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CHANGES IN THE BLOOD-EPIDIDYMIS BARRIER OF THE BROWN NORWAY RAT WITH AGE

by

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A thesis submitted to the Faculty of Graduate Studies and Research in the partial fulfilment of the requirements for the degree of Master of Science.

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Abstract

In aging Brown Norway rats, there is an activation of the immune system represented by a striking increase in the number of halo cells. As the blood-epididymis barrier should protect from immunological attack, we hypothesized that there would be changes in the structure and function of this barrier with age. To test this hypothesis, the immunocytochemical localization of occludin, ZO-1, and E-cadherin, as well as lanthanum nitrate permeability of the blood-epididymis barrier, were done using the epididymides of Brown Norway rats aged 3, 18, and 24 months. Occludin, ZO-1, and E-cadherin immunostaining was observed at the apico-lateral junction between principal cells in the initial segment of 3-month-old animals; with increasing age, occludin and ZO-1 reactivity decreased, while E-cadherin staining increased along the lateral membrane between principal cells. In the caput, corpus, and cauda epididymidis, occludin, ZO-1, and E-cadherin immunostaining showed segment-specific and age-dependent differences in their staining patterns. Occludin and ZO-1 cytoplasmic staining progressively decreased by 24 months, except in the cauda epididymidis where occludin staining slightly increased with age. The E-cadherin reactivity seen in the caput and cauda epididymidis at 3 months spread to the entire lateral plasma membrane between adjacent principal cells by 24 months. The most dramatic changes were seen in the corpus epididymidis with age; the intense E-cadherin cytoplasmic staining that was observed at 3 months was absent by 24 months and no occludin or ZO-1 reactivity was observed in older animals. The greatest penetration of lanthanum nitrate into the blood-epididymis barrier and the lumen was seen in the aging corpus

epididymidis, while there was no barrier permeability in the initial segment or cauda epididymidis of the aged animals. Taken together, these data indicate that there are segment-specific decreases in the structural and functional integrity of the blood-epididymis barrier with age, most notably in the corpus epididymidis.

Résumé

Chez les rats Brown Norway âgés , il y a un déclenchement du système immunitaire qui se manifeste par une augmentation importante du nombre de cellules halos. Puisque la barrière sang-épididyme devrait protéger contre une attaque immunitaire, nous avons envisagé l'hypothèse qu'il y aurait des changements dans la structure et les fonctions de la barrière avec l'âge. Pour tester notre hypothèse, la localisation immunocytochimique de l'occludine, ZO-1 et E-cadherine, ainsi que la perméabilité de la barrière sang-épididyme au nitrate de lanthanum ont été examinées en utilisant des épididymes de rats Brown Norway âgés de 3, 18 et 24 mois. La réaction à l'occludine, ZO-1 et E-cadherine a été observée à la jonction apico-latérale entre les cellules principales du segment initial des animaux âgés de 3 mois; avec l'âge, la réaction à l'occludine et ZO-1 a diminué, alors que pour E-cadherine, la réaction était présente tout le long de la membrane latérale entre les cellules principales. Dans la tête, le corps et la queue de l'épididyme, la réaction à l'occludine, ZO-1 et E-cadherine a démontré des différences spécifiques selon le segment et dépendent de l'âge . La réaction à l'occludine dans le cytoplasme a diminué progressivement avec l'âge dans tous les segments, sauf dans la queue, où elle a augmenté. Pour ZO-1, avec l'âge, on a observé une baisse de l'intensité du marquage dans le cytoplasme des cellules principales à travers tous les segments de l'épididyme. La réaction à E-cadherin dans la tête et la queue de l'épididyme observée à 3 mois s'est étendue le long de la membrane latérale entre les cellules principales lorsque l'animal a atteint 24 mois. Les changements les plus évidents ont été vus dans le corps de l'épididyme

avec l'âge; la réaction intense à E-cadherine observée à 3 mois était absente à 24 mois et il n'y avait pas de réaction à l'occludine ni ZO-1 chez les animaux âgés. La plus grande pénétration du nitrate au lanthanum à travers la barrière sang-épididyme et dans la lumière a été vue dans le corps de l'épididyme à 24 mois, alors que la barrière était imperméable dans le segment initial et la queue des épididymes des animaux âgés. Ces résultats indiquent que selon le segment de l'épididyme, il y a une diminution spécifique de l'integrité structurelle et fonctionelle de la barrière sang-épididyme avec l'âge, spécialement dans le corps de l'épididyme.

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Format of the Thesis

This thesis comprises a paper which is included entirely in the form in which it has been submitted for publication. It is a manuscript-based thesis in compliance with section I. C. of the "Guidelines for Thesis Preparation," Faculty of Graduate Studies and Research, McGill University. These guidelines state that "candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers is mandatory. The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts. The thesis must include the following: (a) a table of contents, (b) an abstract in English and French, (c) an introduction which clearly states the rationale and objectives of the research, and (d) a comprehensive review of the literature (in addition to that covered in the introduction to each paper)."

The Introduction, Chapter 1, contains a general introduction describing the structure and function of the epididymis, the changes in the epididymis with aging and the structure and function of the blood-epididymis barrier. Chapter 1 ends by providing the rationale for the studies presented in this thesis. Chapter 2 will be submitted for publication; all experiments were performed by the candidate except for the lanthanum nitrate perfusions which were performed with the technical help of Valerie Serre. Chapter 3 includes a general discussion of the results.

Acknowledgements

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Chapter 1

INTRODUCTION

A. The Epididymis

1. Structure of the Epididymis

The epididymis is a highly convoluted tubule that connects the efferent ducts, emanating from the testis, to the vas deferens [1-4]. This tissue can be subdivided into several segments: the initial segment, caput, corpus and cauda epididymidis [4]. The head region, consisting of the initial segment and caput epididymidis, is located at the upper pole of the testis. The corpus epididymidis comprises the body of the epididymis and is attached to and follows the medial posterior side of the testis. The tail or cauda epididymidis is found near the lower pole of the testis, connecting the epididymis with the vas deferens [2, 3]. The epididymis is held to the testicular capsule, the tunica albuginea, by connective tissue and is covered by adipose tissue called the epididymal fat pad. This tubule is extremely long and can vary in length from 3 metres in man to 80 metres in horses [1].

2. Histology of the Epididymis

The mammalian epididymis comprises an epididymal tubule and an intertubular space. The epididymal tubule can be divided into two separate compartments: the epithelium and the lumen. The epididymal lumen contains spermatozoa and luminal fluid whose composition varies dramatically from the initial segment to the cauda epididymidis [5]. The diameter of the epididymal lumen is segment-specific. While the lumen is small and circular in the initial segment, it increases in diameter and is characterized by a shape that is increasingly more irregular in the subsequent segments of the epididymis [6].

The duct of the epididymis is lined by a pseudostratified epithelium consisting of several cell types: principal, basal, clear, halo, and narrow cells [4]. The epithelium rests on a basal lamina and a thin lamina propria encircled by a smooth muscle layer. Outside the muscle layer, loose connective tissue is molded about the duct and constitutes the interstitium of the epididymis [2, 3]. Blood vessels, lymphatic vessels, nerve fibres, and variable numbers of fibroblasts and macrophages make up the intertubular space of the epididymis [2, 3].

The segment of the head into which the efferent ducts empty is the initial segment. Principal cells in the initial segment are tall, columnar and show nuclei at different levels of the epithelium. These cells have prominent stereocilia at their apex, which extend into the lumen [4]. Ultrastructurally, the infranuclear region of principal cells is packed with rough endoplasmic reticulum [4]. Structures called sparsely granulated endoplasmic reticulum (CSER), which appear as large, dilated membranous elements are often seen in the apical region [4,7,8]. The supranuclear cytoplasm of principal cells consists of large stacks of Golgi saccules, mitochondria, multivesicular bodies, and smooth surfaced vesicles.

The principal cell is the most abundant cell type in the epididymal epithelium. In rats, principal cells constitute 80% of the total epithelial cell population in the initial segment, and this number decreases to 65% in the cauda epididymidis [4].

In contrast to the principal cells in the initial segment which are tall and columnar, these cells are relatively shorter in the caput, corpus, and cauda epididymidis. The infranuclear cytoplasm contains abundant cisternae of rough endoplasmic reticulum, numerous mitochondria, and lipid droplets. The supranuclear and apical regions consist of a well-developed Golgi apparatus, lysosomes, mitochondria, endosomes, and multivesicular bodies [9, 10]. At the luminal surface between adjacent principal cells, tight junctions are present at their apico-lateral margins. These tight junctions form the blood-epididymis barrier, which prevents the transfer of substances from the general circulation into the epididymal lumen [11, 12].

The second most abundant cell type in the epididymal epithelium is the basal cell; these cells contribute about 10-20% of all the cells [4]. Basal cells are flat, elongated and reside near the base of the epithelium where they contact the basement membrane. These cells are found throughout the epididymis and have been suggested to play a protective role, possibly by preventing electrophilic attack [13].

Clear cells are not found in the initial segment but are present in the rest of the epididymis, where they make up 5-10% of total cells [4]. These cells contain a highly vacuolated apical region and numerous dense granules, above and below their nucleus. Below the round, pale-stained nucleus, the basal region is filled with pale or moderately dense bodies. The clear cells participate in the uptake of luminal components [14] and the disposal of the contents of cytoplasmic droplets detached from spermatozoa [15].

Halo and narrow cells are the least common cells present in the epididymal epithelium, comprising only about 5% of total epithelial cells [4]. Halo cells are present at all levels of the epididymal epithelium. They can be recognized by their dark-stained nucleus surrounded by a pale-stained cytoplasm. Halo cells are observed throughout the epididymis and have been described as lymphocytes [16] or monocytes [17]. It is possible that these cells play a role in the immunological barrier of the male reproductive tract [18]. In contrast, narrow cells are found only in the initial segment and are characterized by their deep-staining cytoplasm and elongated, apical nuclei [4]. These cells may be precursors of clear cells and could be involved in the degradation of endocytosed protein [19].

3. Functions of the Epididymis

The epididymal epithelium has a number of functions. The absorption and secretion of ions, macromolecules, and proteins, as well as the synthesis and metabolism of steroids, and other substances [4], create an appropriate luminal environment for the acquisition of fertilizing ability and motility of spermatozoa [20, 21]. The epididymis is the site for maturation and storage of spermatozoa [21, 22]. The tight junctions between the principal epithelial cells, that form the blood-epididymis barrier, maintain a specialized and changing luminal microenvironment, and may protect spermatozoa from the immune system [4, 5, 11, 23].

a. Absorption

The epididymal epithelium is responsible for the absorption of large volumes of fluid and of particulate matter secreted by the seminiferous tubules [4, 24]. It has been shown that more than 90% of the fluid leaving the testis is taken up by the efferent ducts and proximal segments of the epididymis of many species including the rat [25], the bull, and the boar [26]. The use of micropuncture studies has shown that the fluid absorption that takes place between the rete testis and the caput epididymidis accounts for an eight-fold concentration of spermatozoa. By the time the spermatozoa reach the cauda epididymidis, they have been concentrated twenty-fold [27].

The ionic composition of the epididymal luminal fluid changes dramatically in the different segments of this tissue [28-30]. The absorption of water in the rat epididymis has been suggested to occur by passive diffusion [31]. The driving molecule is chloride in the caput epididymidis, while it is sodium in the cauda epididymidis [32-34]. The transport of these ions is believed to be androgen-dependent [28]. The absorption of different solutes varies along the epididymis. For example, the absorption of the organic molecule L-carnitine, which is proposed to be involved in sperm maturation, is saturable and primarily sodium-independent in the caput epididymidis, while it is purely passive in the cauda epididymidis [35].

Certain proteins disappear between the initial segment and more distal segments of the epididymis suggesting that specific proteins must be taken up by the epididymis [36, 37]. The use of non-specific tracers, such as horseradish peroxidase, has shown that these tracers are endocytosed by epithelial cells in a characteristic manner when they are injected into the lumen [38-40]. At early time intervals, the tracers appear in coated or uncoated pits, large coated vesicles and endosomes. At later time intervals, they are found in multivesicular bodies of increasing densities and eventually in lysosomes. At the electron microscope level, it has been shown that alpha-2-macroglobulin [41] and transferrin [42, 43] are taken up by receptor-mediated endocytosis in rat caput epididymal principal cells. These proteins, like the non-specific tracers, are taken up initially in coated pits and vesicles; subsequently, they are internalized and sequestered within endosomes and multivesicular bodies. At later times, alpha-2-macroglobulin is seen in lysosomes while transferrin is recycled into the epididymal lumen [42, 44].

b. Secretion

The epididymis can secrete a variety of ions, small organic molecules, and glycoproteins into its lumen [4]. The concentrations of ions found in different segments of the epididymis suggest that both potassium and phosphorus ions are the ions that are most actively secreted [28, 30]. While the mechanisms responsible for secretion are unknown, it has been proposed that parathyroid hormone may play a role in regulating the flux of these ions in the epididymis [4].

Three small organic molecules have been shown to be secreted in this tissue: carnitine, inositol, and glycerylphosphorylcholine [4, 45]. The region with the highest ability to take up and secrete carnitine is the distal caput epididymidis

[46]. Glycerylphosphorylcholine is neither taken up by the epididymal epithelium nor does it directly enter the epididymis from the testis like inositol; rather, it is synthesized primarily in the caput epididymidis from blood lipoproteins [47].

Radioautographic studies by Neutra and Leblond [48] have shown that sugars taken up by the epididymis can be synthesized into glycoproteins. Flickinger concluded that, in the mouse, it takes approximately two hours for the events involved in protein synthesis and secretion in epididymal principal cells [49]. It was found, as well, that the synthesis and intracellular transport of proteins occurred faster in the caput and corpus than in the cauda epididymidis [49]. The mechanism by which proteins are secreted in the epididymis remains controversial. The presence of large smooth-surfaced vesicles near the trans (mature) face of the Golgi, and in the supranuclear and apical regions of principal cells in the epididymis, suggests that these vesicles may be secretory granules [4]. An alternative for the secretory granules of the principal cells is the small coated pits [49, 50]. Such vesicles are found near the Golgi and in the apical region.

c. Transport and Maturation of Spermatozoa

As spermatozoa leave the testis, they are immature. It is as they traverse the unique luminal microenvironment that is present in each segment of the epididymis that spermatozoa undergo maturation by acquiring the ability to fertilize eggs and the potential for motility [51-54]. The transit time for the passage of spermatozoa through the epididymis of different mammals is about 10 days [4]. It has been shown that the passage of spermatozoa through some part of the caput epididymidis is essential for the development of fertilizing potential, though there is variation between different species [55].

