Chapter 12

Blood-Testis Barrier, Junctional and Transport Proteins and Spermatogenesis

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Functional Evidence for a Blood-Testis Barrier

The term "blood-testis barrier" appears to have been first used by Chiquoine[1] in an article on effects of cadmium on the testis, but evidence for such a barrier already existed, dating back to the early years of the twentieth century (see ref. 2 for early references). In a number of studies, it was shown that some dyes when injected into animals, stained most tissues, with the notable exceptions of the brain and the seminiferous tubules of the testis. The former observation was rapidly taken up and developed to form the basis for the concept of the blood-brain barrier,[3,4] but it was only with the studies of Kormano[5] that the true significance of the earlier observations on the testis was recognized. He showed that dyes which were excluded from the tubules of adult rats readily penetrated those of prepubertal animals. In addition, Kormano noticed that staining of interstitial cells with acriflavine also fell around the time of puberty, suggesting a change in the blood vessels as well. At about the same time as Kormano's studies, Waites and I showed that testis blood flow measured by indicator dilution with rubidium gave much lower values that with iodoantipyrine, while similar values were obtained in most other organs except brain,[6] suggesting that rubidium was also excluded to some extent from parts of the testis, as it was from the brain.

Also around this time, Waites and I devised techniques for collecting fluid from the rete testis (RTF) of sheep[7,8,9] and from the rete testis and seminiferous tubules (STF) of rats,[10] and we found that both RTF and STF differed appreciably in composition from either blood plasma or testicular lymph collected from a vessel in the spermatic cord. That such differences, especially those for small hydrophilic organic compounds such as inositol[11,12] could be maintained provided further evidence that there was not free communication between the various fluid compartments inside the testis, and this was confirmed in studies on the rate of penetration of various radioactive markers from the bloodstream into RTF in rams[13] or RTF and STF in rats.[14-17]

There are three cell types between the fluid inside the blood vessels and that in the lumina of the seminiferous tubules, namely the endothelial cells lining the blood vessels, the peritubular tissue and the Sertoli cells. These last are the only cells to extend all the way from the peritubular tissue to the lumen of the tubule, with the developing germ cells lying either between the base of a Sertoli cell and the peritubular tissue, or in the intercellular space between a pair of Sertoli cells or in crypts in the luminal surface of a Sertoli cell.[18-20] All three cell types could conceivably influence the rate of entry of substances into the tubules,[21] although most attention has been directed to the Sertoli cells (see next section).

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Other techniques used to estimate the effectiveness of the blood-testis barrier include the measurement of the volume of distribution of a marker known to be excluded from STF, such as Cr-EDTA, inulin or sucrose, and relating this either to the volume of the interstitial tissue[22-24] or to the value obtained when the efferent ducts had been ligated 24 h previously, so that the fluid secreted during that time had been retained in the lumina of the seminiferous tubules.[25] Other studies[26,27] used as a marker, hexamethonium iodide, which has been shown not to penetrate the blood-brain barrier,[28] a zinc complex of carnosine labeled with C-14 and Zn-65,[29] or a biotin tracer.[30] Another approach is to relate the amount of a labeled compound to the amount of Tc-99 or I^{125} labeled albumin appearing in the testis and brain of mice following an intravenous injection.[31-39] From these data, an entry rate (K_i) for the marker can be calculated, but this value in the testis could be influenced by changes in vascular permeability as well as in permeability of the tubular barrier.

The latest development has been the use of magnetic resonance imaging of the testis, before and after intravenous injection of gadopentetate dimeglumine.[40,41] Qualitative evidence for a barrier in young animals is provided by the development of a lumen and the secretion of fluid in the tubules.[42-44]

While most evidence for the involvement of the Sertoli cells is morphological (see next section) it should be remembered that when isolated Sertoli cells are cultured at high density on Matrigel in a two-chamber system, they form a confluent layer, which exhibits barrier properties, as shown by an increase in electrical resistance and directional secretion of a number of substances.[45-47] However, the transepithelial resistance (TER) obtained (usually about 100 ohm.cm^2) was usually much less than that seen with MDCK cells or keratinocytes (100-2000 ohm.cm^2).[48] Nevertheless, treatment of Sertoli cell cultures with FSH and testosterone[45] could raise TER to between 580 and 1200 ohm.cm^2, and the cells were usually obtained from prepubertal rats, in which the barrier would not be fully formed (see below).

Structural Evidence for a Barrier

A Sertoli Cells

The existence of specialized junctions between pairs of Sertoli cells was recognised in the 1960's.[49-53] Their significance became apparent when it was shown that electron opaque markers which were injected into the interstitial tissue or reached there from the blood stream, were restricted from entering the tubules to some extent by the peritububular myoid cells, but almost completely by the specialized junctions between pairs of Sertoli cells. The markers used included colloidal carbon, ferritin, horseradish peroxidase, lanthanum salts,[54-58] and more recently biotin.[30]

Peritubular Myoid Cells

Peritubular myoid cells form a single layer in rodents and several layers in primates around the seminiferous tubules.[59] As long ago as 1901, it was suggested[60] that this cell layer formed "a sort of dialysing membrane which regulates the compostion of the fluid contained in the space that it limits" (une sorte de membrane dialysante qui regle la composition du liquide contenu dans l'espace qu'elle limite). The cells change in shape, structure, marker expression and rate of cell division around the time of puberty[61,62] and respond in culture to endothelin[63,64] which as the name implies, is usually produced by endothelial cells, but in the testis is formed mostly by the Sertoli cells.[65] The myoid cells produce PmodS, a protein which has a powerful influence on several Sertoli cell functions,[66,67] although an effect on the blood-testis barrier has not apparently been examined.

