, 1994; Dunstan, 1996; Fondevila, 2000).

These considerations emphasize the use of canine skin as a clinically relevant research tool for studying the social and intracellular controls of epithelial cell apoptosis in situ under physiological and pathological conditions.

Material and methods

1. Tissue collection

Surgical and autoptic normal canine skin specimens were obtained from 10 male and female short-haired animals of heterogeneous ages. Skin pieces were collected from the dorsal neck and from the back region, fixed and prepared for light and electron microscopic examinations.

2. Basic histologic

Skin pieces were fixed in 10% neutral-buffered formalin, dehydrated and embedded in paraffin wax for light microscopic procedures.

Extensive pilot assays were performed to determine the most sensitive and significative method for the study of follicular cycle-dependent morphological changes and for the detection of apoptotic cells. The following protocol was found to yield optimal results.

We first approached the study of apoptosis in canine follicular biology by carrying out preliminary "aspecific" morphological evaluations in the specimens' serial sections. In particular, we "staged" hair follicles by means of histomorphological staining to determine their state of activity. We then treated our material with basic histological procedures (toluidine blue/safranin technique), TUNEL assay (terminal deoxy-nucleotidyl transferase-mediated d-UTP nick-end-labelling - TUNEL) and electron microscopic techniques.

2.a. Determination of the state of activity of hair follicles

A variation of the Sacpic staining method (Nixon, 1993) was used to determine the cycle stage in longitudinal and cross-sections of hair follicles. This staining produces a high contrast among the principal tissue structures present in the hair follicle, facilitating identification of growing and regressing fibres. The main features of the follicles and fibres examined were: pigmentation, medullation, presence of the "brush" or "club" end, production of the inner root sheath, flattening of the outer root sheath cells, formation of secondary hair germ, presence of the epithelial strand and follicle bulb morphology.

2.b. Differential staining of dead and viable follicular keratinocytes

A simple staining technique, described by Martín-Partido in 1986, used a combination of toluidine blue and safranin to distinguish dead and viable cells in sections of chick embryo tissues fixed in a mixture of glutaraldehyde and formaldehyde and embedded in Spurr's resin (Martín-Partido et al., 1986). This technique yielded satisfactory results even in histological sections of formalin-fixed, paraffin-embedded specimens, allowing blue-staining of the dying cells' pyknotic nuclei to be clearly distinguished from red-staining healthy cells of hair follicles.

The results of the toluidine blue-safranin method were verified in canine timic sections where apoptotic cells usually appear numerous and clearly evident.

3. TUNEL

TUNEL reaction (terminal deoxy-nucleotidyl transferase-mediated d-UTP nick-end- labelling) was performed on paraffin sections as described by Gavrieli et al. (1992), with minor modifications, using a commercially available kit (Apoptag In situ Apoptosis Detection Kit -Intergen).

Tissue sections were treated with 20mg/ml proteinase K for 10 min at room temperature. Endogenous peroxidase was inactivated with 3% H_2O_2 for 5'. After rinsing with Phosphate-Buffered Saline (PBS), the sections were incubated in a TdT solution (Terminal Deoxynucleotidyl Transferase) for 1 h at 37 °C in humidified atmosphere.

The slides were then transferred to a stop/wash buffer for 10 min at room temperature to stop the reaction. Digoxigenin dUTP-labeled DNA compounds were detected by anti-digoxigenin peroxidase-conjugated antibodies (30 min at room temperature). Colour was developed using diaminobenzidine (0.05%) for 5 minutes.

Negative controls were prepared by omitting TdT, according to the manufacturer's protocol.

Positive TUNEL controls were run by comparison with tissue sections from the thymus of dog which normally displays a high degree of spontaneous thymocyte apoptosis.

This method enables apoptotic cells to be recognized due to the combination of the TUNEL signal and the morphological features.