The acquisition of fertilizing potential is thought to be a gradual process. Spermatozoa may first gain the ability to fertilize eggs, and further transit through the tissue may be necessary to acquire the ability to produce complete litters of viable offspring [56-59]. The passage of spermatozoa through the epididymis may be aided by hydrostatic pressure, muscular contraction, and the activity of the cilia present in this initial segment of the excurrent duct system [60]. The pressure within the epididymis is segment-specific; the highest is in the cauda epididymidis [61]. This phenomenon is explained by the active absorption in the initial part of the tissue which contributes to the pressure decrease there [4].

As the spermatozoa travel through the epididymis, a number of morphological and biological changes take place in the sperm. There is a migration of the cytoplasmic droplet from the neck region of the flagellum to the tail until it finally disappears [4]. In addition, there is an increase in the number of disulfide bonds within the nucleus of spermatozoa as they reach the cauda epididymidis [4]. There is also an alteration in cell surface components of spermatozoa [62, 63]. These changes have been proposed to play important roles in sperm-egg recognition and binding as well as gamete fusion [63].

c. Sperm Storage

The major site of sperm storage in the excurrent duct system of mammals is the cauda epididymidis. It has been found that, in some mammalian species, 50 to 80% of the spermatozoa present in the excurrent ducts are located in the cauda epididymidis [22]. After one week without ejaculation, the stored spermatozoa in the cauda epididymidis are two- to three-fold greater than the daily sperm production rate. Frequent ejaculation does not result in a change in the sperm production rate, but does markedly decrease the number of spermatozoa in the cauda epididymidis [22]. The mechanisms responsible for the storage of spermatozoa in the epididymis remain unknown. However, the differences in the morphology and the solute concentrations in this segment of the epididymis are likely to play a role in sperm storage.

B. Aging and the Epididymis

While the literature is filled with studies focussing on the structure, function, and regulation of the epididymis during development [64, 65] and in the adult [66-69], very little information is available regarding the changes taking place in this tissue with age. The Brown Norway rat is a valuable model for the study of aging. This strain of rat has a long life span, does not exhibit many of the age-related pathologies associated with other rat strains, and it does not become obese [70]. However, there are striking changes in the testis of these animals when no disease is apparent. Aging of the testis in the Brown Norway rat is marked by a gradual decrease in the percentage of normal seminiferous tubules [71], total sperm count [70], and the ability of Leydig cells to produce testosterone [72, 73]. Most importantly, the decreases seen in spermatogenesis and steroidogenesis in the Brown Norway rat with age have also been reported in aging human men [74, 75].

The maintenance of the structure and functions of the epididymis is androgen-dependent [4,67, 68]. The two major components entering the epididymis from the testis, spermatozoa and testosterone, decrease with age in the Brown Norway rat. It was, thus, hypothesized by Serre and Robaire [76] that there must be changes in the architecture of the epididymis. The following sections on the "Quantitative Changes in the Epididymis with Age" and the "Histology of the Aging Epididymis" summarize the first, and to my knowledge, only in-depth study on the changes taking place in the aging epididymis of the Brown Norway rat. All of the data presented in these sections on pages 11 to 15 were obtained in our laboratory by Serre and Robaire [76]. The following section highlights the changes in androgen-dependent proteins in the epididymis with age. This study was conducted in our laboratory by Viger and Robaire [77]. The final section briefly outlines the changes in the immunocytochemical localization of glutathione-S-transferases during aging in the Brown Norway epididymis, as described by Mueller, Hermo and Robaire [78].

1. Quantitative Changes in the Epididymis with Age

a. Effect of Age on Epididymal Weight

Although the average weight of the epididymis decreases with age, this decrease is not statistically significantly. However, the average testis weight does decrease from 3 months to 24 months. Furthermore, at all ages, the weights of the testes and epididymides are positively correlated.

b. Effect of Age on Luminal Diameter, Epithelial Height, and Thickness of the Basement Membrane

In the 3-month-old animals, there is a progressive increase in the diameter of the lumen, moving from the proximal to the distal segment of the epididymis. As the lumen becomes wider, the height of the epithelium decreases and the thickness of the basement membrane increases. With increasing age, the luminal diameter tends to narrow. There is a marked increase in the height of the epithelium in the corpus epididymidis with age. The height of the epithelium was not found to be affected by age in the three other regions. In contrast, there is a progressive thickening of the basement membrane in each segment of the epididymis with increasing age.

c. Effect of Age on the Relative Contribution of Cells in the Epithelium

Throughout the epididymis, principal cells are the predominant cell type, followed by basal cells. In the 3-month-old rats, the relative contribution of clear cells increases in the distal segments, contributing to the relative decrease of principal cells. In the young rats, halo cells are rare. With increasing age, there is a decrease in the number of principal cells and basal cells in all segments of the epididymis, of narrow cells in the initial segment, and of clear cells in the corpus epididymidis. This decrease is accompanied by a proportional striking increase in the number of halo cells in each segment. Interestingly, in the caput and proximal cauda epididymidis, the relative number of clear cells does not change but the relative number of principal and basal cells decreases greatly.

2. Histology

a. Initial Segment

The epithelial morphology is not greatly altered with age in the initial segment. The luminal diameter is small and the epithelium is well organized at 3 months of age. Principal cells are tall and columnar with a round nucleus located at the base of the cell and a brush border of microvilli at the apex. At 24 months, occasional intra-epithelial cysts are seen. In these animals, principal cells have lost their columnar appearance and the nuclei are no longer aligned at the base of the epithelium. At 3 months, the basal cells are located at the base of the epithelium between principal cells. As early as 12 months, basal cells emit pseudopods into the thickening basement membrane. There are a few halo cells present in the 3-month-old animals which have a characteristic clear cytoplasm and are found at the base of the epithelium. The number of halo cells increases progressively from 3 to 24 months. In older rats, some halo cells are located more apically, but they are never seen beyond the tight junctions between principal cells. A different type of halo cell, referred to as the "mature" halo cell, is seen in the aged rats. It is large, located at the base of the epithelium, and filled with lysosomes whose content is highly heterogeneous.

b. Caput Epididymidis

The main effects of age in the caput epididymidis are on the number and size of clear cells and on halo cells. The principal and basal cells do not appear to change with age in this segment. The clear cells at 3 and 12 months can be divided into three distinct regions: endosomes (apical), lysosomes (mid), and lipids (basal). The nuclei are round and located in the center of the clear cells. At 18 and 24 months, the nuclei of clear cells are irregular and often found in the apical half of the cell. The upper area of these cells contains small vesicles and multi-vesicular bodies. The dense lysosome and lipid compartments have enlarged and are no longer clearly separated. The electron dense lysosomes appear to contain lipofuscin. Lysosomes and lipid droplets are often fused. When spermatozoa are present in the lumen, the clear cells appear swollen and are bulging into the lumen. When the lumen is filled with cell debris, most clear cells lose their bulging protrusions. Halo cells increase in number and size with age, and can be classified into the two types described for the initial segment.

c. Corpus Epididymidis

The major changes are seen in the principal and halo cells of the corpus epididymidis. At 3 and 12 months, principal cells have lipid droplets in the infranuclear region and a few lysosomes are located mostly in the supranuclear region. At 18 months, there is a striking increase in the size and number of lysosomes. By 24 months, principal cells show a few, giant lysosomes that are usually filled with translucent small vacuoles. The number of halo cells increases progressively with age in this segment of the epididymis. There are no age- or region-specific differences in the morphology of the classical type of halo cell. The "mature" halo cells increase with age and contain a different lysosomal content than that seen in the halo cells in the proximal segments of the epididymis. The lysosomes are filled with translucent vesicles as seen in principal cells of this region. Eosinophils are found only in rats aged 18 months and older. These cells are found in the interstitial tissue, and, sometimes, within the epithelium. There are no major changes in basal, clear, and apical cells with age in the corpus epididymidis.

d. Cauda Epididymidis

The emergence of a localized region with large vacuoles reflects the major effect of age in the proximal cauda epididymidis. In this region at 3 and 12 months, the clear cells have nuclei located at their base, the infranuclear region contains lipid droplets and the supranuclear region has a few lysosomes and endosomes. At 18 and 24 months, the clear cells are located immediately proximal to and within the vacuole region, appear larger, and are filled with dense lysosomes. Lysosome contents are either translucent vesicles or heterogeneous. The principal cells at 18 months have a normal morphology while others contain large vacuoles. Cells that enclose vacuoles have microvilli in the apical region and an irregular nucleus. Endosomes and lysosomes are frequently seen emptying their contents within the large vacuoles. Debris from spermatozoa are seen in endosomes and in a few giant vacuoles at 18 and 24 months. Serial sections of these vacuoles show that entire spermatozoa can be found in vacuoles. No major changes are seen in the epididymal epithelium of the distal cauda region with age.

3. Gene Expression

a. 5α-Reductase

The enzyme 4-ene steroid 5α-reductase catalyzes the conversion of testosterone to its more potent form, dihydrotestosterone (DHT), in many androgen-sensitive target tissues [79, 80]. In the epididymis, the 5α-reduced metabolites of testosterone, DHT and 5α-androstan-3α,17β-diol (3α-diol), are considered the primary regulators of epididymal structure and function. Two rat 5α-reductase transcripts, types 1 and 2, have been identified [81-86]. These isozymes are the products of different genes and have been named type 1 and type 2 according to the chronological order of their identification. The isozymes differ with respect to their biochemical properties, pharmacological characterization, and tissue distribution [81-93]. The mRNA for the type 1 isozyme is found predominantly expressed in peripheral tissues, whereas the mRNA for the type 2 isozyme is found primarily in male reproductive tissues [84]. The major expression of the 5αreductase type 1 mRNA, protein, and enzyme activity is found primarily in the initial segment [90-92]. In contrast, the highest 5α-reductase type 2 mRNA levels are localized to the proximal caput epididymidis [94]. While the 5α-reductase type 1 mRNA expression and enzyme activity are characterized by dramatic increases that occur during postnatal development just before the appearance of spermatozoa in the epididymis [92], 5α-reductase type 2 mRNA expression does not show any significant developmental changes in any epididymal segments [94].

Bilateral orchidectomy results in a decrease of 5α-reductase type 1 mRNA levels in all epididymal segments [90]. High dose exogenous testosterone replacement maintains mRNA levels of this protein at control levels in all regions of the epididymis except in the initial segment, where only partial maintenance is observed [90]. Unilateral orchidectomy [90] and efferent duct ligation [94] cause dramatic decreases of 5α-reductase type 1, selectively in the initial segment of the epididymis. These studies, thus, demonstrate that the primary regulator of 5α-reductase type 1 mRNA expression in the initial segment is a testicular factor [90] while the mRNA expression of this protein in the rest of the epididymis seems to be controlled by circulating androgens [90]. Unilateral efferent duct ligation reveals that 5α-reductase type 2 mRNA levels increase in the initial segment of the ligated side but remain unchanged in the rest of the tissue [94]. Taken together, these experiments demonstrate that 5α-reductase type 1 and type 2 mRNAs are differentially regulated in the rat epididymis.

Relative mRNA concentrations have been assessed in aging Brown Norway rats using Northern blot analysis and specific cDNAs for the rat 5α-reductase isozymes type 1 and type 2 [77]. It was found that in the caput-corpus epididymidis, 5α-reductase type 1 and type 2 mRNA levels decrease significantly by 43% and 33%, respectively, between 6 and 12 months and by 64% and 40%, respectively, between 6 and 30 months of age. No significant change, however, was found in the expression of the 5α-reductase mRNAs in the cauda epididymidis.

The decrease in expression of the 5α-reductases, particularly the type 1 transcript, in the aging Brown Norway rat epididymis strongly suggests that the ability of the aging tissue to produce 5α-reduced androgens is compromised [77]. The selective decrease in the caput-corpus epididymidis (including the initial segment as part of the caput epididymidis) is consistent with the segment-specific nature of the regulation that has been described for 5α-reductase type 1 and type 2 mRNA in the rat epididymis [90, 94].

b. Proteins B/C (retinoic acid binding protein) and D/E (acidic epididymal glycoprotein)

Proteins B/C and D/E are epididymis-specific proteins secreted by principal cells and dependent on androgens for their expression [95-104]. They are not detected in blood serum or testicular extracts and are still found after ligation of the efferent ducts [104]. Protein B/C is not glycosylated and does not interact with epididymal spermatozoa directly [99]. This protein is postulated to function as a retinoic acid binding protein [100]. In contrast, protein D/E is a glycoprotein that does adhere to the head of epididymal spermatozoa [100, 101]. Protein D/E , also known as acidic epididymal glycoprotein, may play a role in the fertilization process [101].

Northern blot analysis has shown that mRNA levels for protein B/C are abundant as a fraction of total RNA in the efferent duct, initial segment, and caput epididymidis. However, no observable transcripts of this protein were detected in the cauda epididymidis [102]. The mRNA levels for protein D/E are elevated in the caput and cauda epididymidis, detectable in the initial segment, and absent in the efferent ducts [102]. Immunostaining for protein B/C is only observed over tubules throughout the caput epididymidis. In this region, every tubule appears to express the protein [103]. In sharp contrast, protein D/E shows no hybridization-positive cells within the efferent ducts, initial segment, and proximal caput epididymidis. There is a weak hybridization signal near the end of the caput epididymidis and it becomes uniformly strong in the corpus and cauda epididymidis [103].

Bilateral orchidectomy results in significant decreases in protein B/C and protein D/E mRNA levels. Androgen replacement, however, completely prevents this decline. Unilateral orchidectomy and efferent duct ligation have no effect on mRNA levels encoding protein B/C and protein D/E [99]. These results suggest that these proteins are regulated by androgens at the mRNA levels. Non-androgen testicular factors do not contribute to the expression of these genes in the epididymis.