The peritubular myoid cells prevented the passage of larger electron-opaque markers like colloidal carbon or thorium, and lanthanum penetrated the myoid cell layer in only about 15% of the tubules in rodent testes.[54,55] However, in primate testes, the peritubular cells have much less effect in restricting the penetration of markers.[56]

Nevertheless, the myoid cells may have an important influence in restricting the entry of retinoic acid (RA) into the tubules. Less than 1% of the RA in the testis is derived from plasma RA, much less than in any other tissue studied.[68] This may be due to the presence in the myoid cells of the RA-degrading enzymes Cyp 26 a1, Cyp 26 b1 and Cyp 26c1,[69] while the first stage of the formation of RA from retinol occurs in the Sertoli cells. It has been known for many years that spermatogenesis is arrested in Vitamin A-deficient animals, and retinoic acid is effective in restoring sperm production only in pharmacological doses (10 mg/week compared with 0.1 to 0.2 mg/week for retinol).[70] The restricted entry of retinoic acid may explain this difference. The myoid cells also contain high levels of cellular retinol binding protein,[71,72] which is probably involved in the transport of retinol into the tubules (see below).

Endothelial Cells

The endothelial cells in the testis are unusual for an endocrine tissue in that they are unfenestrated,[74-76] although in the human testis, some capillaries in the lamina propria do have fenestrations.[77] Endothelial cells in the rat testis also have a much lower density of vesicles than vessels in other tissues, except brain,[78] suggesting that vesicular transport is less important in these tissues than elsewhere in the body.

Structural Constituents of the Sertoli Cell Junctions

In recent times, a large amount of information has appeared about the constituent proteins of the Sertoli cell junctions which constitute that part of the blood-testis barrier (Fig. 1). The main components include occludin, one or more of the claudins, zonula occludens (ZO), and junctional adhesion molecules (JAM's).Occludin, claudin-11 and JAM-1 are transmembrane proteins, the extracellular parts of which join with similar structure on an adjacent Sertoli cell to form a tight junction. In the cytoplasm of the cells, the intracellular tails of the occludin, claudin and JAM molecules are joined to ZO-1 and ZO-2 molecules, which in turn are linked to actin chains.[48]

Occludin is a 60 to 65 kDa protein with four transmembrane domains, one intracellular and two extracellular loops, and is present in the tight junctions between Sertoli cells in rats and mice, but not guinea pig or human.[79] In mice which carry a null mutation of the occludin gene, the testes initially develop normally, but by 40 to 60 weeks of age, the tubules become atrophic, with complete loss of germ cells.[80] Therefore, it is rather surprising that occludin first appears in the fetal testis at about day 13 pc (post-coitus), long before spermatogenesis is initiated, suggesting that occludin has functions other than the establishment of the barrier. In postnatal rats, at about day 5, the reaction for occludin becomes more intense and is then located along the lateral plasma membrane of the Sertoli cells. Then at day 14, the reaction appears as intense focal bands close to the base of the epithelium, near the presumed sites of the tight junctions which are forming at about that time.[81] Injection into rats of a 22-amino acid synthetic peptide corresponding to the second extracellular loop of occludin perturbs the blood-testis barrier and disrupts spermatogenesis.[82]

Claudins are a family of more than 20 proteins, about 22 kDa in size,[83] and claudin-11 is present at tight junctions between Sertoli cells in testes, but again appears first during fetal life. Its concentration in the testis reaches a peak at about 6 days of age, and then appears to decline, probably due to the appearance of claudin-negative germ cells.[84] Claudin-11 null male mice are sterile, and tight junctions appear to be absent in these animals as judged by freeze-fracture.[85] Claudin-5, which is found only in endothelial cells,[86] is present in endothelial cells in the rat testes,[87] but as mice null for this peptide die within a few days of birth,[88] it has not been possible to study the effect of lack of this protein on spermatogenesis.

Integrins are thought to be involved in junctions between testicular cells and extracellular matrix,[48] but there is evidence[89] that integrin $\alpha 6\ \beta 1$ is also present in Sertoli-Sertoli cell junctions, especially at certain stages of spermatogenesis, but also in Sertoli cell-only testes.[90] In testis explants, the development of this suprabasal integrin occurred only in the presence of FSH.[90]