4. Ultrastructural studies

Small pieces of tissue specimens were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.4, for 5 hours at 4 °C, post fixed in phosphate-buffered 1%

Osmium tetroxide for 3 hours, dehydrated in graded ethanol and embedded in Epon 812. Semithin sections were stained with methylene blue/Azur II solution and examined by means of light microscopy. Ultrathin longitudinal and cross-sections of at least 50 hair follicles in different stages of the cycle were double-stained with uranyl acetate and lead citrate (Reynolds, 1963) and then observed in a Philips EM208 electron microscope at 80 KV.

Results

Basic histology

The sequence of events which took place during the apoptotic process (nuclear and cytoplasmic condensation, cell fragmentation and phagocytosis by neighbouring cells) was clearly revealed by the use of the toluidine blue-safranin dichromic technique.

The nucleus or nuclear debris in dead cells was stained a deep blue while in healthy cells the cytoplasm and nuclear chromatin stained a pale red.

The early recognisable stage of cells undergoing apoptosis involved separation of the plasma membrane from that of the adjacent cells and nuclear and cytoplasmic hyperchromatism related to cellular condensation (individualization and condensation). A sort of extensive villous transformation of the cell surface, attributable to cellular blebbing, was detected in early apoptotic phases (hairy cells) (Fig. 1).

Apoptotic nuclei were contracted and round-shaped or appeared as massive chromatin deposits in form of characteristic dense crescent-shaped areas lining the nuclear side of the nuclear envelope.

Cellular fragmentation resulted in the formation of apoptotic bodies looking like small, spherical or roughly ovoid acidophilic globules with or without pyknotic nuclear remnants. Apoptotic bodies occurred both singly and in clusters; they were sometimes surrounded by clear halos and varied considerably in size (Figs. 2, 3). Nuclear remains present in some fragments aided recognition of the apoptotic bodies.

Scattered, shrunken apoptotic keratinocytes and apoptotic bodies were mostly detected in the outer root sheath (ORS) and in the epithelial strand (ES) of regressing hair follicles (Fig. 4).

Several apoptotic bodies were also observed in the ORS of anagen hair follicles (Fig. 5).

There wasn't any evident difference in the results between animals of different age, sex or breed.

TUNEL

TUNEL- positive cells showed a variable reactivity depending on the stage of the process: several keratinocytes displayed a mild anular, peripheral staining while other cells were strongly reactive and condensed (Fig. 6).

Catagen was associated with the largest number of apoptotic keratinocytes in the residual outer root sheath and in the entire epithelial strand (Fig. 6) of the involuting hair bulb.

Several cornified TUNEL-positive cells were observed in the inner root sheath (IRS) and in the hair medulla of growing fibres.

Fibroblasts of the dermal papilla were never seen to be TUNEL-reactive in any stage of the hair cycle.

The exact spatial relationship of the bodies to adjoining intact tissue cells was often difficult or impossible to define. However, the numerous apoptotic