It is crucial to look at the expression of specific androgen-dependent genes in the aging Brown Norway epididymis, as these genes are the ultimate targets of androgen action. Steady-state mRNA concentrations do not show any significant changes in the expression of proteins B/C and D/E in the aging Brown Norway rat epididymis [77]. This unaltered expression of the highly androgen-stimulated epididymal proteins B/C and D/E with age in the Brown Norway rats suggests that the androgen receptors remain functional. In fact, the androgen receptor mRNA expression did not change significantly in the aging epididymis [77]. This finding suggests that despite possible deficiencies in the production of active androgen in the aging epididymis through decreased 5α-reductase expression, the potential of the tissue to respond to exogenously administered testosterone remains intact [77].

c. Clusterin (Sulfated Glycoprotein-2; SGP-2)

Clusterin was first isolated from ram rete testes, and it has been shown to promote sperm aggregation in vitro [105]. It is a glycoprotein heterodimer consisting of two 40 kD subunits joined by a unique five disulfide bond motif [106]. As spermatozoa leave the seminiferous tubules and enter the rete testis, testicular clusterin is released from the sperm and is taken up by the epithelium of the rete testis and efferent ducts. This high molecular weight form of clusterin is subsequently replaced by a lower molecular weight form that binds to the sperm membrane and is secreted by the caput epididymidis [107]. Clusterin is secreted by the principal cells in the caput epididymidis, and it binds to spermatozoa as they transit through the epididymis [107, 108].

In addition, many roles have been ascribed to clusterin. This glycoprotein is part of the fluid phase membrane attack complex (SC5b-9) and functions as a complement inhibitor which prevents complement mediated cytolysis in vitro [107]. Clusterin may have a function in lipid transport between cells [109], and in induction of cell aggregation [105]. It is expressed in high concentrations in dying cells of the prostate after androgen withdrawal [110] and T-lymphocytes [111] during a later phase of active cell death.

Clusterin mRNA levels are highest in the caput followed by the initial segment, the cauda, and the corpus epididymidis [112]. Bilateral orchidectomy and testosterone replacement studies have revealed that in the initial segment and the caput epididymidis, these treatments have no effect on clusterin mRNA concentrations. In the corpus and cauda epididymidis, bilateral orchidectomy results in 3.5 and 9.4 increases, respectively, in clusterin mRNA concentrations which are suppressed by testosterone replacement [112]. The regulation of clusterin mRNA concentrations is, therefore, segment-specific in the epididymis and testicular factors such as testosterone are involved in this regulation.

The changes seen in clusterin mRNA concentrations are in marked contrast to the changes that have been reported in clusterin protein levels [113, 114]. After bilateral orchidectomy, the level of clusterin in the caput epididymidis, as measured by radioimmunoassay, decreases slowly with time; in the corpus and cauda epididymidis, there is no change in clusterin protein level [113]. These results suggest that whereas clusterin transcription or mRNA stability is modulated by androgens in the corpus and cauda epididymidis, the translation of clusterin mRNA or the stability of the protein is regulated by factors other than androgens [114].

During aging in the Brown Norway rat, mRNA concentrations for clusterin do not change in the caput-corpus epididymidis. However, in the cauda epididymidis, there is more than a doubling of the mRNA between 12 and 18 months, followed by a drop at 24 months to values less than 50% those seen in young adult rats [77].

Since clusterin mRNA levels are androgen insensitive in the caput-corpus epididymidis [112], the finding that caput-corpus epididymal clusterin mRNA levels do not change significantly with age, despite a fall in testosterone levels, is consistent with the lack of androgenic regulation of clusterin in this region of the epididymis. The clusterin mRNA levels are repressed by testosterone in the cauda epididymidis [112]; thus, the marked increase in clusterin mRNA levels at 18 months may be a reflection of decreased serum testosterone levels [70, 73, 77]. However, serum testosterone levels are not dramatically reduced until 24 months of age [70, 73]. Therefore, it is also possible that the increase in clusterin mRNA levels at 18 months may be an early marker of cellular damage in this region of the epididymis [77]. The possibility exists that the period between 18 and 24 months is the time during which apoptotic cell death is prevalent in the cauda epididymidis [77]. As a difference exists in clusterin mRNA and protein levels in the adult epididymis [112-114] , and it is suggested that clusterin protein levels are dependent on factors other than androgens, it would be of interest to determine if clusterin mRNA and protein levels also differ with age.

4. Localization of Glutathione-S-Transferases (GSTs) With Age

Aging is associated with an overall increase in oxidative stress and thus free radical production [115-117]. The accumulation of free radicals during aging leads to loss of function in various tissues [118-120]. In order to minimize the negative effects of oxygen damage, aerobic organisms have developed a variety of antioxidant defenses [115, 121-123]. One of these is the family of glutathione-S-transferases (GSTs). The GSTs are isoenzymes that catalyze the conjugation of glutathione to a variety of electrophiles, and protect cellular constituents from electrophilic and oxidative attack [124-128].

GSTs can be grouped into five classes based on the degree of amino acid homology of their subunits [129]. The alpha gene family consists of the Ya, Yc, and Yk subunits, the mu gene family is made up of Yb_1, Yb_2 , Yb_3 , Yb_4, Yn, and Yo subunits, while the pi gene family is made up of the Yf subunit. Immunocytochemical localization studies of GST subunits Yf [130], Yo [131], Ya, Yc, Yb_1 and Yb_2[132], have confirmed that these proteins have a distinctive pattern of expression in the rat epididymis. In addition, it has been determined that GSTs are differentially expressed in the compartments of the testis and regions of the epididymis of the rat [133, 134].

With age, principal cells of all epididymal regions, except the proximal cauda region, show no change in GST expression at all ages examined [78]. At 24 months, the principal cells that become greatly enlarged and vacuolated in the cauda epididymidis, show a different GST immunostaining pattern than the principal cells that maintain a normal appearance [78]. The vacuolated principal cells are unreactive for Yo, Yb_1, Yb_2, and Yc, while adjacent normal-appearing principal cells maintain the same intensity of expression as seen in 3-month controls. In contrast, vacuolated principal cells are reactive for the Ya subunit, while adjacent normal principal cells are unreactive [78]. The Ya subunit, being a member of the alpha family of GSTs (Ya, Yc), displays non-selenium dependent glutathione peroxidase activity [135, 136]; Ya plays a major role in protecting tissues from endogenous organic hydroperoxides produced during oxidative stress.

These data indicate that selective changes occur in the expression of GSTs at 24 months in principal cells having both a normal and a vacuolated appearance in the proximal cauda epididymidis [78]. The underlying mechanism responsible for these changes with age is unresolved, but it is suggested that they lose the ability to handle oxidative stress.

C. The Blood-Epididymis Barrier

1. Structure of the Blood-Epididymis Barrier

The epididymal junctional complex between adjacent principal cells is composed of apically located gap, adherens, and tight junctions. Tight junctions present between adjacent principal epithelial cells at their luminal surface form the blood-epididymis barrier [137]. The epididymal tight junctions are highly developed among other epithelial cell contacts [138]. They form a continuous zonule around the cell, sealing the spaces between the epithelial cells, so that the luminal space and the intercellular spaces become separate physiological compartments [139].

Changes in the structure of the junctional complex have been observed at the electron microscope when comparing the initial segment to the other segments of the epididymis. In the initial segment, the tight junction spans a considerable length of the apical plasma membrane and has few desmosomes [137]. In the other regions of the epididymis, the span of merging plasma membranes is considerably reduced but has numerous desmosomes in the apical region [137].

The tight junctions begin to form at the time of differentiation of the Wolffian duct [140]. The development of the blood-epididymis barrier in immature rats has been examined with the electron microscope using lanthanum nitrate as an electron opaque tracer [139]. While lanthanum nitrate can penetrate the adherens and gap junctions of the junctional complex, the tight junctions block the passage of this tracer. It has been shown that the postnatal development of the blood-epididymis barrier is gradual and that its formation is virtually complete by day 21 in Sprague-Dawley rats [139].

2. Functions of the Blood-Epididymis Barrier

The composition of epididymal luminal fluid is distinctly different from that of blood plasma. The blood-epididymis barrier keeps the two fluids in separate compartments [24]. The blood-epididymis barrier also maintains a specialized luminal microenvironment for the maturing spermatozoa by restricting the passage of a number of ions, solutes, and macromolecules across its epithelium [12, 24]. For instance, molecules such as inositol and carnitine can be concentrated ten- to a hundred-fold in the lumen of the caput epididymidis, while others such as inulin, L-glucose, and bovine serum albumin are effectively excluded [4, 5]. The blood-epididymis barrier carefully controls the microenvironment so that the spermatozoa are bathed in an appropriate fluid milieu at each stage of maturation as they travel through each segment of the epididymis [5].

This barrier also serves as an extension of the blood-testis barrier. Spermatozoa are immunogenic; they contain proteins on their surfaces which would be recognized as foreign if they were to enter the epididymal epithelium [4] . This barrier thus protects the spermatozoa from autoimmune attack by blocking the passage of spermatozoa into the epithelium of the epididymis.

3. Junctional Proteins

The junctional complex includes several well-defined structures including gap junctions, desmosomes, adherens junctions, and tight junctions. Gap junctions, made up of proteins termed connexins, mediate communication between cells by allowing small molecules to pass from cytoplasm to cytoplasm of neighbouring cells, thereby metabolically and electrically coupling them together [141]. Desmosomes are the "button-like" points of intercellular contact that provide anchoring points for intermediate filaments [142]. Adherens junctions form a continuous belt and hold neighbouring cells together through a family of calcium-dependent cell-cell adhesion molecules, called cadherins, that are linked to actin and myosin filaments [143]. The cadherins have also been implicated in the formation and maintenance of tight junctions [143-148].

The tight junction is the most apical component of the junctional complex, and its molecular structure has been the subject of recent investigations. Several proteins have been implicated in the makeup of the tight junction including occludin [149, 151], ZO-1 [150, 151], ZO-2 [152], cingulin [153], 7H6, [154] and several phosphoproteins that are still being characterized. Occludin was the only integral membrane protein that was localized to tight junctions until very recently. Novel integral membrane proteins, claudin-1 and -2, have been shown to localize to the tight junction as well [155] but little is known about these proteins at this time. Based on the distance from the plasma membrane, tight junction peripheral membrane proteins can be subclassified into two categories [151]. The first class includes ZO-1 and ZO-2, which are localized in the immediate vicinity of plasma membranes. The second class includes cingulin and 7H6 antigen, which are localized more than 40 nanometres from the plasma membranes.

a. E-cadherin

E-cadherin is a 120 kD, calcium-dependent homotypic cell adhesion glycoprotein [144-148]. Cadherins have been implicated in the formation and maintenance of tight junctions between epithelial cells of the kidney [146], intestine [147], and liver [148]. The formation of cellular junctions involves the interactions of cell adhesion proteins followed by the addition of junctional proteins which assemble into tight and gap junctions [143-145]. E-cadherin may, therefore, be involved in the formation and maintenance of the blood-epididymis barrier and cell-cell adhesion in the epididymis.

The presence of E-cadherin has previously been reported in the rat epididymis [156]. In this tissue, E-cadherin is localized to the principal cells of the entire epididymis. At the electron microscope, E-cadherin is observed between the lateral plasma membranes of adjacent principal cells, both in the region of the junctional complex and in the deeper lying areas [137]. Steady-state mRNA levels of E-cadherin are almost four times higher in the caput and corpus epididymidis than those in the initial segment and cauda epididymidis [156].

The formation and maintenance of tight junctions in the caput epididymidis appear to be regulated by gonadal hormones [157]. The bilateral orchidectomy of 20-day-old mice results in a decrease of the tight junction network within 2-4 days. This network is subsequently reinitiated spontaneously in the days following orchidectomy [157]. It has also shown that E-cadherin mRNA concentrations in the epididymis are dependent on circulating levels of androgens [156]. After replacement with testosterone, a dose-dependent maintenance of mRNA concentration of E-cadherin is observed throughout the epididymis of orchidectomized rats [156]. After unilateral orchidectomy, it was found that 14 days after this surgery, no differences are observed in the concentrations of epididymal E-cadherin mRNA between the ipsi- and contralateral sides [156]. Together, these experiments show that E-cadherin is regulated by circulating androgens in the epididymis.

b. Occludin

An understanding of the tight junction-associated integral membrane proteins was lacking until occludin was identified. Electron microscopic studies have shown that occludin is an adhesion molecule working at the tight junction to obliterate the intercellular space [158]. This occludin function has also been directly proven by an experiment in which the tight junction barrier function was induced by transfection with occludin cDNA [159]. Occludin has a molecular mass of ~65 kD; it was first isolated from a chicken using monoclonal antibodies [149]. Mammalian homologues of occludin were soon identified, and it was found that the amino acid sequences of human, murine, and canine occludins are very closely related (90% identity) although they diverge considerably from those of chicken [160]. However, despite this interspecies diversity in the occludin sequence, the basic structure appears to have been conserved. Mammalian, as well as chicken occludins, contain four transmembrane domains, a long carboxyl-terminal cytoplasmic domain, a short amino-terminal cytoplasmic domain, two extracellular loops, and one intracellular turn [159, 160].

The function of occludin is thought to be regulated by its association with undercoat-cytoplasmic proteins, especially ZO-1. The long carboxyl-terminal cytoplasmic domain of occludin is responsible for the direct interaction of occludin with ZO-1 [161]. Association with ZO-1 may also be important for localization of occludin to tight junctions. In one study, carboxyl-terminal deletions of occludin failed to localize occludin to the tight junction [161]. However, in another study, a large carboxyl-terminal deletion of occludin was found at the tight junction but in a discontinuous pattern [162].

Phosphorylation of occludin occurs on serine and threonine residues and the degree of phosphorylation may affect the localization of occludin in the cell [158]. The less phosphorylated forms (smaller sizes) are found in the basolateral membrane, cytosol, and tight junction, whereas the more heavily phosphorylated form (larger size) is concentrated exclusively at tight junctions [163].

c. Zonula Occludens 1 (ZO-1) / Zonula Occludens 2 (ZO-2)

ZO-1 has a molecular mass of 220 kD and is a tight junction-associated peripheral membrane protein [150]. This molecule is localized in the immediate vicinity of the plasma membrane of tight junctions in epithelial and endothelial cells. However, in cells lacking tight junctions, such as fibroblasts and cardiac muscle cells, ZO-1 colocalizes with cadherins [165, 166]. ZO-2 with a molecular mass of 160 kD was identified as a ZO-1 binding protein by immunoprecipitation [152]. ZO-2 appears to be more restricted to tight junctions. Tight junctions among various cell types can differ significantly, and multiple isoforms of ZO-1 and ZO-2 may contribute to these differences [167, 168].