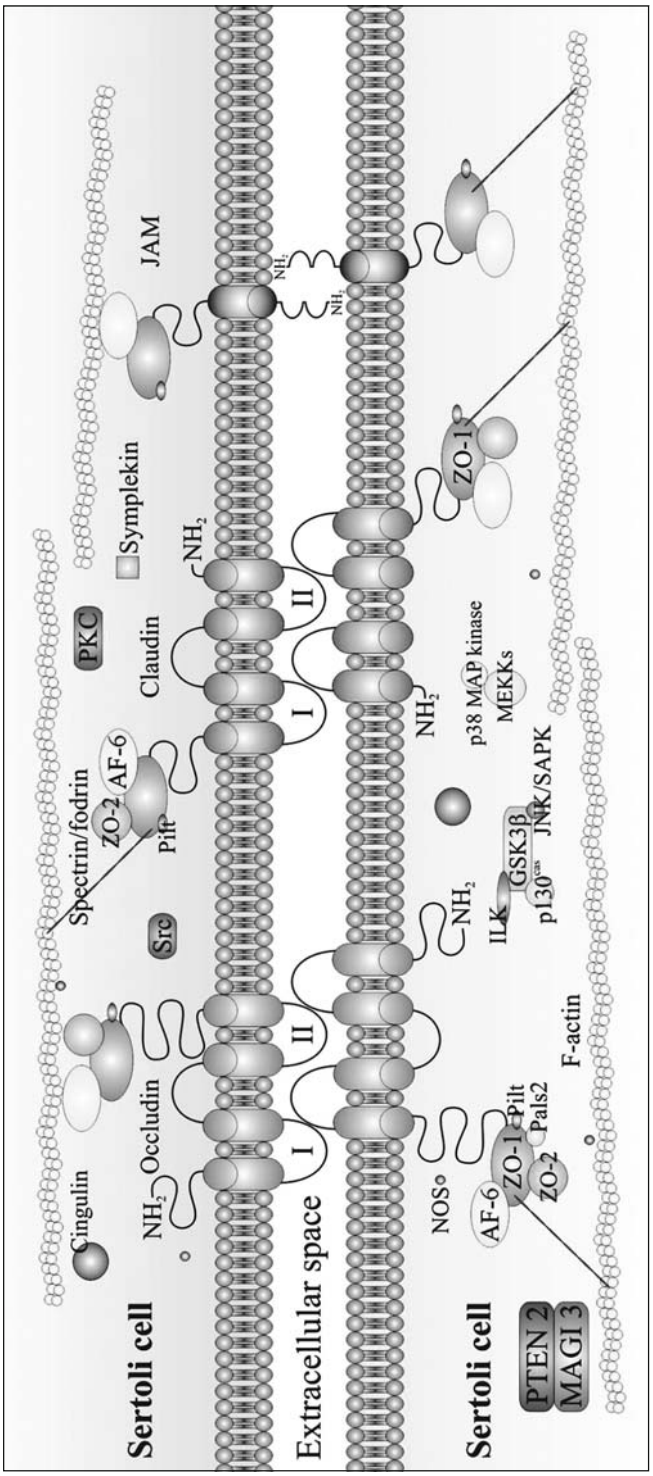

Figure 1. A diagram illustrating the molecular architecture of the three multiprotein complexes found at the Sertoli-Sertoli cell junctions of the blood-testis barrier. The three complexes are: (1) Occludin-ZO1/ZO2; (2) claudin-ZO1/ZO2; and (3) JAM-ZO1. Also shown are the peripheral membrane proteins known to regulate Sertoli cell tight junction dynamics. Reproduced with permission from: Mruk DD, Cheng CY. Endocrin Rev 25:747-806, ©2004 The Endocrine Society.[48]

Transport Proteins and the Blood-Testis Barrier

Transferrin

Iron is transported into the germ cells inside the blood-testis barrier by a mechanism involving a specific transport protein, transferrin. In the blood, iron is carried bound to transferrin secreted by the liver, and on reaching the testis, this complex binds to transferrin receptors on the basal surface of the Sertoli cells.[91] The iron-transferrin complex is then internalised and dissociated, the apo-transferrin returned to the interstitial extracellular fluid and the iron is complexed to transferrin produced inside the Sertoli cell and secreted into the space between the Sertoli cell and the germ cells (Fig. 2). How the iron is moved across the Sertoli cell is still uncertain, but may involve a ferritin-like molecule.[92,93] Sertoli cells in a bicameral culture system synthesize and secrete transferrin,[94] and iron from basally applied human transferrin is transported through rat Sertoli cells and appears in the apical compartment bound to rat transferrin.[95] Nevertheless, the concentration of transferrin in seminiferous tubule fluid is less than one-twentieth of that in interstitial extracellular fluid or blood plasma.[96]

Other elements besides iron are bound by transferrin, and this may be important in causing the accumulation inside the tubules of potentially mutagenic radioactive substances like indium[97,98] and plutonium.[99]

Transferrin production by Sertoli cells is greater if the cells are derived from 17 day old rather than 10 day old rats,[100] is reduced following hypophysectomy and not restored by testosterone treatment.[101] It is stimulated by FSH,[102] cytokines,[103] a factor PmodS produced by the peritubular myoid cells,[104] and heregulins, which may also come from the same source.[105] The presence of germ cells in the tubule may also have an effect on transferrin production by the Sertoli cells,[105,106] although different results were obtained when the germ cells were depleted with methoxyacetic acid.[107] Sertoli cells also secrete an copper-transporting protein, ceruloplasmin,[108] but it is not known whether this substance is involved in copper transport into the tubules.

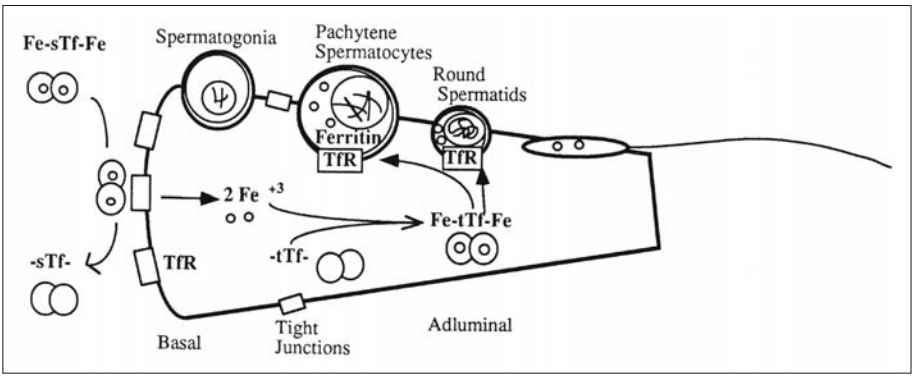

Figure 2. A diagram illustrating the role of transferrin in the transport of iron and other metals into the seminiferous tubules. Diferric serum transferrin (Fe-sTF-Fe) binds to a transferrin receptor on the basal surface of the Sertoli cell. The transferrin-ferric ion-transferrin receptor complex is internalized into special compartments in the cell, acidified and broken down. The apotransferrin and the transferrin receptor are recycled to the cell surface, and the iron is moved through the cell to newly synthesized testicular transferrin (tTf) or is incorporated into ferritin in the Sertoli cell. The testicular transferrin with the ferric ions is released into the intercellular space between the Sertoli and germ cell and then binds to transferrin receptors on the surface of the germ cells. The net result is transport of ferric ions from the basal surface of the Sertoli cell to the adluminal compartment of the tubule. Reproduced with permission from: Sylvester SR, Griswold MD. J Androl 1994; 15:381-385, ©1994 American Society of Andrology.[93]