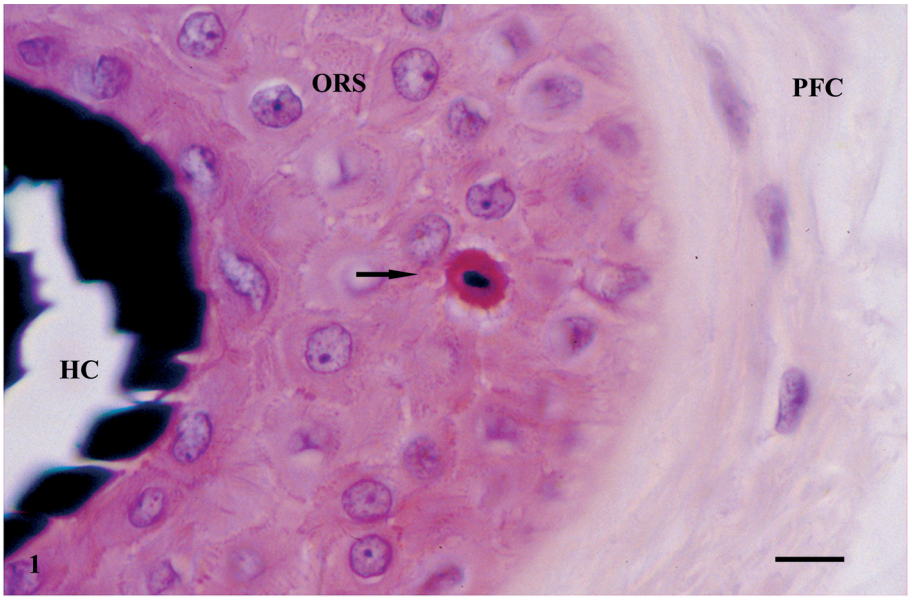


Fig. 1. Early apoptotic keratinocyte (arrow) in the outer root sheath of a cross-sectioned anagen hair follicle. The apoptotic cell shows nuclear and cytoplasmic hyperchromatism and villous transformation of its surface. ORS, outer root sheath; HC, hair canal; PFC, perifollicular connective tissue. Toluidine blue/Safranin. Bar: 10 μm.

bodies were seemingly located inside adjacent viable keratinocytes which sometimes showed a deformed profile and indented nuclei.

Engulfment by "professional" phagocytic cells was never observed.

No differences were detected between dogs of different age, sex or breed.

Ultrastructure

Even after a prolonged search, the early stages of apoptosis were never detected. On the contrary, membrane-bound apoptotic bodies phagocytosed and digested by the surrounding resident keratinocytes were widespread.

Apoptotic bodies were single or multiple and varied greatly in size. Some of them contained one or more nuclear fragments, whereas others only contained cytoplasmic elements. Nuclear components, when present, did not appear consistently related to the size of the apoptotic body.

Characteristically, nuclear fragments often showed

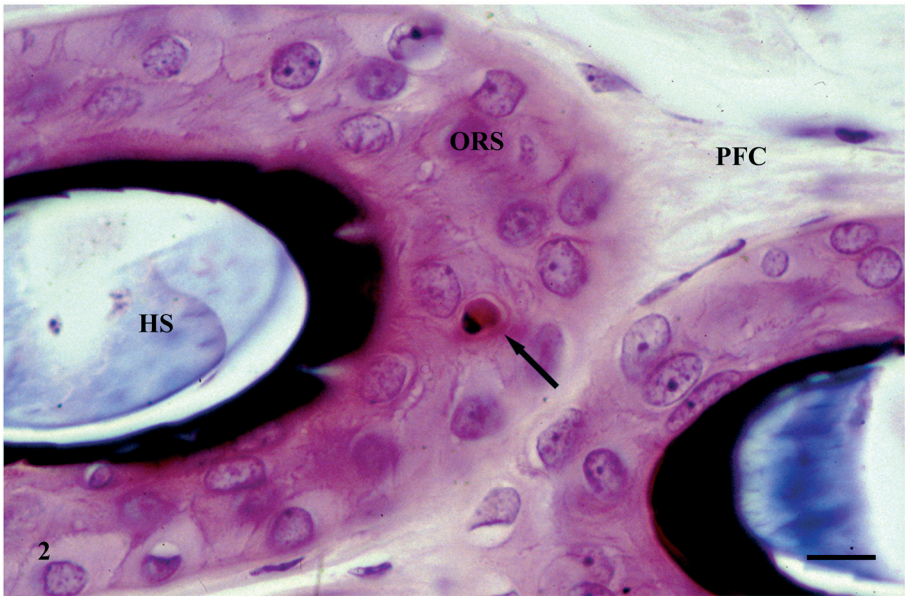


Fig. 2. Large apoptotic body (arrow) in the outer root sheath of a cross-sectioned anagen hair follicle, characterized by chromatin cap-shaped margination and peripherial clear halo. ORS, outer root sheath; HS, hair shaft; PFC, perifollicular connective tissue. Toluidine blue/Safranin. Bar: 10 μm.