Both ZO-1 and ZO-2 belong to the membrane-associated guanylate kinase

(MAGUK) family of proteins [150]. Members of this family are often found at sites of cell-cell contact and may function to couple extracellular signalling pathways with the cytoskeleton [150, 169]. MAGUK family members share several conserved motifs including an SH3 domain, guanylate kinase domain, and PDZ domain [169]. The PDZ domain is named after the three proteins in which this domain was first recognized: PSD-95, Dlg, and ZO-1. The SH3 domain is likely to be important for interaction with other signalling molecules or the cytoskeleton [169]. The PDZ domains appear to interact with the carboxyl-terminal cytoplasmic tail of transmembrane proteins, and this may be the mechanism of ZO-1/occludin interactions [169].

d. Cingulin / 7H6

Cingulin and 7H6 have not been as extensively studied and are not as well characterized as occludin and ZO-1. Cingulin is a 140 kD protein located in the junctional regions of epithelial cells from a variety of epithelial tissues including the chicken intestine, kidney, liver, pancreas, retina [170], human normal and neoplastic colon [171], and rat epididymis [172]. Cingulin appears to be composed of two intertwined peptides. This protein is localized in close proximity to the vinculin-rich cytoskeleton belt associated with adherens junctions of chick embryonic kidney cells and in the tight junction of chicken intestine [170].

7H6 is a 155 kD protein within the tight junction of hepatocytes and epithelial cells. 7H6 antigen is recognized by a specific monoclonal antibody that was generated to a bile canaliculus-rich membrane fraction from liver [154]. The limited data suggest that 7H6 may function to regulate paracellular permeability [173].

D. Hypothesis of the Present Study

There are striking changes in the epididymis of the Brown Norway rat with age. The presence of a large number of halo cells, which may be monocytes or lymphocytes, suggests that the immune system is activated in the aging epididymis of these animals. A functional blood-epididymis barrier is responsible for protecting spermatozoa from autoimmune attack. Thus, in the present study we look at the structural integrity of the blood-epididymis barrier by analysing the distribution of the tight junctional proteins, occludin and ZO-1, in the adult and aging Brown Norway rat epididymis. We will assess whether there is a change in E-cadherin, the androgen-dependent, possible regulator of tight junctions, as there is a decrease in androgens in the aging Brown Norway rats. Finally, to examine the functional integrity of the blood-epididymis barrier of the Brown Norway rat with age, a lanthanum nitrate study will be performed.

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Chapter 2

SEGMENT-SPECIFIC CHANGES IN THE EXPRESSION OF JUNCTIONAL PROTEINS AND THE PERMEABILITY OF THE BLOOD-EPIDIDYMIS BARRIER WITH AGE

Introduction

The blood-epididymis barrier is formed between principal epithelial cells by tight junctions found in the apical region [1]. This physical barrier creates the unique luminal microenvironment in each segment of the epididymis that is necessary for proper sperm maturation [2]. In addition, the blood-epididymis barrier may protect spermatozoa from autoimmune attack by physically blocking the passage of immunogenic spermatozoa from the lumen through the epididymal epithelium [3].

Several studies have focussed on the identification of tight junctional proteins and how they interact to maintain the structure of tight junctions in various epithelia [4, 5]. However, few have focussed on the tight junctions of the blood-epididymis barrier. Occludin, a ~65 kD integral membrane protein, has been identified as being responsible for sealing the adjacent plasma membranes of tight junctions together [6]. At the molecular level, ZO-1, a peripheral membrane protein, has been found to be directly associated with the carboxyl terminus of occludin in the tight junction [7]. Together, occludin and ZO-1 have been postulated to maintain the structural integrity of the tight junction [4, 5, 7]. While there are several other tight junction-associated proteins such as ZO-2 [8], cingulin [9], and 7H6 [10], ZO-1 binding to occludin has been shown to be important in targeting occludin to the tight junction and anchoring occludin at the extracellular seal [7, 11].

The cadherins, a family of calcium-dependent cell adhesion glycoproteins joining together adjacent cells within tissues [12-15], have been implicated in the formation and maintenance of tight junctions between epithelial cells of many tissues including the kidney [16] , intestine [17] and liver [18]. In the adult Sprague-Dawley rat, Cyr et al (1993) have shown that E-cadherin is present in the cytoplasm of principal cells of the epididymal epithelium, and that its expression is dependent on serum androgen levels [19]. Studies by Suzuki and Nagano [20] have also suggested that the formation and maintenance of tight junctions in the caput epididymidis relies on gonadal hormones.

The epididymal tight junctions are among the most highly developed contacts between mammalian epithelial cells [21]. Tight junctions, adherens junctions, and gap junctions are present in the epididymal junctional complex [22]. However, studies using electron-opaque tracers, including lanthanum nitrate, have shown that, in the adult rat, the tight junction is the only one that does not allow the passage of the tracer [1]. Thus, in situations where the function of the blood-epididymis barrier is altered, such as in immature rats before the development of the barrier is complete [23], lanthanum nitrate easily permeates the tight junction and enters the lumen.

To date, the blood-epididymis barrier has been investigated during development [23] and in the adult rat [1], but the effects of aging on this barrier are unknown. In addition, the effects of age on tight junction structure or function have not been investigated previously in any tissues, except the testis

[24]. In the aging Brown Norway rat testis, the typical Sertoli-Sertoli junctions were rarely seen and were replaced by focal contact points [24]. Lanthanum nitrate was able to penetrate both the basal and adluminal compartments of the seminiferous epithelium of the aging testis [24]. The Brown Norway rat has become a valuable model for the study of aging. It does not exhibit many of the age-related pathologies seen in other rat strains, nor does it become obese during its long life span [25]. The male reproductive system of the Brown Norway rat undergoes dramatic changes with age while the rat remains completely healthy. There is a decrease in spermatogenesis and steroidogenesis [25-27] accompanied by marked histological alterations [28]. There is also an apparent decrease during aging in the quality of sperm with respect to progeny outcome [29]. Importantly, these changes are similar to those observed in the human male reproductive system with age [30, 31].

There are striking transformations in the epididymis of the Brown Norway rat with age [32]. The presence of a large number of halo cells, which may be lymphocytes [33] or monocytes [34], suggests that the immune system is activated in the aging epididymis of these animals. In the present study, we determined if there were changes in the structural integrity of the blood-epididymis barrier with age by analysing the distribution of the tight junctional proteins occludin and ZO-1 in the adult and aging Brown Norway rat epididymis. As there is a decrease in androgens with age in the Brown Norway rat, the localization of E-cadherin, a possible regulator of tight junctions, was also compared in the epididymis during aging. Finally, to examine the functional integrity of the blood-epididymis barrier in the aging Brown Norway rat, resistance to penetration by lanthanum nitrate was assessed. We report segment-specific changes in the localization and expression of occludin, ZO-1, and E-cadherin in the aging Brown Norway rat epididymis consistent with the changes seen in lanthanum nitrate penetration.

Materials and Methods

Animals

Brown Norway rats aged 3, 18, and 24 months were purchased from the National Institute on Aging, Bethesda, MD and supplied by Charles River Breeding Laboratories (Wilmington, MA). Animals were housed at the McGill University McIntyre Animal Centre in a temperature (22°C) and light (14L:10D) controlled room with rat food and water available *ad libitum*. We followed the policies set forth by the facility Animal Care Committee at McGill University as well as those described in the "Guide to the Care and Use of Experimental Animals" prepared by the Canadian Council on Animal Care.

Immunocytochemistry

Brown Norway rats aged 3 (n=6), 18 (n=6), and 24 (n=6) months were perfused with Bouin's solution for 10 minutes. While the group of 24-month-old animals contained regressed and non-regressed testes, the immunostaining patterns were consistent in all of these aged animals with the three antibodies used. Retrograde perfusions through the abdominal aorta were used to fix the initial segment and caput epididymidis and prograde perfusions were used to fix the corpus and cauda epididymidis. The epididymides were removed, cut along their longitudinal axis and immersed in Bouin's solution for 24 hours. The tissues were then dehydrated and embedded in paraffin. Tissues were cut into 5 µm-thick sections and mounted on glass slides. Subsequently, tissues were rehydrated through graded concentrations of ethanol, including 70% alcohol with 1% hydrogen peroxide for 10 minutes to remove endogenous peroxidase activity, and 70% alcohol with 1% lithium carbonate for 5 minutes to remove residual picric acid. The sections were then incubated in 300 mM glycine for 5 minutes to block free aldehydes and washed in 1M phosphate buffered saline (PBS) at pH 7.4.

Three antibodies were used in this study: a rabbit polyclonal anti-occludin antibody from Zymed Laboratories, Inc. (San Francisco, CA), a rat monoclonal anti-ZO-1 antibody from Chemicon Laboratories Inc. (Temecula, CA), and a mouse monoclonal anti-E-cadherin antibody from Transduction Laboratories (Lexington, Kentucky). Each antibody was used in conjunction with the appropriate Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Incubations with each of the primary antibodies were done for 18 hours at 4°C. The working dilutions were 1:540 for occludin and 1:100 for ZO-1 and E-cadherin. Normal appropriate animal sera and omission of the primary antibody served as negative controls. Subsequently, the sections were incubated with a peroxidase DAB substrate kit (Vector Laboratories, Burlingame, CA). Epididymal sections were then washed in 1M PBS, counterstained for 30 seconds with 0.1% methylene blue, dehydrated in solutions containing graded concentrations of ethanol, immersed in xylene, and mounted in Permount. Sections were then examined using the light microscope (Leiz Wetzlar, Laborlux D, Montreal, Canada) for immunoperoxidase activity.

To assess the intensity of staining in occludin, ZO-1, and E-cadherin at 3, 18, and 24 months (Table 1), four animals were studied for each age group. Four different slides per animal were examined for each antibody. Slides were coded and examined without knowledge of the identity of the sample in order to eliminate any potential observer bias. At least 150 tubules were examined for each segment in every age group.

Lanthanum Nitrate Tracer Study

Brown Norway rats aged 3 (n= 6) and 24 months (n=6) were anaesthetized with an i.m. injection of a cocktail of ketamine hydrochloride (Ketalean®, MTC Pharmaceuticals, Cambridge, ON), xylazine (Rompun®, Bayer Inc., Etobicoke, ON), acepromazine maleate (Atravet®, Ayerst Laboratories, Montreal, QC), and 0.9% sodium chloride (Baxter Corporation, Toronto, ON). This cocktail is often used because it decreases the risk of respiratory depression which can occur with sodium pentobarbital, especially in aged animals. While the group of 24-month-old animals contained rats having both regressed and non-regressed testes, the results of the lanthanum nitrate study were consistent in all of these aged animals. Retrograde and prograde perfusions were used for the reasons cited above. The fixative consisted of 5% glutaraldehyde buffered with 0.16 M collidine buffer (pH 7.4) containing a final concentration of 2% lanthanum nitrate (Marivac, Nova Scotia, Canada) and 2.5% polyvinylpyrollidone (PVP), at a final pH of 7.3. The solution was filtered through a Millipore filter using a 5 micron membrane (MicronSep Membrane Filters, Westborough, MA) prior to use. After perfusion, the epididymides were removed, cut into 1-mm^3 pieces and left either in the same fixative or in the same fixative without lanthanum for 2 hours at 4°C. The tissues were washed quickly 3 times in a 0.16M collidine buffer with or without 2% lanthanum nitrate and 2% sucrose, pH 7.4, and were subsequently washed 3 times in 0.1M sodium cacodylate buffer with or without 2% lanthanum nitrate and 2% sucrose, pH 7.4 (this last wash was done to remove any toxic collidine which remained). The reason lanthanum was omitted from the immersion solutions in some cases was to confirm that the observed path of tracer was due exclusively to extravasated lanthanum. Tissues were postfixed in 1% osmium tetroxide containing the same cacodylate buffer mixture used for washing, stained en bloc with uranyl acetate, and embedded in epoxy resin. Thin (75 nm) sections were cut using an ultramicrotome; some sections were unstained and others were routinely stained with uranyl acetate and lead citrate, and examined with the Philips 410 electron microscope.

Results

Immunocytochemistry

Initial Segment

Occludin (Fig. 1A), ZO-1 (Fig. 1B), and E-cadherin (Fig. 1C) immunostaining was observed at the apico-lateral junction between adjacent principal cells in the entire initial segment of the 3-month-old animals. While ZO-1 (Fig. 1B) and E-cadherin (Fig. 1C) were present as a punctate reaction at the apico-lateral junction of adjacent principal cells, occludin was localized to the same region but was more extensively distributed, spanning part of the apico-lateral membrane between the principal cells (Fig. 1A). Occludin and ZO-1 immunostaining was not seen in any other cell types in this segment, while narrow cells and apical cells were immunoreactive to E-cadherin (not shown).

With age, the immunostaining pattern of occludin and ZO-1 differed from that of E-cadherin. At 18 months, occludin immunostaining became more diffuse around the apico-lateral junction and decreased in intensity (Table 1). The punctate reaction of ZO-1 was no longer seen at every junction of principal cells at 18 months (Table 1). By 24 months, occludin immunostaining was dramatically reduced with a faint reaction at the apex between adjacent principal cells (Fig. 1D); ZO-1 punctate reactivity between principal cells was virtually absent (Fig. 1E and Table 1). While occludin and ZO-1 reactivity decreased with age in the entire initial segment of the Brown Norway rat, E-cadherin immunostaining increased in intensity (Table 1) and was observed along the entire lateral membrane between principal cells at 24 months (Fig. 1F). No other cell types showed occludin or ZO-1 reactivity while only apical cells continued to show E-cadherin reactivity at 24 months (not shown). For all three markers, the initial segment exhibited a unique staining pattern; cytoplasmic staining was never seen in this segment at any age for any of the junctional proteins examined.