Another divalent metal transporter DMT1 (Slc1 1a2) is also present in the Sertoli cells of the rat testis, although it is not primarily responsible for translocating iron across the epithelium, but in intracellular handling of iron during spermatogenesis.[109,110]

P-Glycoprotein

P-glycoprotein (Pgp) is the product of the multidrug resistance 1 gene (MDR1 or ABC B1 in humans, and mdr 1a (sometimes called mdr 3) and 1b in mice and rats). It was originally identified in cancer cells which had become resistant to chemotherapeutic drugs.[111-114] Subsequently, it was found that this protein was present in a number of normal tissues, and especially in the endothelial cells of the brain and testis.[115-118] It is also present in other cells in the testis, including Leydig cells, macrophages, peritubular cells, Sertoli cells and late spermatids, although not detectable in spermatogonia, spermatocytes or early spermatids.[119,120] However, the relative concentrations in the various cell types has apparently not yet been determined and another group has detected mdr 1 in germ cells, probably spermatogonia, in rats, as well as in endothelial cells in the testis.[121] In endothelial cells from brain, Pgp is expressed only on the luminal surface, consistent with a role in protecting the brain from circulating lipophilic molecules which would otherwise cross the blood-brain barrier. However, in endothelial cells in the testis, Pgp is expressed on both luminal and abluminal sufaces, which suggests that it acts to exclude substrates of the transporter from the endothelial cells themselves.[122] A mRNA from a related gene mdr 2 is also present in Sertoli cells, but at a lower concentration than in liver.[109]

The testes and brains of mice in which the gene for mdr-1a has been deleted accumulate more ivermectin, digoxin, cyclosporin A, ondasetron, loperamide and vinblastine than controls.[123-126] In other studies,[127,128] similar results were obtained with amitriptyline and some of its metabolites, but not with fluoxetine. In mice in which both mdr 1a and 1b have been knocked out, the entry of the anti-Parkinson drug budipine into the testes and brains was enhanced.[129] In these double knockout mice, the penetration of the steroids, corticosterone, cortisol, aldosterone and progesterone into the testes was also enhanced,[130] although cortisol[131] or prednisolone[132] entry into the testis was unaffected in mdr 1a single knockout mice. Pgp also transports HIV protease inhibitors (HPI) used in the treatment of AIDS[133] and pharmacological inhibition of the transporter enhances the penetration of the HPI nelfinavir into the testes of mice treated with LY-335979, a potent Pgp inhibitor, as well as in mdr-1a knockout mice.[134] The penetration of saquinivir, another HPI into the testes of mice was also enhanced by treatment of the animals with another inhibitor of Pgp, GF120918.[135] However, treatment of mice with a variety of Pgp inhibitors failed to increase the penetration of vinblastine into either testis or brain,[136] and vincristine enters seminiferous tubule fluid reasonably rapidly,[137] although it is a substrate for both Pgp and MRP.[126] The closely related efflux pump, breast cancer resistance protein (BCRP) is also found in the endothelial cells and peritubular myoid cells in the testis,[120] but the structurally related protein encoded by the cystic fibrosis gene is not found in endothelial cells, but is expressed in spermatids in a stage-specific fashion.[121,138]

Multidrug Resistance Protein

Multidrug resistance proteins (MRP) are other members of the ATP-binding cassette superfamily distantly related to Pgp. MRP1 is present in high concentrations in testes[139] and is localized to the Leydig and Sertoli cells in human and mice,[120,140] but cannot be detected in endothelial cells in the rat testis.[141] Mice lacking the gene for this protein are much more sensitive to the damaging effects of etoposide phosphate[141] and methoxychlor[142] than normal mice, suggesting that it acts to exclude these drugs from the seminiferous tubules. MRP1 is also involved in glutathione-mediated transport of sulfated estrogens, and it has been suggested that the high levels of MRP1 in the Leydig cells may be responsible for the efflux of the hydrophilic sulfated conjugates from the cell.[143] The anticancer drug methotrexate, which is transported out of cells by MRP, but poorly by Pgp,[144] is virtually excluded from seminferous tubule fluid.[145]

Other Transport Mechanisms

Endothelial cells in the testis contain high levels of γ-glutamyl transpeptidase,[118,146] an enzyme usually associated with amino acid transport, and it has also been shown that endothelial cells of the larger blood vessels in the rat testis transport leucine with transport kinetics similar to those of brain and much lower than for other tissues.[147] There is also a large amino acid transporter present in rat testis as well as brain and heart, but not other tissues.[148] Endothelial cells in the rat testis also contain an endothelial barrier antigen (EBA), previously thought to be confined to nervous tissue,[149] and an isoform GLUT-1 of the glucose transporter family, usually associated with brain and retina.[118]

The peritubular cells in the mouse testis contain a specialized transporter protein involved in urea movement across plasma membranes, UT-A5, the levels of which are not related to the stage of spermatogenesis in adults but are coordinated with the stage of testis development, increasing around 15 days post partum.[150] In rat Sertoli cells, there are also 4 other urea transporters, UT-A 1, 2, 3 and 4 present at all stages of spermatogenesis, and UT-B is present at stages II and III. UT-A3 was also present in some interstitial cells. Flux of urea across the walls of isolated perfused seminiferous tubules is inhibited by phloretin.[151] It is interesting that there is some evidence for the active accumulation of radioactively labeled urea inside the seminiferous tubules of rats.[152]

Evidence has recently been presented for the presence of a family of saturable nucleoside transporters in isolated Sertoli cells, as primary cultures or as polarized layers on Matrigel, some of which is sodium-dependent and can be inhibited with nitrobenzylthioinosine.[153]