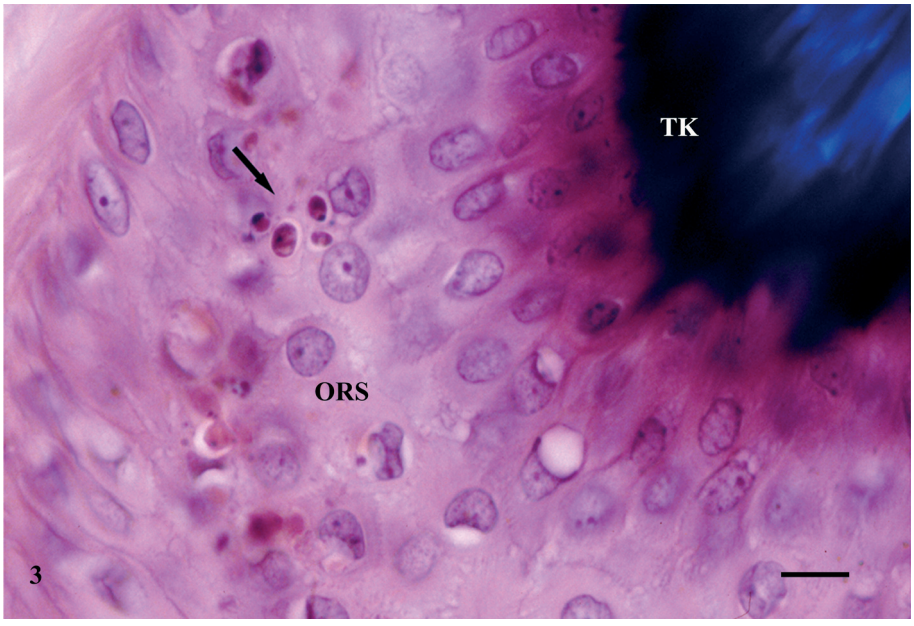


Fig. 3. Cluster of different-sized apoptotic bodies (arrow) in the outer root sheath of a regressing hair follicle. ORS, outer root sheath; TK, trichilemmal keratinization. Toluidine blue/Safranin. Bar: 10 μm.

very electron-dense, condensed chromatin. This either occupied the entire cut surface (Fig. 7) or was arranged in crescentic, highly contracted caps (Fig. 8).

The organelles of the ingested apoptotic bodies appeared to be either intact or markedly degraded within phagolysosomes of viable engulfing cells (Fig. 9). Apoptotic bodies exhibiting degenerative changes showed breaks in the plasma membrane, vacuolation, electron-lucent mitochondria with focal matrix densities, dilation of rough reticular endoplasmic cisternae and chromatolysis.

Free extracellular bodies were never observed, but they always appeared inside the cytoplasm of neighbouring keratinocytes.

Large, condensed cell remains containing clumped tonofilament bundles and devoid of nuclear fragments were sometimes seen (Fig. 10).

The highest concentration of apoptotic bodies was

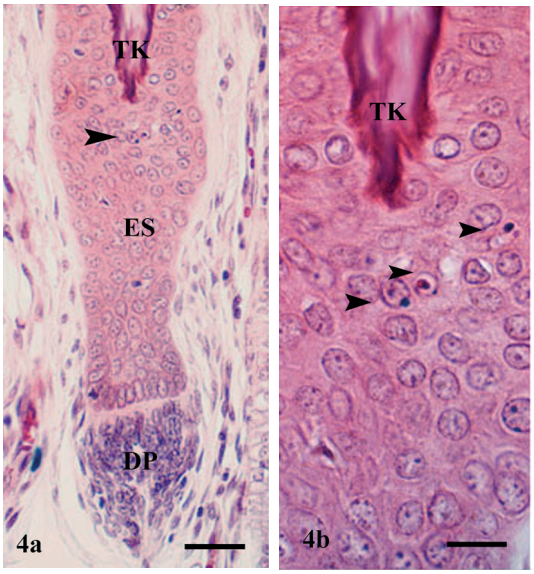


Fig. 4. Catagen hair follicle, longitudinal section. Apoptotic bodies are present in the epithelial strand (arrowheads). TK: trichilemmal keratinization; ES: epithelial strand; DP: dermal papilla. Bar: a, 50 μm; b, 25 μm.