Caput Epididymidis

in contrast to the initial segment at 3 months, where occludin, ZO-1, and E-cadherin exhibited a similar staining pattern, the reactivity to these junctional proteins differed in the caput epididymidis for all three markers. At 3 months, the occludin immunostaining was dramatic in the caput epididymidis (Fig. 2A). Occludin immunostaining intensity in the caput epididymidis was the highest of all the epididymal segments (Table 1). There was a surprising reactivity with a grainy appearance in the entire cytoplasm of the principal cells, and it appeared stronger at the base of the epithelium (Fig. 2A). An intense, concentrated staining was observed at the apical margin of the principal cells (Fig. 2A). ZO-1 was not abundantly expressed in the caput epididymidis at 3 months (Fig. 2B and Table 1). There was a uniform staining of the cytoplasm of the principal cells (Fig. 2B). In contrast to the ZO-1 punctate reaction seen at the apico-lateral junction between all principal cells in the initial segment at 3 months (Fig. 1B), ZO-1 punctate staining was only observed at the apico-lateral junction of few principal cells (Fig. 2B and Table 1). E-cadherin immunostaining at 3 months in the caput epididymidis consisted of both cytoplasmic staining of principal cells and a punctate reactivity at their apico-lateral margins (Fig. 2C); the punctate reactivity was greater in the proximal caput epididymidis than in the distal region. There was no ZO-1 or occludin immunoreactivity in any other cell types in the caput epididymidis at 3 months, however, the top of clear cells were immunoreactive to E-cadherin (not shown).

As in the initial segment, in the caput epididymidis there was a decreased reactivity of occludin and ZO-1 at 18 months along with an increase in E-cadherin immunostaining (Table 1). By 24 months, occludin immunostaining had greatly diminished in the cytoplasm of principal cells (Fig. 2D and Table 1). The occludin immunostaining at the apex of the principal cells appeared more intense than in the rest of the cytoplasm but was diffuse in appearance (Fig. 2D). At 24 months, ZO-1 reactivity was absent; a rare punctate reaction at the apico-lateral junction of principal cells was observed only a few times throughout the entire segment (Fig. 2E and Table 1). Again, E-cadherin exhibited a change in its staining pattern. The cytoplasmic staining of principal cells was absent at 24 months while immunoreactivity was observed as a punctate stain at the margin of principal cells at their apex (Fig. 2F). The E-cadherin reactivity spread to the entire lateral membrane of principal cells in addition to the punctate reactivity (Fig. 2F). The staining of the lateral membranes was most intense in the proximal caput epididymidis. This E-cadherin immunostaining pattern at 24 months is similar to that seen for this marker in the initial segment of the aged animals (Fig. 1F). With age, there was a gradual decrease in the E-cadherin immunoreactivity of clear cells at their apex such that, at 24 months, the majority of clear cells were unreactive (not shown); a few clear cells still showed intense reactivity. All other cell types did not show any immunostaining for occludin, ZO-1 or E-cadherin.

Corpus Epididymidis

The corpus epididymidis showed the most dramatic alterations in immunostaining for occludin, ZO-1, and E-cadherin with age. The staining patterns were identical in the proximal and distal corpus epididymidis. However, the intensity of staining was always greater in the distal corpus epididymidis than in the proximal region for all the markers studied. The occludin reactivity is the same as the staining pattern seen in the caput epididymidis at 3 months but with a much lower intensity (Fig. 3A and 2A). A grainy cytoplasmic staining and an intense band at the apex of the principal cells was seen in the corpus epididymidis (Fig. 3A). The most intense cytoplasmic staining for ZO-1 and E-cadherin was observed in the corpus epididymidis at 3 months (Fig. 3B and 3C). However, ZO-1 also showed a punctate reactivity at the apico-lateral margin of some principal cells (Fig. 3B), which was not seen for E-cadherin at 3 months (Fig. 3C). As in the caput epididymidis, immunostaining for occludin and ZO-1 was not seen in any other cell types; the clear cells, however, exhibited an intense E-cadherin immunoreactivity along the apex of these cells (not shown).

The most striking change seen in the corpus epididymidis was that occludin, ZO-1, and E-cadherin reactivity all decreased progressively with age (Table 1). This was the only segment of the epididymis where E-cadherin immunostaining was absent at 24 months (Fig. 3F); the intense cytoplasmic staining seen at 3 months had disappeared. It should be noted though that an occasional punctate reactivity at the apico-lateral junction of principal cells was observed. In addition, the majority of clear cells no longer exhibited E-cadherin immunoreactivity at 24 months (not shown).

Cauda Epididymidis

The cauda epididymidis showed yet another staining pattern for occludin, ZO-1, and E-cadherin. At 3 months, occludin immunoreactivity in the cauda epididymidis (Fig. 4A) exhibited a similar staining pattern as that seen in the caput (Fig. 2A) and corpus (Fig. 3A) epididymidis. The grainy cytoplasmic staining was observed and, while the apical band at the top of the principal cells was more intense than the rest of the cytoplasm (Fig. 4A), it was not as distinctive as in the other segments. A cytoplasmic reactivity to ZO-1 was seen in the cauda epididymidis at 3 months (Fig. 4B). The ZO-1 immunostaining seen in the cytoplasm (Fig. 4B) was much more intense than that seen for E-cadherin (Fig. 4C and Table 1). E-cadherin immunostaining was the only one that differed in the proximal and distal cauda epididymidis at 3 months. In the proximal cauda epididymidis, E-cadherin reactivity was only cytoplasmic (not shown). In contrast to the staining seen for E-cadherin in the other segments of the epididymis at 3 months, E-cadherin immunostaining was observed at the basolateral membrane between adjacent principal cells in the distal cauda epididymidis (Fig. 4C). An immunoreactivity to occludin or ZO-1 was not seen for any other cell types in the cauda epididymidis. However, as in the caput, corpus, and cauda epididymidis, clear cells continued to show E-cadherin immunostaining at their apical border, most intensely in the proximal cauda epididymidis (not shown).

With age, each junctional protein exhibited a different change in its immunostaining pattern. By 24 months, the occludin immunoreactivity progressively increased in intensity (Fig. 4D and Table 1). However, ZO-1 immunostaining decreased to become absent by 24 months (Fig. 4E). This is the only segment where occludin and ZO-1 did not change in a coordinate manner. Finally, E-cadherin immunoreactivity was now seen at the basolateral membrane of principal cells of the entire cauda epididymidis but had spread to stain the entire lateral membrane between principal cells (Fig. 4F and Table 1). At 24 months, the clear cell E-cadherin immunoreactivity was absent (not shown).

Lanthanum Nitrate Tracer Study

As reported in previous studies [1, 23], lanthanum nitrate entered the capillaries and subsequently crossed into the epididymal epithelium where it was seen between adjacent principal cells. In the initial segment, the electron-opaque tracer was consistently stopped at the tight junction at 3 (Fig. 5A) and 24 months (Fig. 5B). While the tight junction obstructed the passage of lanthanum nitrate into the lumen in the caput epididymidis at 3 months (Fig. 5C), the tracer was occasionally seen entering the first fusion points of the tight junction. At 24 months, lanthanum nitrate often entered the tight junction leaflets of the caput epididymidis and, in addition, the tracer could be seen, at times, in the junction as well as within the lumen as dark granules (Fig. 5D).

While lanthanum nitrate did not penetrate the tight junction in the corpus epididymidis at 3 months (Fig. 6A), there was a striking amount of tracer that was able to cross the blood-epididymis barrier and enter the lumen in this segment at 24 months; this permeability was observed consistently (Fig. 6B). In contrast, lanthanum nitrate was blocked at the tight junction of both 3- (Fig. 6C) and 24-month-old (Fig. 6D) animals in the cauda epididymidis, in a manner similar to what was seen in the initial segment at both ages (Fig. 5A and 5B).

Discussion

The results from the present study show that there are segment-specific changes in the expression of the junctional proteins occludin, ZO-1, and E-cadherin in the adult Brown Norway rat epididymis. In addition, there are progressive alterations in the distribution and reactivity of these proteins with age (Table 1 and Fig. 7). The segment-specific penetration of lanthanum nitrate into the blood-epididymis barrier and into the lumen with age is consistent with the changes seen in the expression of the junctional proteins.

In the initial segment, occludin, ZO-1, and E-cadherin co-localize to the apico-lateral junction of adjacent principal cells. While the immunoreactivity to occludin and ZO-1 decreases with age in the initial segment, the lateral membrane between adjacent principal cells stains intensely for E-cadherin with increasing age. The initial segment exhibits a unique staining pattern in that cytoplasmic staining is not seen in this segment as in the caput, corpus, and cauda epididymidis for any of these junctional proteins. The junctional complex in the initial segment has been previously shown to span a considerable length of the apical plasma membranes with few desmosomes [22]. In contrast, in the rest of the epididymis the span of merging plasma membranes is considerably reduced, and there are numerous desmosomes in the apical region [22]. This difference in the junctional complex may be one of the reasons for the differing distribution of occludin, ZO-1, and E-cadherin in the initial segment.

The blood-epididymis barrier maintains the ability to block the passage of lanthanum nitrate in the initial segment of the aging animals despite the decrease in occludin and ZO-1 reactivity. Interestingly, Saitou et al [35] have recently shown that tight junctions in occludin-deficient epithelial cells are able to function as a primary barrier to the diffusion of a low molecular mass tracer through the paracellular pathway. These findings indicate there may be unidentified tight junction integral membrane proteins that can form strand structures, recruit ZO-1, and function as a barrier without occludin [35]. Furuse et al [36] have recently discovered two novel integral membrane proteins, termed claudin-1 and -2, that localize at tight junctions but bear no sequence similarities to occludin. Claudin-1 or -2 can reconstitute tight junctions and recruit occludin in fibroblasts [37]. The possibility, therefore, exists that there may be other junctional proteins that enable the tight junction to block the passage of lanthanum nitrate despite the decrease in occludin and ZO-1. Alternatively, the amount of occludin and ZO-1 remaining in the aged animals may be sufficient to inhibit the passage of the tracer, especially since E-cadherin is still present and shows a greater intensity in the initial segment of the aged animals.

In contrast to the initial segment, occludin, ZO-1, and E-cadherin all exhibit some cytoplasmic staining in the caput, corpus, and cauda epididymidis of the adult Brown Norway rats. E-cadherin cytoplasmic reactivity has been previously seen in the epididymis of adult male Sprague-Dawley rats [19]. It has been suggested that the cytoplasmic localization of E-cadherin may reflect the synthesis and processing of this protein in the endoplasmic reticulum and Golgi apparatus, and its transport via vesicles within the cytoplasm toward the lateral membranes [19]. Recently, however, a study on the mechanisms of epithelial cell-cell adhesion reported that insertion of preassembled E-cadherin membrane-insertion structures from the cytoplasm into the membrane at cell-cell contacts was never seen [38]. It was suggested that E-cadherin originates by de novo aggregation at sites of cell-cell contact [38]. Cadherins are known to associate with the actin-based cytoskeleton via the catenin family [39, 40], and occludin has also been shown to bind to the cytoskeleton via ZO-1 [41]. It is likely, therefore, that the cytoplasmic staining we are seeing for E-cadherin and ZO-1 is a result of the binding of these proteins to the cytoskeleton in the cytoplasm.

The most dramatic age-dependent changes seen in the present study occur in the corpus epididymidis. The loss of occludin, ZO-1, and E-cadherin with age is most pronounced in this segment of the epididymis and is reflected by the loss of the functional integrity of the blood-epididymis barrier. Thus, it appears that the major decrease in occludin, ZO-1, and E-cadherin leads to the change in permeability of the blood-epididymis barrier of the Brown Norway rat with age. Interestingly, a study by Kimura et al [42], which looked at the expression of occludin, ZO-1, and E-cadherin in cancers of the human digestive tract, suggested that expression of occludin and ZO-1 may not be enough to form a tight junction. The authors postulated that perhaps both intercellular adhesion, mediated by E-cadherin, and expression of occludin and ZO-1 may be required for normal tight junction formation [42]. It appears that maintenance of tight junctions is also dependent on the presence of E-cadherin, occludin, and ZO-1 in the epididymis. Cyr et al [19] have shown that bilateral orchidectomy followed by low dose testosterone replacement is able to maintain mRNA concentrations of E-cadherin in all regions of the epididymis except in the corpus epididymidis region of adult Sprague-Dawley rats. As androgen levels are decreased with age in Brown Norway rats [25,26], our data support the possibility that the corpus epididymidis requires more androgen than other segments of the epididymis to maintain E-cadherin expression.

The most striking changes that have been reported in aging Brown Norway rats seem to occur in the corpus epididymidis, especially in the distal region. There is a large increase in the size and number of lysosomes with age in this segment. By 24 months, principal cells show a few giant lysosomes that are usually filled with translucent small vacuoles [32] , suggesting that there is extensive degradation and breakdown of substances in this segment. The activation of the immune system is most pronounced in the aging corpus epididymidis [32]. In addition to an increase in halo cells, Serre et al [32] have seen eosinophils in the interstitial tissue and within the epithelium of the corpus epididymidis of rats aged 18 months and older. Finally, the expression of certain subunits of the glutathione-S-transferases, involved in protecting cellular constituents from electrophilic and oxidative attack, were found to change their immunoreactivity patterns only in the distal regions of the epididymis [43]. Together these data point to a loss in the ability to handle oxidative stress in the corpus epididymidis of the aging Brown Norway rats.

The segment-specific alterations in the expression of occludin, ZO-1, and E-cadherin and the changes in the penetration of lanthanum nitrate into the blood-epididymis barrier with age indicate that there are both structural and functional changes taking place. The fact that the blood-epididymis barrier is responsible for creating the unique luminal microenvironment present in each segment of the epididymis [2] raises questions as to the ability of the barrier to maintain this function with age. The penetration of lanthanum nitrate into the lumen of the aged animals in the caput and corpus epididymidis suggests that there is a greater permeability of the blood-epididymis barrier in these segments with age.

The regulation of junctional proteins is currently under extensive investigation. Phosphorylation has been suggested to be a possible mechanism by which occludin and ZO-1 localization and function are regulated [44-46]. Tyrosine phosphorylation may also cause an increase in tight junction permeability [45] . The Rac subfamily of small G proteins has been shown to regulate the cadherin-based cell-cell adhesion but not the formation of tight junctions [47]; the Rho subfamily has been shown to be necessary for both functions [47, 48]. It is, therefore tempting to speculate that the changes with age in the expression of E-cadherin, occludin, and ZO-1 in the epididymis are due, in part, to a change in the phosphorylation of these proteins.