Binding proteins may also be important in the regulating the entry of retinol into the tubules. Homogenates of rat testis bind more retinol and retinoic acid (RA) than any other tissue examined,[154] but in vivo, very little RA enters the tubules from blood.[68] Both myoid and Sertoli cells in the testis contain a cellular retinol-binding protein (CRBP).[155-157] The Sertoli cells also contain a number of retinoic acid receptors.[68,69] Retinol circulates in the plasma bound to a retinol-binding protein (RBP), a 21 kDa protein which normally is present as a 76kDa 1:1 complex with transthyretin. This complex in the testis is confined to the interstitial tissue.[158] When retinol bound to RBP was injected into the testis[158] or under the capsule,[159] it appeared in the tubules only after at least 30 minutes, whereas tritiated retinol injected mixed with albumin, spread rapidly throughout the testis. Early studies could not detect any interaction of RBP with cells in or on the seminiferous tubules.[158] Nevertheless, both peritubular myoid and Sertoli cells appear to be involved in the transport of retinoids to the germ cells. Both cell types in culture are able to accumulate retinol from serum RBP by a saturable and competable process, which involves recognition of the retinol-RBP complex at the cell surface, with subsequent internalization of the retinol but not the RBP. The first step involves the myoid cells, which bind the retinol inside the cell to newly formed CRBP, and the new complex is released into the space between the myoid cells and the Sertoli cells. The latter then take up just the retinol and complex it with new CRBP, before releasing the complex again to reach the germ cells.[71,72,160]

Sertoli cells also contain a prostaglandin D_2 synthetase, which also binds retinoic acid but not retinol.[161] This protein is secreted into rete testis fluid,[162] but its role in the transport of retinoids into the tubules in not yet clear.

Factors Affecting Blood-Testis Barrier Function

Age and Hormones

As already mentioned, studies on the penetration of certain dyes into the seminiferous tubules showed that these dyes were excluded only from the tubules of rats older than about 20 days.[5] Subsequently, it was shown that electron-opaque markers injected into the interstitial tissue of the testes of rats entered the tubules freely up to 16 days of age, but between 16 and 19 days , the occluding junctions between the Sertoli cells appear and the tracers are effectively

prevented from reaching the tubular lumen.[163] In immature rat testes, occluding junctions, as demonstrated by freeze-fracture, are absent, although gap junctions are present. Furthermore, perfusion with hypertonic lithium chloride caused the cells outside the Sertoli cells junctions in adult testes to shrivel, with no effect on those inside the junctions, whereas in the testes of 13 day old rats, cellular shrinkage occurred throughout the tubules.[164] Shrinkage of adluminal cells in response to exposure to a hypertonic solution decreased between 14 and 18 days of age.[43] Similarly in guinea pigs, Sertoli cell junctional complexes appeared around 15 days after birth[165] and in mice at about 16 days.[166] In rats around 15 days of age, the barrier appears only in those parts of the tubule where germ cells have reached pachytene.[167]

In seasonal breeders such as mink,[168,169] viscacha,[170] and Djungarian hamsters[171] electron opaque markers are excluded by the Sertoli cell junctions during the breeding season, but during testicular regression, the tracer penetrates throughout the tubules. In the study on mink, the exclusion of the marker from the tubule was associated with the presence of a tubular lumen, rather than any particular type of germ cells. At the other end of life, the barrier in 24 month old rats was grossly deficient, with associated failure in spermatogenesis.[172] The development of the barrier in young rats and mice can be retarded by the neonatal administrations of diethylstilboestrol.[173,174]

The development of a lumen in the tubules is more gradual, beginning at around 10 days after birth, and with the diameter continuing to increase slowly to day 30 and then more rapidly to around day 50.[22,43] Fluid secretion per unit weight of testis also continued to increase until about 45 days of age,[42,44] and the volume of distribution of Cr-EDTA, which is normally excluded from the tubules, continues to fall until after 30 days of age,[22] so the functional barrier appears to develop more gradually than the anatomical one.

The development of transepithelial electrical resistance (TER) in two-compartment Sertoli cell cultures is delayed by FSH for several days, and once established is decreased and then returns to control levels or increases. Testosterone alone caused a rapid increase in TER, and testosterone and FSH together resulted in the highest TER levels. Dihydrotestosterone was more effective than testosterone, whereas estradiol was without effect.[45] Dibutryl cyclic adenosine monophosphate (cAMP) in low concentrations stimulated TER development, whereas higher doses were inhibitory. Cholera toxin mimicked the FSH effects.[175] The effect of cAMP on the Sertoli cell tight junctions is probably mediated by a proteasome-sensitive ubiquitination of occludin.[47] TGF-β3 also regulates blood-testis barrier dynamics, probably by determining the steady-state levels of occludin and ZO-1 via the p38 MAP kinase signaling pathway.[176] Tumour necrosis factor α injected directly into rat testes caused a temporary disruption of the blood-testis barrier, by reducing the levels of occludin, zonula occludens-1 and N-cadherin.[177]

Testosterone, acting through its receptor in the Sertoli cells, regulates the expression of claudin-3, which encodes a transient component of newly formed tight junctions. Sertoli cell-specific ablation of androgen receptor results in increased permeability of the barrier to biotin.[30] The effect of androgen withdrawal on the Sertoli cell junctions was studied either by hypophysectomy or by treatment of rats with ethane dimethane sulfonate to destroy the Leydig cells. These treatments led to degeneration of germ cells and the formation of numerous basally-located vacuoles, formed by multiple focal dilations of the intercellular space associated with the junctional complexes. As this occurred also in Sertoli cell-only testes, produced by fetal irradiation, it cannot be explained by spaces left by degenerating germ cells.[178] The expression of occludin is also reduced by treatment of rats with the anti-androgen, flutamide.[179] In an intratesticular androgen suppression model, using subcutaneous implants of testosterone and estrogen to suppress LH secretion and hence endogenous androgen production, the adherens junctions between the Sertoli cells and spermatids can be disrupted, without affecting blood-testis barrier integrity.[180]