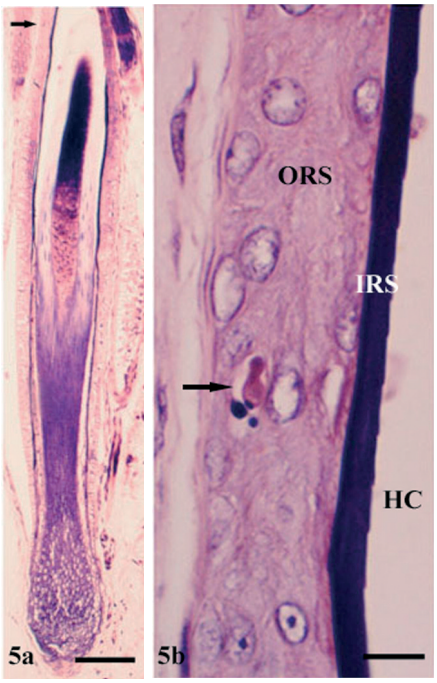
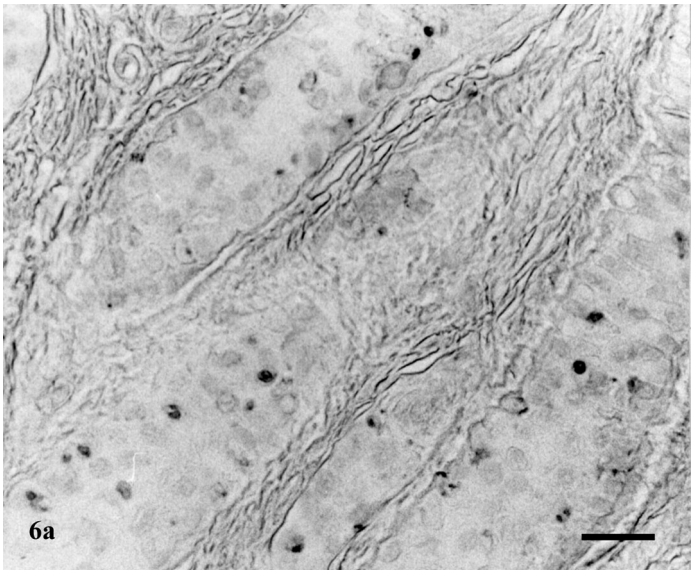


Fig. 5. Anagen hair follicle, longitudinal section. Apoptotic bodies are seen in the outer root sheath (arrow). ORS: outer root sheath; IRS: inner root sheath; HC: hair canal. Bar: a, 100 μm; b, 10 μm.



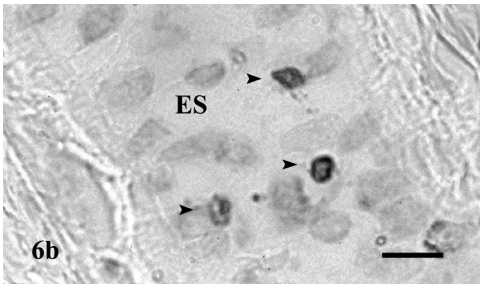


Fig. 6. Tunel-reactive apoptotic keratinocytes in the epithelial strand of longitudinal-sectioned regressing hair follicles (arrowheads). ES: epithelial strand. Tunel. Bar: a, 20 μm; b, 10 μm.

observed in the outer root sheath epithelium; however, a precise localization with transmission electron microscopy was often very difficult to achieve due to the small surface examined.

Discussion

The results reported in this work are consistent with previous findings showing the presence of apoptotic keratinocytes in both growing and regressing canine hair follicles by means of combined histological, TUNEL and ultrastructural procedures.

According to the observations of Lindner et al. (1997), Soma et al. (1998) and Kishimoto et al. (1997), apoptotic bodies were seen in the IRS, the ORS and the medulla of growing fibres.