In summary, we have demonstrated that there are segment-specific changes in the localization and distribution of occludin, ZO-1, and E-cadherin in the aging Brown Norway epididymis, and that the permeability of the blood-epididymis barrier is also affected in a region-specific manner with age. The permeability of the blood-epididymis barrier increases with age in the caput and corpus epididymidis. The present study is one of the first to provide information about the effects of age on the structure and function of tight junctions in any tissue.

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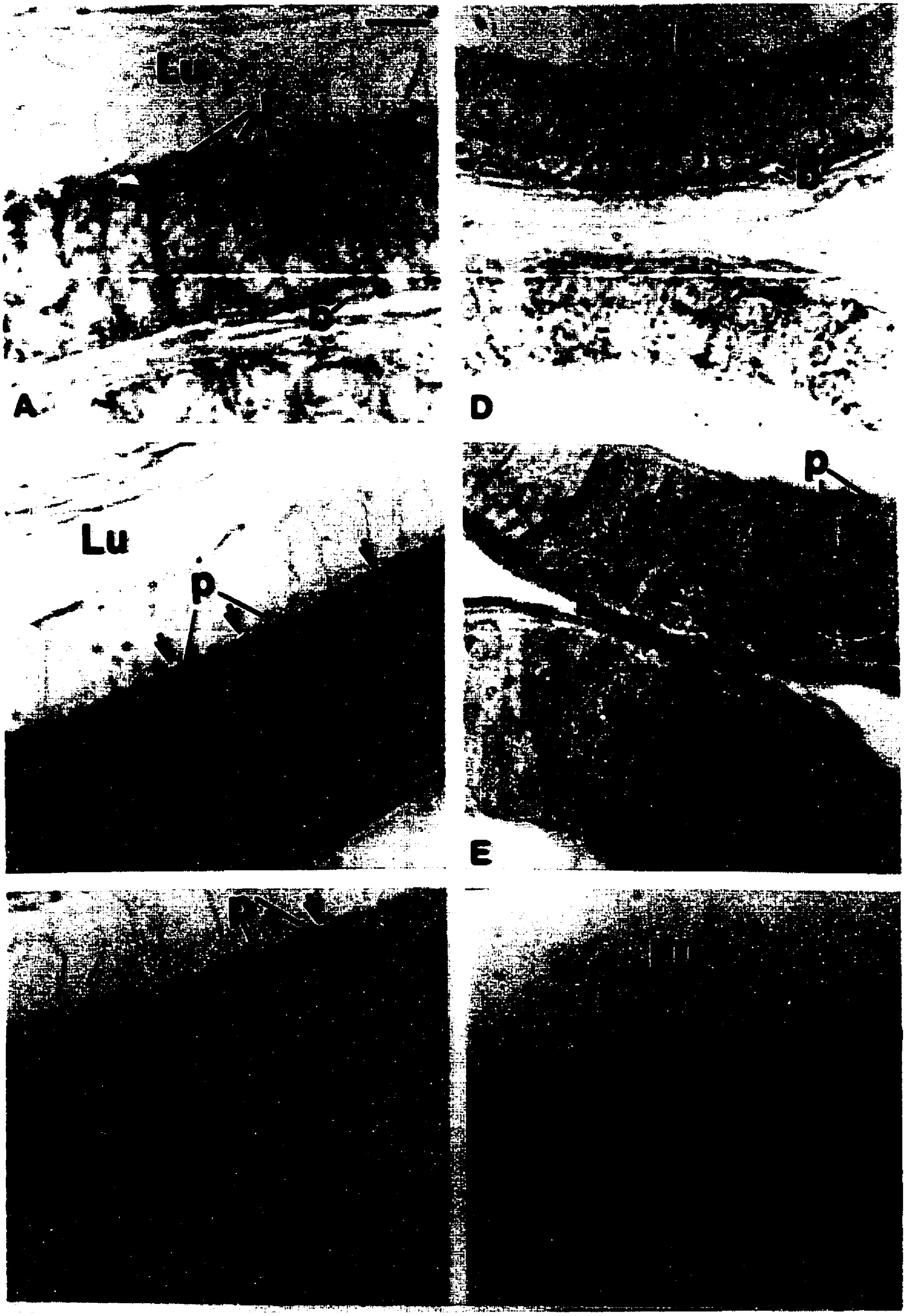
Figure 1. Light micrographs showing the effects of age on the immunostaining pattern of occludin **(A, D)**, ZO-1 **(B, E)**, and E-cadherin **(C, F)** in the initial segment of the Brown Norway rat. **A, B, C)** 3 months; **D, E, F)** 24 months. P, principal cell; b, basal cell; Lu, lumen, IT, interstitium. The arrows indicate the punctate immunostaining seen in the initial segment. Note that E-cadherin reactivity is observed along the entire lateral membrane of principal cells at 24 months **(F)**. Scale bar **A-F** = 3 μm.


Lu
b
A
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D
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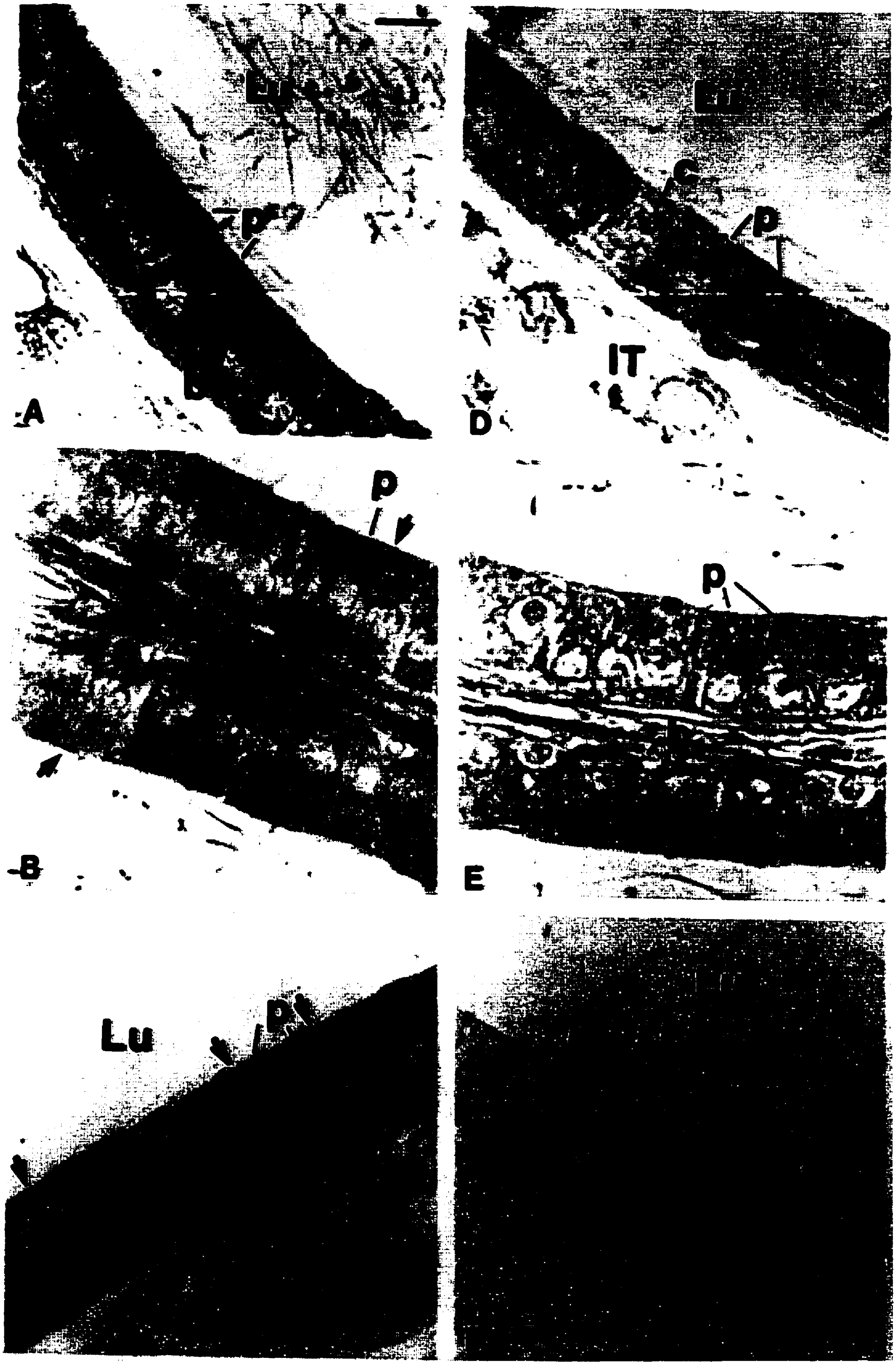
p
p
E
p
Lu

Figure. 2. Light micrographs showing the effects of age on the immunostaining pattern of occludin **(A, D)**, ZO-1 **(B, E)**, and E-cadherin **(C, F)** in the caput epididymidis of the Brown Norway rat. **A, B, C)** 3 months; **D, E, F)** 24 months. P, principal cell; b, basal cell; c, clear cell; Lu, lumen; IT, interstitium. The arrows indicate the punctate reactions; at 24 months, E-cadherin staining spreads to the entire lateral membrane as shown by the double arrows **(F)**. Scale bar **A-F** = 3 μm.


Lu
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A
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Lu
P

Figure 3. Light micrographs showing the effects of age on the immunostaining pattern of occludin **(A, D)**, ZO-1 **(B, E)**, and E-cadherin **(C, F)** in the corpus epididymidis of the Brown Norway rat. **A, B, C)** 3 months; **D, E, F)** 24 months. P, principal cell; b, basal cell; c, clear cell; Lu, lumen, IT, interstitium. Arrows show the apical band of occludin staining at 3 months **(A)**, and the punctate ZO-1 reactivity seen at 3 months **(B)**. Scale bar **A-F** = 3 μm.

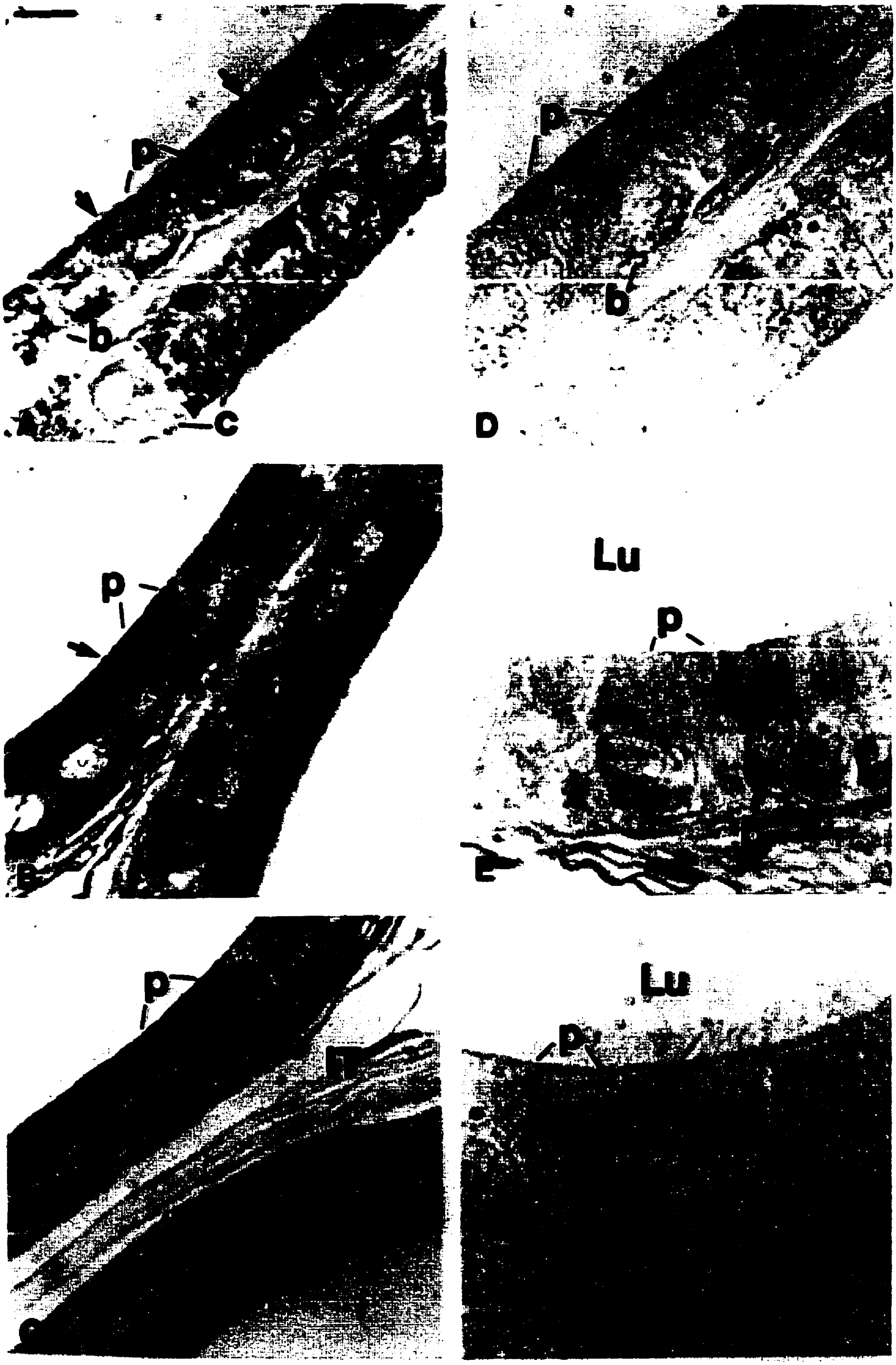
p
b
c
p
b
D
p
Lu
p
p
m
Lu
p

Figure 4. Light micrographs showing the effects of age on the immunostaining pattern of occludin **(A, D)**, ZO-1 **(B, E)**, and E-cadherin **(C, F)** in the cauda epididymidis of the Brown Norway rat. **A, B, C)** 3 months; **D, E, F)** 24 months. P, principal cell; b, basal cell; c, clear cell; Lu, lumen, IT, interstitium. The arrows in **(A)** and **(D)** show the apical band of occludin reactivity. In **(C),** the E-cadherin reactivity at the basolateral membrane of the distal cauda epididymidis is indicated by the arrows; at 24 months **(F)**, E-cadherin staining has spread to the entire lateral membrane between principal cells of the cauda epididymidis (arrows). Scale bar **A-F** = 3 μm.