The Sertoli cell barrier to lanthanum develops normally in rats treated in utero with busulfan but at a later age around 30 days of age, at the time of the appearance of the first zygotene and pachytene cells in these animals.[181] However, in prenatally irradiated rats, tight junctions, as detected by freeze fracture, were extensive by 3 months of age, although their ability to block

the penetration of markers was not examined.[167] It is probably relevant that the fluid inside the Sertoli cell only tubules of prenatally irradiated or busufan-treated rats was plasma-like in its potassium content, in contrast to the high potassium of normal fluid.[16,183]

Vitamin A Deficiency

In rats made Vitamin A-deficient from weaning (20 days old), Sertoli cell junctions were intact and complete spermatogenesis was maintained up to 80 days of age. However, by 90 days, lanthanum could penetrate through the junctions and by 100 days severe regression of spermatogenesis had occurred.[184] Different results were obtained by Ismail and Morales,[185] who found that the junctions remained impermeable to lanthanum, even when spermatogenesis had failed in rats 104 days old, deficient since 20 days old. In a later study, following long-term deprivation of Vitamin A, the Sertoli cell junctions became permeable to lanthanum when spermatogenesis was arrested and remained so even when spermatogenesis was first reinitiated. Spermatocytes normally found in the adluminal compartment were apoptotic, while spermatocytes normally found in the basal compartment remained normal.[186]

Tissue and Blood Pressures

If the efferent ducts leading from the testis to the epididymis are ligated close to the testis, the fluid normally secreted by the Sertoli cells to transport the immotile spermatozoa is retained inside the seminiferous tubules. These become progressively distended for between 24 and 36 h in rats, so that the testis becomes enlarged and turgid. Then the testis weight falls again and eventually by 21 days, spermatogenesis is completely deranged.[42] During this time the blood-testis barrier, judged by the ratio of the space of distribution of Cr-EDTA to the measured volume of the interstitial tissue remained normal during the phase of fluid accumulation, but increased sharply as testis weight begins to fall again, indicating breakdown of the barrier. Surprisingly, by the time testis weight had returned to control levels, the barrier appeared to be functioning again, and it remained functional even when spermatogenesis was completely disrupted up to 3 weeks later.[24,187] One author[188] found that lanthanum penetrated more readily through the Sertoli cell junctions as early as 24 h after efferent duct ligation. However, other studies with electron opaque markers gave contradictory results.[188-191]

In chronically hypertensive rats, the penetration of sucrose and 2-methyl-4-chlorophenoxyacetic acid into the testis is reduced, while that of the highly permeable antipyrine is unaffected.[192] In rats with testicular degeneration induced by epinephrine, the barrier remains able to exclude lanthanum.[193]

Cadmium and Other Toxic Substances

The testes of most mammals are extremely sensitive to the effects of cadmium salts, in doses which have little effect on other tissues. Early observations[1] concentrated attention on the blood vessels in the testis, and there is no doubt that testis blood flow is reduced in rats as a result of increases in vascular permeability as early as several hours after a single injection of cadmium chloride.[6] Later studies showed that permeability of the blood-testis barrier to rubidium probably preceded the changes in vascular permeability.[194] In guinea pigs on the other hand, increased staining of the interstitial tissue with acriflavine injected subcutaneously occurred before an increase in staining of the seminiferous tubules.[195] However, lower doses of cadmium affect spermatogenesis without noticeable changes in the vascular system, and these effects can be reduced by coadministration of zinc salts.[196]

Exposure of bicameral Sertoli cell cultures to cadmium salts caused a progressive and dose-dependent drop in TER.[197,198] The expression of occludin is decreased and u-plasminogen activator is increased in the presence of cadmium.[198] Treatment of rats with low doses of cadmium chloride caused changes in the tight junction-associated microfilaments in the Sertoli cells by 24 h after injection, although no changes were found after 4 h.[199] The fall in TER in the presence of cadmium was reduced if testosterone and FSH were added.[198] The disruption

of the barrier is associated with a transient increase in testicular TGF-β2 and 3 and the phosphorylated p38 mitogen activated protein (MAP) kinase, concomitant with a loss of occludin and ZO-1 from the barrier site.[200] There is also a surge in α_2-macroglobulin at the Sertoli-Sertoli cell junctions at the time of disruption of the barrier.[201]

It is interesting that there are some strains of mice whose testes are much more resistant to the effects of cadmium, and this is associated with reduced transport of cadmium into the testes. The cadmium transporter is saturable and can be competitively inhibited by zinc, but not calcium, and appears not to be associated with any tubular cells, but is probably located in the endothelial cells.[36]

The integrity of the blood-testis barrier is altered by intratesticular treatment of rats with cytochalasin D, a known microfilament inhibitor.[202] Evidence for this was obtained from studies on the penetration of electron-opaque markers, from the effects of perfusion with hypertonic solutions and from the entry of radioactive inulin into seminiferous tubular fluid.