Apoptotic cell clusters observed during anagen may reflect a possible mechanism devoted to regulating cellular turnover balancing proliferative events and, as previously hypotesized by Lindner et al. (1997), the strategy of mantaining the inner root sheath at a constant length by deleting surplus cells.

As reported in the previous literature, the number of apoptotic cells in regressing hair follicles was by far higher in the ORS and in the epithelial strand than in any other follicle region or cycle phase.

The remarkable concentration of apoptotic keratinocytes in catagen hair follicles lends strong support to previous considerations that this process represents the major driving force of hair follicle epithelium remodelling and involution. As proposed by Lindner et al. (1997), the simultaneous deletion of keratinocytes in many different areas may explain the extraordinary speed of hair follicle involution during

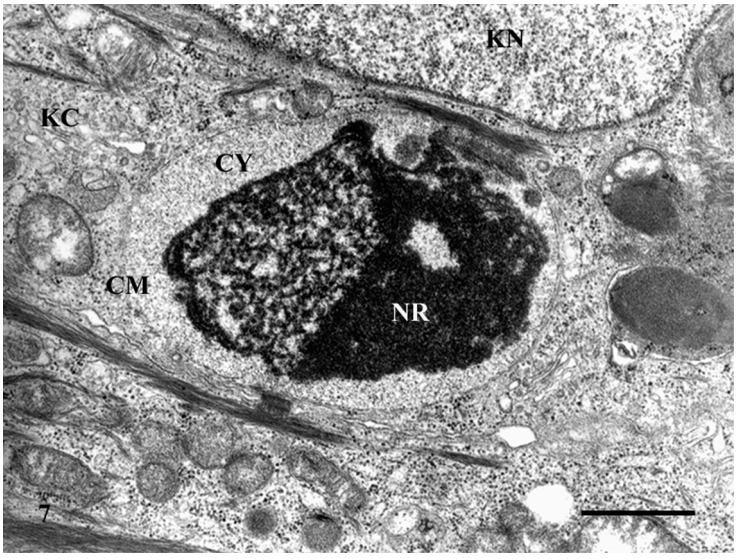


Fig. 7. Phagocytosed apoptotic body showing a clear-cut cellular membrane (CM), delimiting fine granular cytoplasm (CY) and strongly electron-dense nuclear remain (NR). KC: keratinocyte cytoplasm; KN: keratinocyte nucleus. TEM. Bar: 1 μm.

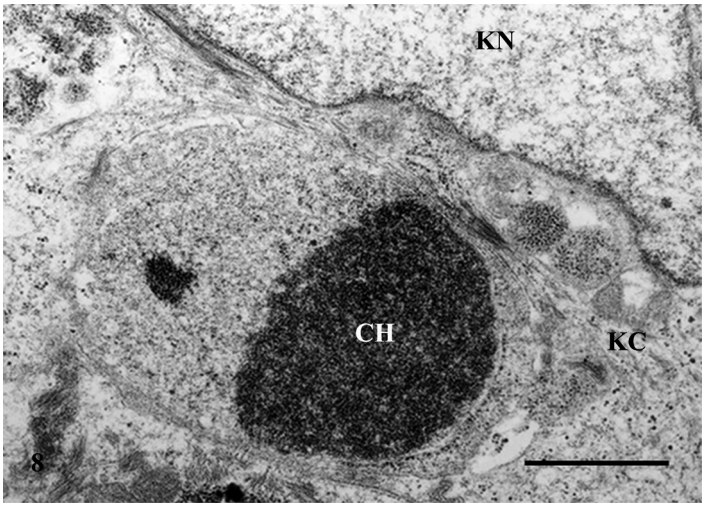


Fig. 8. Intracellular apoptotic body with crescent-shaped chromatin (CH) margination. KN: keratinocyte nucleus; KC: keratinocyte cytoplasm. TEM. Bar: 1 μm.

which it shortens more than two-thirds of its initial length.

Basic histology, TUNEL stain and ultrastructural examination confirmed the presence of extensive apoptosis of the follicular epithelial cells evolving through the stereotyped morphological sequence of nuclear and cytoplasmic condensation, cellular fragmentation and phagocytosis of apoptotic bodies.