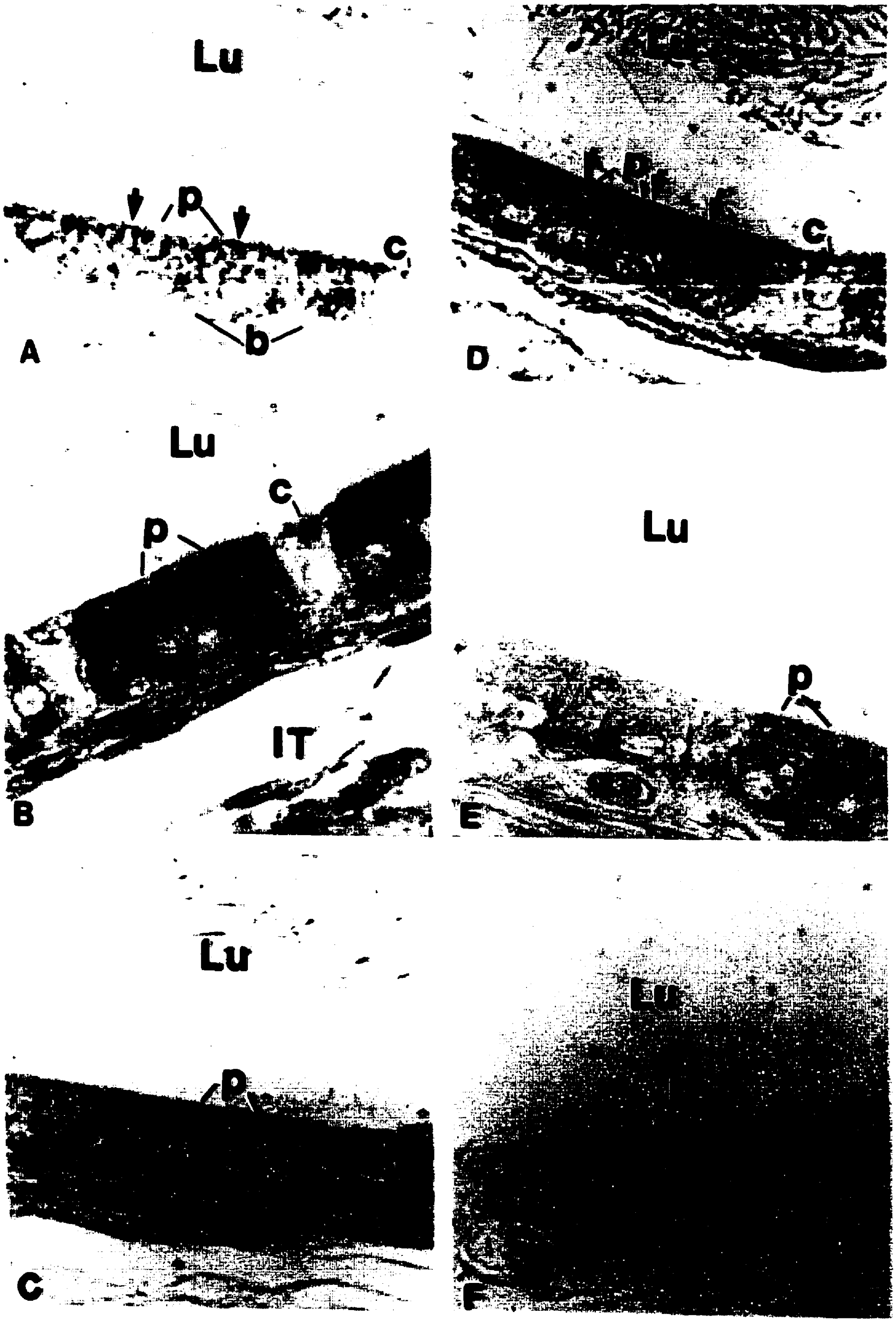
Lu
p
c
b
A
Lu
c
p
IT
B
Lu
p
C
p
c
D
Lu
p
E
Lu
F

Figure 5. Electron micrographs showing the effects of age on the permeability of the blood-epididymis barrier to lanthanum nitrate in the initial segment **(A, B)** and caput epididymidis **(C, D)** of the Brown Norway rat. **A, C)** 3 months; **B, D)** 24 months. P, principal cell, Lu, lumen. The curved arrows indicate where the passage of lanthanum nitrate is occluded **(A,B,C)**, and where it enters the tight junction to enter the lumen in the caput epididymidis at 24 months **(D)**. The small, straight arrow shows lanthanum nitrate in the lumen **(D)**. Scale bar **A-D** = 0.3 μm.

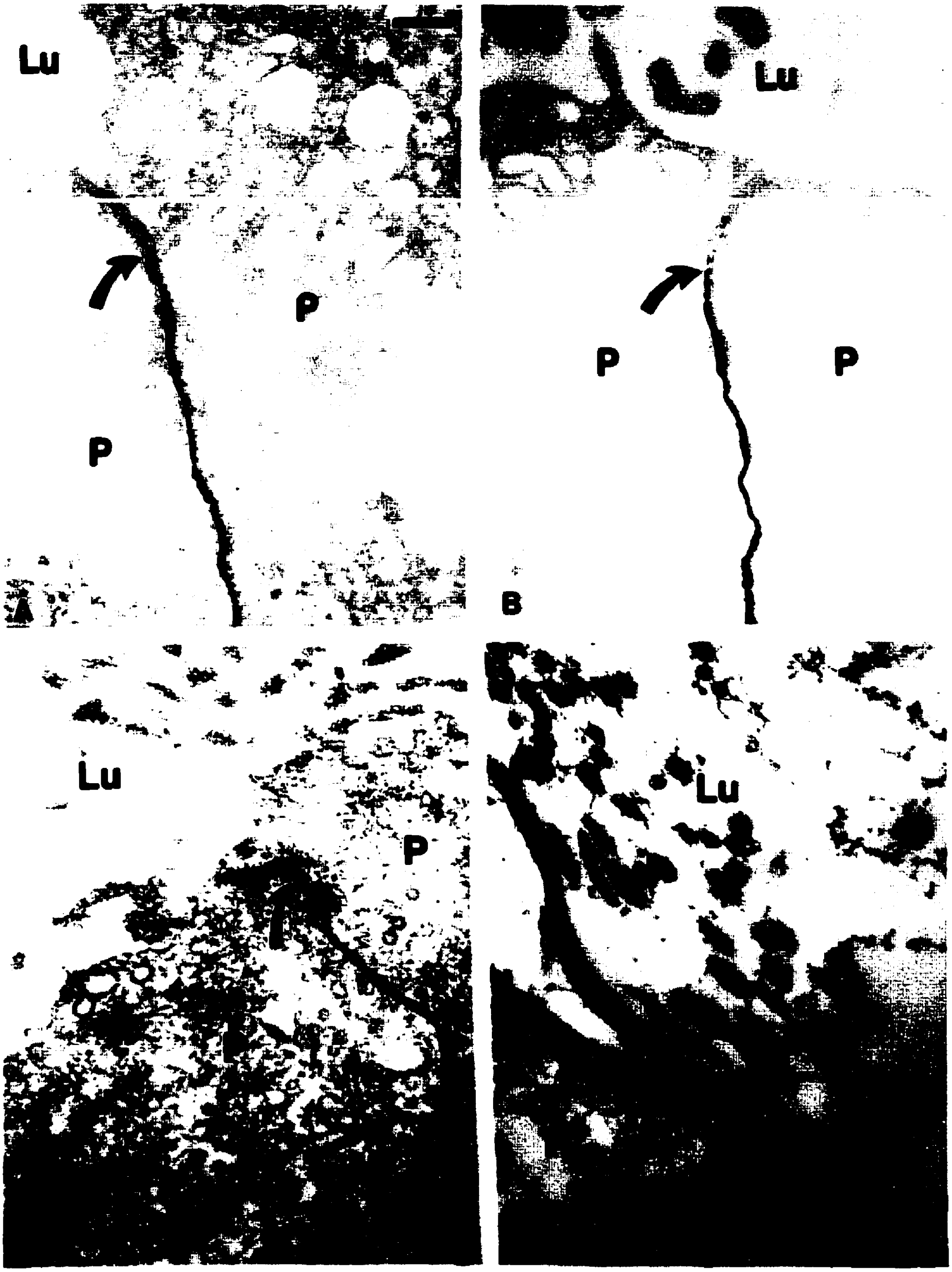
Lu
P
P
A
Lu
P
P
B
Lu
P
Lu

Figure 6. Electron micrographs showing the effects of age on the permeability of the blood-epididymis barrier to lanthanum nitrate in the corpus **(A, B)** and cauda epididymidis **(C, D)** of the Brown Norway rat. **A, C)** 3 months; **B, D)** 24 months. P, principal cell, Lu, lumen. The curved arrows indicate where the passage of lanthanum nitrate is occluded **(A,C,D)**, and where it enters the tight junction to enter the lumen in the corpus epididymidis at 24 months **(B)**. The small, straight arrows show lanthanum nitrate in the lumen **(B)**. Scale bar **A-D** = 0.3 μm.

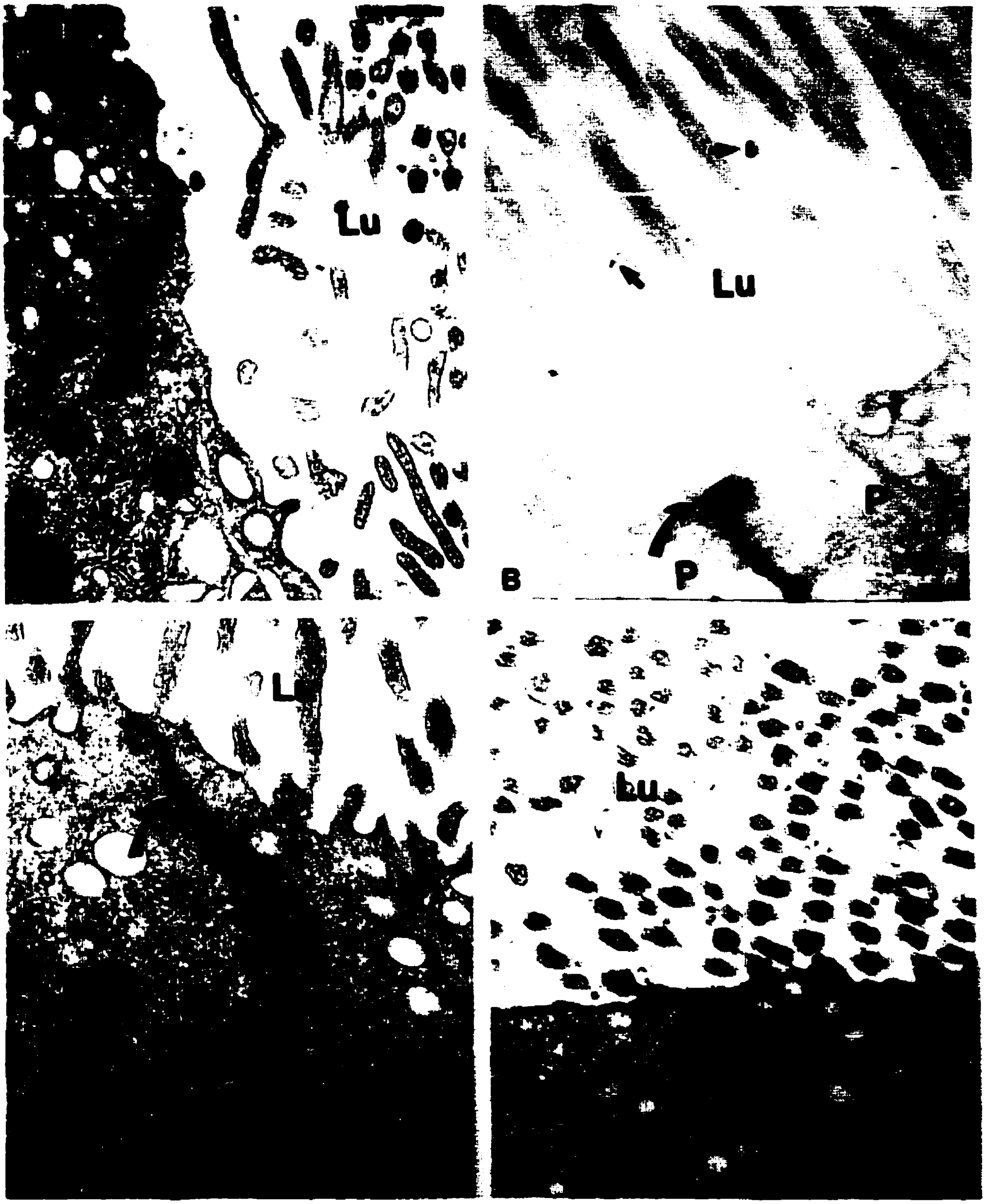
Lu
Lu
B
P
P
Lu
Lu

Figure 7. Diagrammatic representation of occludin, ZO-1, and E-cadherin immunostaining along the length of the epididymis at 3 and 24 months. ● represent occludin reactivity, ✱ symbolize ZO-1 reactivity, ▲ represent E-cadherin reactivity, and the thick black lines represent lanthanum nitrate while ● show lanthanum nitrate in the lumen. Note that the staining pattern shown for E-cadherin in the cauda epididymidis at 3 months is representative of the distal cauda epididymidis. The most striking changes are observed in the corpus epididymidis with age.