Another substance which has been shown to disrupt the blood-testis barrier is glycerol when injected into the testes of rats. These animals showed increased entry of radioactive inulin and albumin into seminiferous tubular and rete testis fluids,[203] and also disrupted tight junction-associated actin microfilaments, occludin and microtubules in the Sertoli cells.[204]

Other substances which appear to affect the blood-testis barrier include hexanedione,[205] *cis*-platinum,[206] sarin,[26] and DEET[27] but stainless steel corrosion products affects spermatogenesis without apparently interfering with the blood-testis barrier.[207] Other treatments such as bisphenol A[208] or Adjudin (AF 2364)[48] disrupt the junctions between Sertoli cells and spermatids without affecting the blood-testis barrier. Freunds complete adjuvant injected into guinea pigs 7 days previously increased the entry of horseradish peroxidase into the seminiferous tubules.[209]

Temperature and Cryptorchidism

The entry of radioactive albumin into rete testis fluid of rats was unaffected during or following heating of the testes, but the entry of K, Rb, Na, lysine and some steroids was increased during heating.[210] The entry of Cr-EDTA into the tubules was not affected when spermatogenesis had been disrupted in rats by local heating of their testes.[23] In surgically-induced cryptorchidism in rats, the blood-testis barrier appears to remain intact,[211,212] but in spontaneous cases in humans, the penetration of lanthanum between the Sertoli cells depended on the extent of the loss of germ cells.[213] In other conditions of spermatogenic cycle breakdown in humans, lanthanum entry is increased in maturation arrest and in irregular hypospermatogenesis, but in germ cell aplasia the barrier remains efficient.[214]

Mutants and Hybrids

The blood-testis barrier is less efficient in Tfm and Sxr mice, but normal in Mo^{vbr}/Y and Gy/Y mutants.[215] There are defects in both the germ cells and in the blood-testis barrier in *as*-mutant rats, as demonstrated by the distribution of cytochrome-c in the testis, as well as from studies involving spermatogonial transplantation.[216] The blood-testis barrier is deficient in hybrids between blue and silver foxes, and spermatogenesis is arrested at early pachytene.[217]

Significance of the Blood-Testis Barrier

As has already been discussed,[218] there are several obvious consequences of the operation of the blood-testis barrier. The first is immunological. The barrier isolates the developing germ cells from circulating antibodies in the bloodstream. It also means that the body's immunological system does not "see" the haploid germ cells, and therefore a male can be immunized against his own spermatozoa.[219] However, the isolation is not complete and Tung[220] has concluded that "tissue barriers and antigen sequestration are important but not sufficient to protect germ cell antigens and prevent experimental allergic orchitis". Some germ cells outside the barrier can certainly provoke an immunological reaction,[221] even

peritubular cells,[222] leading to autoimmune orchitis.[220,223] Furthermore, mice immunized with syngeneic testis antigen have IgG deposits surrounding cells at the periphery of about half the tubule cross-sections, particularly those at stage 7 to 12. Also sera from testis-immune orchidectomized donors are able to transfer IgG passively into the testes of normal syngeneic recipients in an antigen-specific manner,[221] although there is evidence that the rete testis and tubuli recti are the sites of the earliest and most frequent lesions.[224] Therefore, other factors must be involved in making rodent testes, but not those of sheep[225] or monkeys,[226] immunologically privileged sites. Possible factors have been discussed recently by Hedger.[227]

The second effect of the barrier relates to the endocrine system. Peptide hormones such as FSH and LH do not instantaneously pass from the blood even into the extracellular interstitial fluid, so that the Leydig cells begin to respond to a rise in blood LH even before there is any change in the LH levels in the immediate vicinity of these cells.[228] FSH on the other hand acts principally on the Sertoli cells, and therefore must penetrate both the endothelial cell and peritubular cell layers. This is probably less important as the concentration of FSH does not seem to show such pronounced peaks as LH does,[229] and therefore changes in its concentrations in blood are more likely to be reflected in the concentrations at the basal surface of the Sertoli cells.

The situation with steroids is less clear cut. Because of their relatively high lipid solubility, they should pass more readily through the barrier than the hydrophilic peptides, but there is some evidence,[218,230] that the concentration of testosterone in RTF and STF does not change as much as that in blood. This may suggest that there is a transport system for steroids in the tubules, but no further evidence for this idea has been presented. It is clear from the relative concentrations inside and outside the tubules that the androgen-binding protein secreted by the Sertoli cells preferentially inside the barrier certainly does not produce a higher concentration of the total (free plus bound) steroid there. In fact the concentration of free testosterone may be appreciably lower in STF. Conjugated steroids, which are produced in large amounts in the testes of some species such as pig[231] and horse[232] tend to be less lipophilic than the free steroids and therefore remain in higher concentrations in the interstitial extracellular fluid than inside the barrier.

Glucose is transported across the barrier by a transport system the capacity of which appears to be less than the capacity of the Sertoli cells to convert the sugar to lactate. The consequence of this is that there is very little glucose in the fluid inside the tubules[8-12] and the developing germ cells prefer to metabolize lactate even in vitro.[233]

An interesting recent development has been the identification of a number of specific transport proteins for xenobiotics in various cells in the testis. These transporters, Pgp and MDR have important consequences in determining whether a particular toxicant will affect spermatogenesis, but in the case of transferrin, it may result in the accumulation of mutagenic substances in the environment of the germ cells.

One of the most interesting aspects of the function of the blood-testis barrier is the fact that it cannot remain closed all the time, but must open at different points along each tubule at specific times in the spermatogenic cycle to allow developing spermatocytes to pass from the basal to the adluminal compartment.[57] How this is achieved is still a matter of debate. Four theories have been advanced to explain this phenomenon: zipper, intermediate compartment, repetitive removal of membrane segments and junction restructuring. However, junction disassembly and reassembly seems to be the most likely explanation.[48] Opening of the Sertoli-Sertoli cell junctions in a limited part of the tubule must occur without affecting the Sertoli-Sertoli cell junctions elsewhere in that tubule or Sertoli cell-germ cell adherens junctions in the same and other parts of the tubule. It appears that cytokines may be involved[234] and α_2-macroglobulin also appears to play a part.[48,201] One of the most intriguing questions which remains to be answered is how spermatogonia injected into the lumen of a single seminiferous tubule either directly or via the rete testis[235] can pass between pairs of Sertoli cells to take up a position adjacent to the peritubular tissue and repopulate that area of the tubules with developing germ cells. The recipient animals

have usually been treated with busulfan or irradiation to eliminate endogenous spermatogenesis, but nothing appears to be known about the efficacy of the barrier in these animals. It is interesting that transmissible leukemic cells, when injected together with testicular cells into the tubules through the rete in normal rats can reach the intertubular tissue where they resume their uncontrolled multiplication and make the recipient animals leukemic.[236]

It has also been repeatedly stated that the specialized environment created by the barrier may be necessary for the germ cells to proceed through meiosis. However, just what these conditions are has yet to be defined, but the fact that spermatogenesis can proceed, albeit to a limited extent, in aggregates of testicular cells encased in alginate[237] may indicate that as long as Sertoli and germ cells are in reasonably close association, that is sufficient.