The early stages of keratinocyte apoptosis (blebbing and condensation) were rarely observed with light

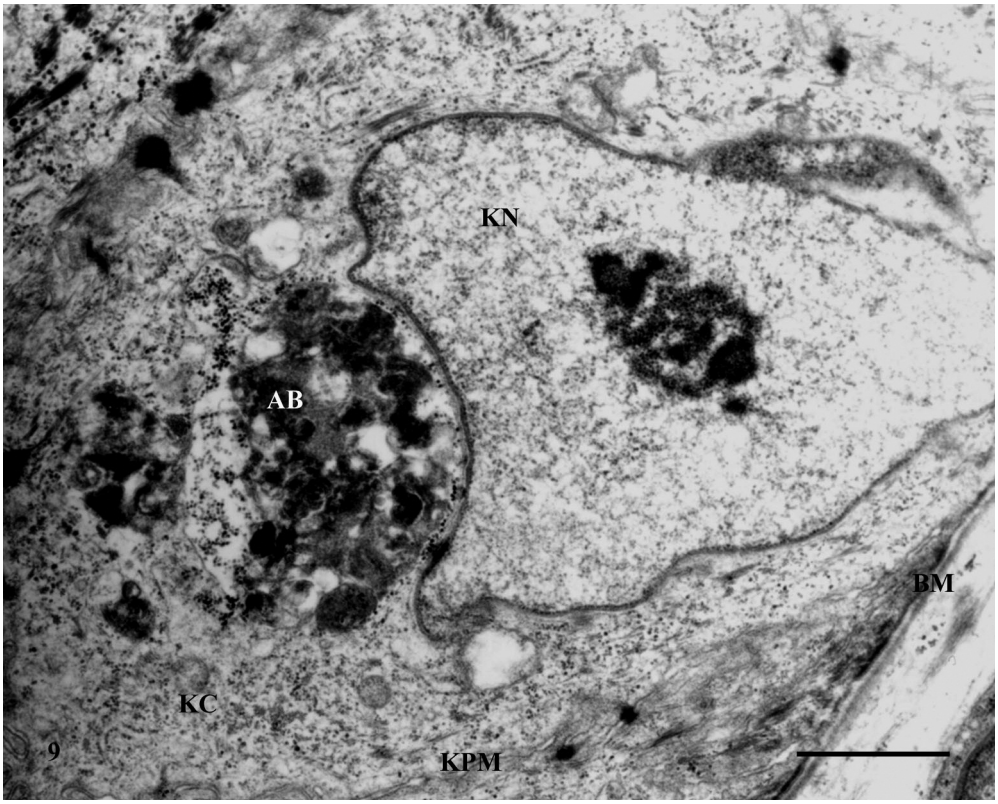


Fig. 9. Apoptotic body exhibiting advanced degenerative changes. AB: apoptotic body; KN: keratinocyte nucleus; KC: keratinocyte cytoplasm; KPM: keratinocyte plasma membrane; BM: basal membrane.TEM. Bar: 1 μm.