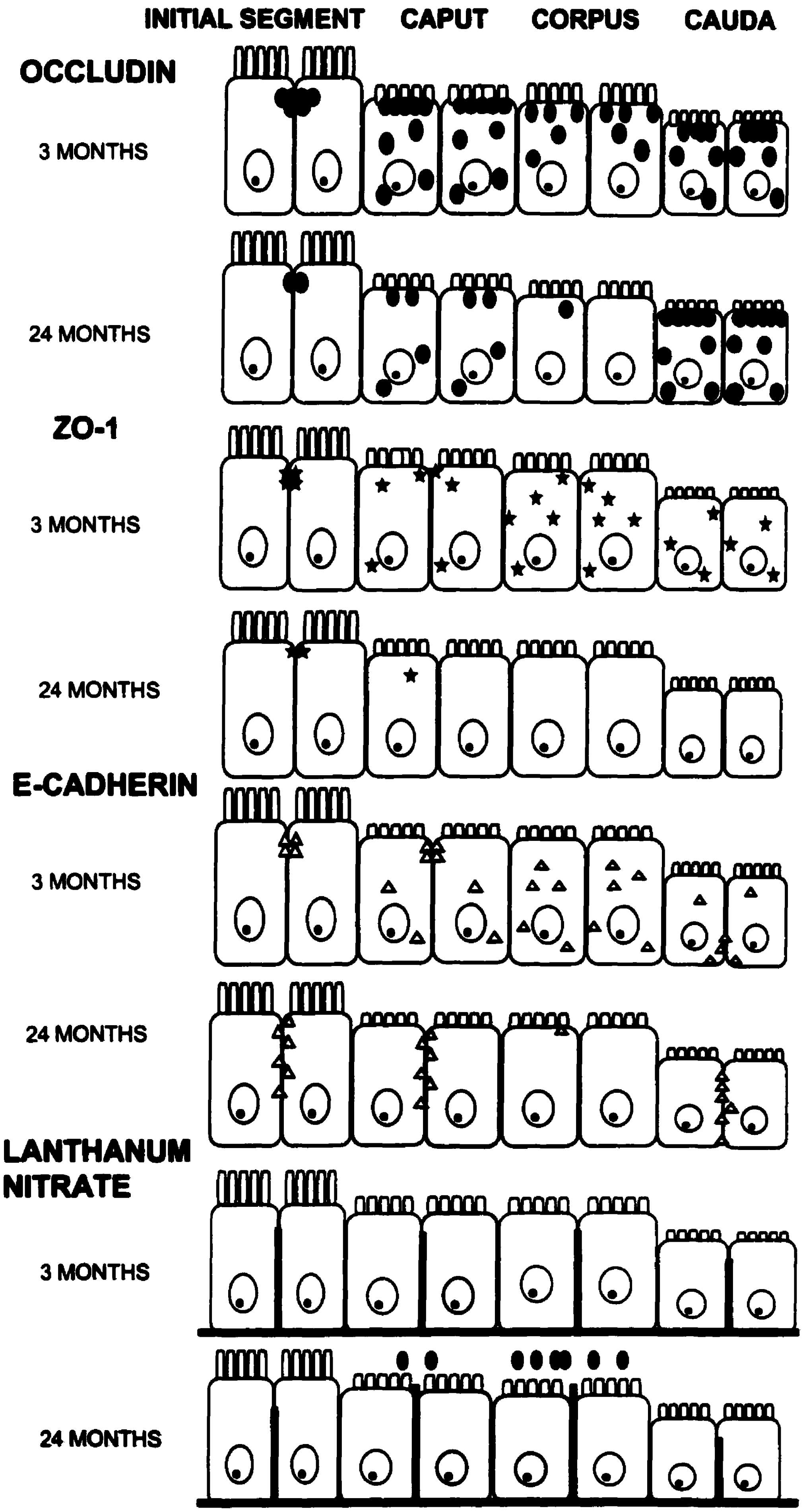
INITIAL SEGMENT
CAPUT
CORPUS
CAUDA
OCCLUDIN
3 MONTHS
24 MONTHS
ZO-1
3 MONTHS
24 MONTHS
E-CADHERIN
3 MONTHS
24 MONTHS
LANTHANUM NITRATE
3 MONTHS
24 MONTHS

TABLE 1. Immunostaining of occludin, ZO-1 and E-cadherin of principal cells in the epididymis of the Brown Norway rat at 3, 18, and 24 months

Segment	Junctional	3 months		18 months		24 months	
	Protein	Proximal	Distal	Proximal	Distal	Proximal	Distal
Initial Segment	**Occludin**						
	Junction	+++[a]	+++	++	++	+	+
	Cytoplasm	-[a]	-	-	-	-	-
	ZO-1						
	Junction	++	++	+	+	+/-[a]	+/-
	Cytoplasm	-	-	-	-	-	-
	E-cadherin						
	Junction	++	++	++	++	+++	+++
	Cytoplasm	-	-	-	-	-	-
Caput	**Occludin**						
	Junction	+++++	+++++	+++	+++	++	++
	Cytoplasm	++++	++++	+++	+++	+	+
	ZO-1						
	Junction	+	+	+	+	+/-	+/-
	Cytoplasm	++	++	+	+	-	-
	E-cadherin						
	Junction	++	+	++	+	+++	++
	Cytoplasm	++	++	+	+	-	-
Corpus	**Occludin**						
	Junction	++	+++	+	++	-	+/-
	Cytoplasm	+	++	+	+	-	-
	ZO-1						
	Junction	+/ -	+	-	+/-	-	-
	Cytoplasm	++	+++	+	++	-	-
	E-cadherin						
	Junction	-	-	-	-	-	+/-
	Cytoplasm	++	++++	+	+++	-	-
Cauda	**Occludin**						
	Junction	++++	++++	++++	++++	+++++	+++++
	Cytoplasm	+++	+++	+++	+++	++++	++++
	ZO-1						
	Junction	+/-	+/-	-	-	-	-
	Cytoplasm	++	++	+	+	-	-
	E-cadherin						
	Junction[b]	-	++	++	+++	+++	+++
	Cytoplasm	+	+	+	+	-	-

[a] The number of plus signs (+) is directly proportional to the strength of the reaction. The plus/minus sign (+/-) indicates that the reaction is faint and not present in all principal cells while the minus signs (-) indicate the absence of a reaction.

[b] This row represents the staining at the basolateral membrane between principal cells.

Chapter 3

DISCUSSION

Tight junctions constitute the only continuous, circumferential physical barrier between epithelial cells [1-5]. These structures form the apical barrier to the paracellular movement of water, solutes and immune cells in polarized epithelia [1-5]. In the past decade, the molecular architecture of tight junctions has become a rapidly expanding field. In recent months, several new proteins including claudin-1, claudin-2 [6], and ZO-3 [7], have been localized to tight junctions. The exact roles played by these proteins are unknown and illustrate the complexity of tight junctions. In addition, the regulation of tight junctional proteins and the signalling pathways involved in the assembly and the permeability of the paracellular barrier are still in the initial stages of study, making this a fascinating area at this time.

Molecular architecture of tight junctions

The junctional proteins that were selected for this study provide important clues about the organization of tight junctions in the epididymis. Recent studies have focussed on the role of ZO-1, a member of the membrane-associated guanylate kinase homologs (MAGUKs), within the epithelial tight junction [8,9]. Epitope-tagged fragments of ZO-1 have been introduced into cultured Madin Darby Canine Kidney (MDCK) cells to identify the critical domains necessary for the interactions of ZO-1, ZO-2, occludin, and F-actin [8]. In addition, a combination of in vitro and in vivo binding assays have been performed that reveal that both ZO-2 and occludin interact with specific domains within the N-terminal half (MAGUK-like) of ZO-1, whereas the unique proline-rich C-terminal half of ZO-1 cosediments with

F-actin. These observations suggest that one functional role of ZO-1 is to organize components of the tight junction and to link them to the cortical actin cytoskeleton [8]. Thus, our localization of ZO-1 to both the apico-lateral tight junctions between principal cells in some segments of the epididymis and within the cytoplasm of the caput, corpus, and cauda epididymidis, seems to be consistent with the different functions of ZO-1. An immunocytochemical study at the level of the electron microscope would prove useful in localizing ZO-1 and the other junctional proteins more precisely.

The Blood-Testis Barrier and Aging

The effects of age on blood-tissue barriers are absent from the literature. Within the male reproductive system, the changes in the seminiferous epithelium and the blood-testis barrier in the aging Brown Norway rats have been recently examined . We have shown that by 24 months, a majority of seminiferous tubules are fully regressed, virtually devoid of germ cells, and contain large intercellular spaces [10]. These regressed tubules also show a complete loss of cyclical variations of the organelles of the Sertoli cells. Most importantly, while lanthanum nitrate is stopped by the blood-testis barrier in the young rats, the tracer permeates the basal and adluminal compartments in the fully regressed tubules of the aged rats, extends between Sertoli cell processes, and enters the intercellular spaces and lumen [10]. Interestingly, the typical Sertoli-Sertoli junctions of the blood-testis barrier in the young animal are rarely seen at 24 months and are replaced by focal contact points between Sertoli cell processes. Whether these focal contact points are tight junctions is unknown at this time. In contrast, while there are many histological changes taking place in the Brown Norway rat epididymis with age [11], the principal epididymal cells maintain their general architecture, and there is no change in the localization of tight junctions in the aged animals.

Freeze-fracture studies done using seasonal breeders such as the mink (*Mustela vison*) suggest that the blood-testis barrier and the blood-epididymis barrier are regulated in different manners [12-16]. Electron-opaque tracers such as lanthanum nitrate and horseradish peroxidase were intravascularly infused in these animals at different times during their reproductive cycles. It was found that the blood-testis barrier becomes permeable to these tracers during the inactive spermatogenic phase [12-14]. In contrast, the blood-epididymis barrier is able to block the passage of these tracers at all times [15,16]. The reason for such a difference in the integrity of these barriers remains unknown. However, this model could prove to be useful in understanding the different factors involved in the maintenance of each of these barriers.

Factors affecting the blood-epididymis barrier

The permeability of the blood-epididymis barrier has been estimated following a number of insults. In vivo micropuncture has been a common method used to monitor the integrity of the barrier. Following various treatments, animals have been administered intravenous radiolabeled inulin. Subsequently, the amount of radioactivity in the luminal fluid compared to that of the blood-plasma is measured at various times after beginning the intravenous infusion [17-22]. This test shows whether the tight junctions have become more leaky to inulin, a compound that is considerably restricted from entering the epididymal lumen from blood.

Studies by Turner and Howards [21] have shown that a number of insults, including vasectomy, varicocele, and estradiol treatment do not adversely affect the rate of entry of radiolabeled inulin into the lumen of the cauda epididiymidis. Hoffer and Hinton [22] showed that the blood-epididymis barrier remains intact following treatment with gossypol, an oral male contraceptive, by measuring the rate of inulin entry into the lumen of the caput and cauda epididymidis and by electron microscopic examination of the tight junctions following systemic lanthanum perfusions. Thus, these studies suggest that the integrity of the blood-epididymis barrier remains intact following insults that result in male infertility. At this time, aging appears to be one of the only factors affecting the permeability of the blood-epididymis barrier, although few studies in the literature have focussed on this topic.

Aging and the Functions of the Blood-Epididymis Barrier

A disruption of tight junctions in the epididymis with age can lead to serious consequences. The activation of the immune system, represented by the greatest increase in halo cells with age, occurs in the corpus epididymidis where we have also seen the biggest decrease in junctional proteins and the most penetration of lanthanum nitrate. This suggests that one of the main functions of the blood-epididymis barrier, to protect spermatozoa from autoimmune attack, may be altered with age.

Furthermore, the disruption of the blood-epididymis barrier in the caput and corpus epididymidis in the aged Brown Norway rats suggests that there may be a change in the luminal contents in these segments. Such changes may have an effect on the maturation of spermatozoa during aging. Serre et al [23] have found that, with age, the quality of spermatozoa appears to be affected. There is a significant increase in preimplantation loss in litters fathered by aged male rats compared with young rats [23]. In addition, a decrease in fetal weight is seen with advancing paternal age along with a dramatic increase in the number of neonatal deaths of pups in litters fathered by 24-month-old rats [23]. Many factors are likely to be involved in the changing quality of spermatozoa during aging of Brown Norway rats [23-28]. The exposure to an inadequate environment during maturation in the aged epididymis due to alterations of the blood-epididymis barrier may be one of the reasons for the changes seen in fertility with increasing paternal age. It seems clear that the distal corpus epididymidis should be the segment to focus on since the most striking age-related changes are seen here.

Cell-Cell Communication in the Epididymis

In addition to the junctional complex located between adjacent principal cells, there are other points of cell-cell communication between the different cell types in the epididymis. An immunocytochemical study found that connexin-43, one of the most predominant gap junction proteins, is localized to the base of the epididymal epithelium between principal and basal cells [29]. In the initial segment only, connexin-43 immunostaining is observed apically between adjacent principal cells after bilateral orchidectomy [29]. This result suggests that the intracellular targeting of connexin-43 to the principal-principal cell interface in the initial segment is regulated by androgens under normal conditions [29]. While the androgen-dependence of E-cadherin has been shown in the epididymis [30], it would be of interest to perform bilateral orchidectomy and androgen replacement, in both young and old rats, to determine the effects of androgens on occludin and ZO-1.

The immunocytochemical localization study of connexin-43 showed that there was no immunostaining between clear and basal cells [29] despite the abundance of clear cells in the corpus and cauda epididymidis. These results suggest that there are no connexin-43 containing gap junctions present between basal-clear cells or between principal-clear cells. The findings in our study also indicate that there are no tight junctions consisting of occludin and ZO-1 between clear-clear cells, clear-basal cells or basal-basal cells. However, it is possible that gap junctions containing different connexins may be present between these cell types; as occludin and ZO-1 are present in most tight junctions examined in various tissues [31-35], it is possible, although less likely, that tight junctions made up of different proteins are present between other epididymal cell types.

Intercellular communication between epididymal cells is poorly understood.

It has been reported that different connexin-containing gap junctions allow molecules of different sizes and charge to pass between neighbouring cells [36]. At this time, if there are different connexins present in the epididymis, they have yet to be elucidated and the methods by which the other cell types communicate remain unknown. The passage of lanthanum nitrate between principal-basal cells and principal-clear cells in the adult animals illustrates that the same type of tight junctions that are present between adjacent principal cells are not found between other cell types. In addition, lanthanum nitrate continues to penetrate principal-basal and principal-clear cells in aged animals.

As we begin to gain more knowledge about the functions of basal and clear cells, the ways in which these cells communicate will follow. For now, many questions remain about cell-cell communication in the epididymis. For instance, the importance of the leakiness of clear cells into the lumen is unknown; perhaps it is necessary for the passage of certain substances into and out of the epithelium. Also, it is not known why spermatozoa do not enter the epithelium via clear cells if there are no tight junctions to keep them out. The answers to these and other questions will lead to a better understanding of the epididymis, its functions, and its regulation.

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