One fascinating possibility is that retinoic acid (RA), not derived from blood but newly formed from retinol by a two-stage process, may be involved in the switch of the germ cells from mitosis to meiosis in the testis. The first stage of this conversion involves the Sertoli cells and the second the germ cells.[69,238] RA has been shown to cause the germ cells in the fetal ovary to enter meiosis, while in the fetal testis, meiosis is inhibited by destruction of RA by Cyp26 b1[239,240] the same enzyme that in the myoid cells, prevents the entry of RA into the tubules.[68] A premeiotic germ cell-specific cytoplasmic protein encoded by the RA-responsive gene Stra8 is present in only less than half the tubule cross-sections in a mouse testis,[241] although unfortunately these authors did not identify the stage of spermatogenesis at which this protein was expressed. It is interesting that in mice in which the gene for $p27^{kip1}$ is knocked out, spermatocytes were often arrested at preleptotene[242] and this mitotic inhibitor can be induced in cultured Sertoli cells by RA.[243] Furthermore, the expression of mRNA for CRBP is highest in spermatogenic stage IX to XIV, when most of the mitoses in the tubules occur. Preleptotene spermatocytes appear first at stage VII but CRBP mRNA rises significantly only in stage VIII,[157] when the meiotic DNA synthesis is occurring. The expression of mRNA for the retinoic acid receptor RARα is also highest at stage VIII in the rat testis, and this receptor is present in preleptotene spermatocytes as well as in round spermatids.[244] This receptor is required for synchronization of the spermatogenic cycle, and in its absence, preleptotene spermatocytes do not proceed to leptotene in the first, second and third waves.[245,246] However, others have shown that in mice lacking plasma RBP, Vitamin A deficiency does not delay the entry of preleptotene spermatocytes into meiosis, while spermatogenesis is blocked by delayed or arrested differentiation of spermatogonia.[247-250] This suggests that Vitamin A may have several functions in the testis, and furthermore, there may be important difference between mice and rats in the responses of their testes to Vitamin A deficiency.[247]

The observation that when spermatogenesis is restored in previously Vitamin A-deficient rats, spermatocytes progress to pachytene but then degenerate until the barrier is reformed,[186] would add emphasis to the need for the barrier for complete meiosis. Likewise, the finding that the barrier is disrupted by the injection of a 22-amino acid peptide corresponding to the second loop of occludin, accompanied by a cessation of spermatogenesis[82] would strongly emphasise the importance of the barrier for spermatogenesis.

However, as already mentioned, there are a number of conditions in which spermatogenesis is disrupted but the barrier function appears to be intact, suggesting that other factors are also important for normal sperm production. Nevertheless, the blood-testis barrier remains an important factor in the physiology of the testis, in particular in relation to spermatogenesis.

Future Directions

There are a number of lines of research on the blood-testis barrier which could yield important results in the future. First, possible roles of the endothelial and peritubular cells in regulating entry of substances into the testis or of influencing the Sertoli cell barrier need reevaluating. This is because of the many peculiarities of the testicular endothelial cells, many of which they share with brain endothelial cells, the site of the blood-brain barrier[3,4] and the recent demonstration of transport systems for urea in the peritubular cells. Studies on endothelial cells should

now be possible following the recent demonstration that these cells can be isolated from rat testes, and that when cocultured with interstitial cells, the endothelial cells enhance the production of testosterone.[251] Techniques for isolation and culture of peritubular cells have been available for some years.[63,64] While cocultures of peritubular and Sertoli cells have been used[252] to study basement membrane gene expression, and the effect of proteins from pachytene spermatocytes[253] and spermatids[254] have been used to study their effects on secretion by Sertoli cells, no-one appears to have used cocultures of peritubular or germ cells and Sertoli cells in bicameral chambers (as illustrated in Fig. 1B,C in ref. 46) to study the effects of other cells on barrier function.

However, probably the most interesting problem in this area is the mechanism by which the Sertoli cell barrier is opened and closed again to allow the passage of the developing germ cells. Various theories have been advanced[48] but more evidence is needed on local factors controlling the distribution of this process in relation to spatially and temporally determined stages of spermatogenesis. Related to this problem is the need for an explanation of the occurrence in fetal testes of the structural proteins associated with the Sertoli-Sertoli cell junctions, occludin and claudin.

One new area of interest in relation to the blood-testis barrier is the involvement of specific transport proteins, such as Pgp and MDR. These may have important toxicological consequences in determining whether a particular compound disrupts spermatogenesis. It is conceivable that toxins could either be normally excluded or concentrated inside the tubules by these transporters, and further information on their distribution and specificity is needed. This may be particularly important for the disruptors of the barrier, cadmium salts and glycerol, and studies on the transport of these substances should be undertaken.

Finally, there is the old question of the role of the barrier in creating the conditions necessary for meiosis which needs further study. Recent progress in stem cell transplantation[235] and in vitro spermatogenesis[237] may provide the tools for further study of this fascinating problem.

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