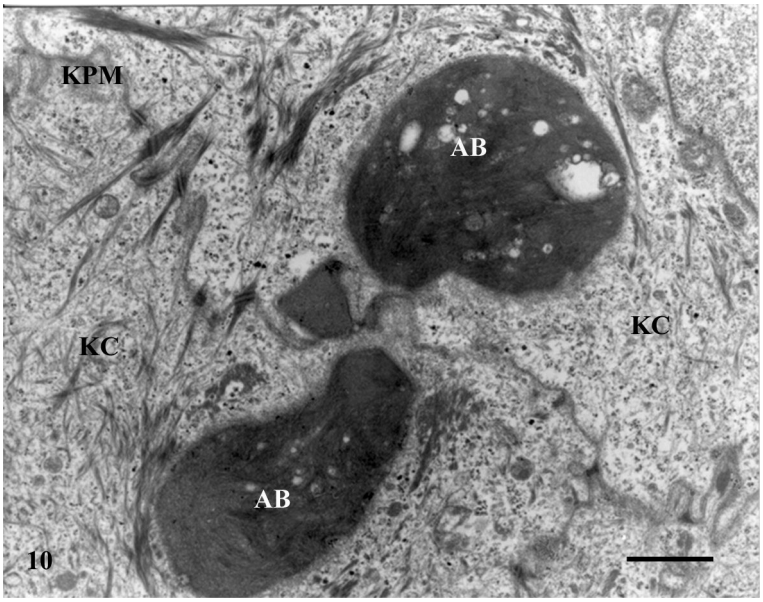


Fig. 10. Apoptotic bodies devoid of nuclear remains characterized by a dense filamentous cytoplasmic matrix associated to sparse fragmented and degenerated cellular organules. AB: apoptotic body; KC: keratinocyte cytoplasm; KPM: keratinocyte plasma membrane. TEM. Bar: 1 μm.

microscopy and never by transmission electron microscopy, as expected for such extremely fleeting phenomena which take place in only a few minutes. Furthermore, the close contact between adjacent keratinocytes undoubtedly represents a spatial limitation to the blebbing of the dying cells. It should also be emphasized that the narrow surface of a thin section examined during ultrastructural examination certainly contributes to reducing the probability of observing very transient and rare events.

On the other hand, a far greater number of apoptotic bodies appeared inside viable neighbouring keratinocytes showing that, once formed, they are avidly phagocytosed and the ranks are rapidly closed with inappreciable modifications in follicular architecture. The significant proportion of phagocytosed bodies showing evidence of advanced lysosomal digestion accounts for the rapidity and neatness of the apoptotic process.

Concerning the observation of large, condensed, tonofilament-rich cell remains arising from apoptosis of follicular keratinocytes, we believe they could be ascribed to filamentous cells as described by Hashimoto (1976) and by Weedon et al. (1979) for epidermal keratinocytes. The rigidity imparted to the epithelial cell cytoplasm by the abundance of tonofilament bundles, restricts surface convolution (straitjacket effect) and gives rise to small blebs that are virtually devoid of tonofilaments. Most tonofilaments aggregate in a circumscribed area of the cell that initially resists further subdivision. The unusually large apoptotic bodies containing predominantly clumped intermediate filaments probably derive from final fragmentation of this area.

Additional studies would be necessary to elucidate the question of whether keratinocyte terminal differentiation and apoptosis share several molecular steps. Because accepting terminal differentiation as a form of apoptosis could challenge the entire concept of apoptosis, it is difficult to determine the real significance of TUNEL reactivity of the cornified IRS cells and medullary cells described in this work.

In conclusion, it should be emphasized that the *in situ* identification of cells undergoing apoptosis by light and electron microscopy is, in general, a difficult and tedious task. The apoptotic process, in fact, is limited to only a few hours and apoptotic bodies are seen for a very short time before they are phagocytosed and digested.

The evidence of apoptosis in spontaneously cycling hair follicles is even more difficult in that the major concentration of apoptotic cells has been reported in catagen hair follicles. Unfortunately, only a very small percentage (4-7%) of spontaneously cycling hair follicles in normal canine skin is in catagen at any given time, due to the short duration and to the unpredictable and non-synchronized starting time of this phase (Scott et al., 1995).

In spite of the analytical and technical difficulties, the comparison of our results with those reported in the previous literature (Weedon and Strutton, 1980; Tamada et al., 1994; Kishimoto et al., 1997; Lindner et al., 1997; Matsuo et al., 1998; Soma et al., 1998), lead us to conclude that apoptosis involvement in canine follicular biology has patterns of distribution that are similar to those reported for other mammalian species.

Because a defective regulation of apoptosis is believed to play an essential role in initiating several alopecic dermatopathies in dogs, the ambitious target of mapping follicular apoptosis and decoding its molecular control could provide one of the missing links between developmental and pathological aspects of hair growth and lay the foundation for innovative pharmacological therapy.

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