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Expression of connexin 43 in human testis

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Abstract In order to further characterize the Sertoli cell state of differentiation, we investigated the expression of connexin 43 (cx43) protein in the testis of adult men both with normal spermatogenesis and associated with spermatogenic impairment, since cx43 is first expressed during puberty. Cx43 protein was found as a single 43-kDa band on western blots of extracts of normal human testicular material. Cx43 immunoreactivity was generally present between Leydig cells. Within the normal seminiferous epithelium cx43 immunoreactivity was localized between adjacent Sertoli cells, except at stages II and III of the seminiferous epithelial cycle when primary spermatocytes cross from the basal to the adluminal compartment suggesting a stage-dependent Sertoli cell function. While testes with hypospermatogenesis and spermatogenic arrest at the level of round spermatids or spermatocytes revealed a staining pattern similar to that of normal adult testis, the seminiferous tubules showing spermatogenic arrest at the level of spermatogonia and Sertoli-cell-only syndrome were completely immunonegative. We therefore assume that severe spermatogenic impairment is associated with a population of Sertoli cells exhibiting a stage of differentiation deficiency.

Introduction

Gap junctional protein channels are localized in the plasma membranes of adjacent cells. Each channel consists of two hemichannels which are formed by a hexameric assembly of connexins, a so-called connexon. The capacity to assemble channels with different connexins presents the opportunity for cells to form functionally diverse gap junctions with tissue-specific regulatory properties. Gap junctions are selectively permeable to a variety of molecules. The permeability is limited by molecular size (<1 kDa; Simpson et al. 1977) and electronegativity (Flagg-Newton et al. 1979). In non-excitable cells, gap junctions are thought to be involved in the regulation of development and differentiation (Bennett et al. 1991). The tissue-specific distribution of connexins suggests various functions in different tissues (Beyer et al. 1987, 1989; Yancey et al. 1989; Hendrix et al. 1992).

It is assumed that, within the seminiferous epithelium, structure, development, and metabolism of germ cells are regulated by paracrine communication pathways mediated by Sertoli cells (review by Jegou 1993). Sertoli cells form gap junctions with spermatogonia (McGinley et al. 1979) and are in contact with each other via a junctional complex of adhering, gap, and tight junctions constituting the anatomical basis of the blood–testis barrier (reviews by Dym and Fawcett 1970; Russell and Peterson 1985; Pelletier and Byers 1992). In the normal adult seminiferous epithelium, primary spermatocytes migrate from the basal to the adluminal compartment. This migration is associated with a modulation of the Sertoli-Sertoli cell junctions (Russell 1977; Dym and Cavicchia 1978; Connell 1980; Pelletier 1986; Bergmann et al. 1989). These data suggest the presence of gap junctional cell-to-cell communications constituting the basis of coordination between formation and breakdown of the Sertoli-Sertoli cell junctions and the germ cell differentiation.

So far, five types of connexins have been demonstrated in rodent testes (Table 1). Applying immunohistochemistry, connexin (cx)37 was solely found in endothelia of blood vessels (Tan et al. 1996). While Sertoli cells express cx26, cx32, cx33, and cx43 (Risley et al. 1992; Pelletier 1995; Tan et al. 1996), Leydig cells are solely immunopositive for cx43 (Risley et al. 1992; Perez-Armendariz et al. 1994; Pelletier 1995; Tan et al. 1996)

This paper is dedicated to Prof. Dr. Karl-Heinz Wille on his 65th birthday

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Table 1 Connexins investigated so far in the testis. (*cx* Connexin, *NB* northern blot, *WB* western blot, *IHC* immunohistochemistry, *PCT* patch-clamp technique, *Lc* Leydig cells, *Sc* Sertoli cells, *ec* endothelial cells, *pc* peritubular cells)

Connexins	Species	Methods	Testicular localization	References
cx26	Mouse, rat	NB, WB	Testis	Risley et al. 1992
		IHC	Sc	
cx32	Mouse, rat	NB, WB	Testis	Risley et al. 1992
		IHC	Sc	
cx33	Rat	NB	Testis	Haeflinger et al. 1992
	Xenopus, rat	NB, PCT	Testis	Chang et al. 1996
	Rat	IHC	Sc	Tan et al. 1996
cx37	Rat	NB	Testis	Haeflinger et al. 1992
	Xenopus, rat	NB, PCT	Testis	Chang et al. 1996
	Rat	IHC	ec of blood vessels	Tan et al. 1996
cx43	Mouse, rat	NB, WB	Testis	Kadle et al. 1991
	Mouse, rat	NB, WB	Testis	Risley et al. 1992
		IHC	Lc, Sc, pc	
	Mouse	WB	Testis	Perez-Armendariz et al. 1994
		IHC	Lc	
	Mouse	PCT	Lc	Varanda and de Carvalho 1994
	Guinea pig, mink	IHC	Lc, Sc	Pelletier 1995
	Xenopus, rat	NB, PCT	Testis	Chang et al. 1996
	Rat	NB, WB	Testis	Cyr et al. 1996
	Rat	IHC	Lc, Sc	Tan et al. 1996
	Mouse	WB, IHC	Sc (42GPA9 cell line)	Lablack et al. 1998

as are peritubular cells (Risley et al. 1992). Recently, cx43 has, in addition, been identified in cultured Sertoli cells by immunofluorescence (Lablack et al. 1998). Fetal testes of cx43 knock-out mice exhibit a 50% reduction of germ cells (Juneja et al. 1996). However, the involvement of cx43 in spermatogenesis remains unclear, since the newborns of these mice die because of a heart defect.

In rat testis, cx43 occurs in Sertoli cells together with the pubertal onset of spermatogenesis and is involved in intercellular communication and, therefore, plays a vital role in the regulation of spermatogenesis (Risley et al. 1992). In order to further characterize the Sertoli cell state of differentiation, we investigated the expression of cx43 protein, for the first time, in the testis of adult men both with normal spermatogenesis and associated with spermatogenic defects. Our data demonstrate the involvement of cx43 in the regulation of normal human spermatogenesis as found in other species and add further insight into the Sertoli cell state of differentiation associated with severe spermatogenic impairment. The absence of cx43 is associated with an arrest of spermatogenesis at the level of spermatogonia and Sertoli-cell-only (SCO) syndrome.

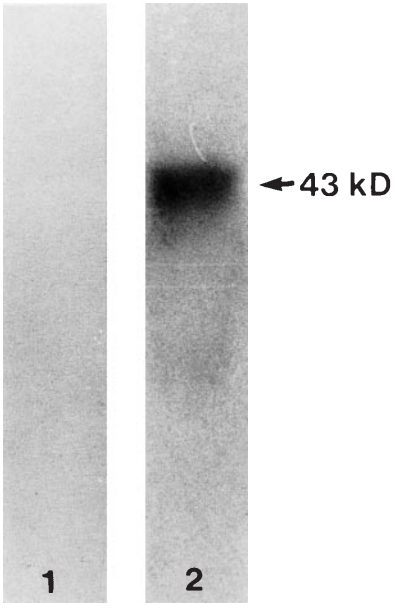

Fig. 1 The monoclonal antibody against human connexin 43 (cx43) revealed a single immunoreactive band at 43 kDa on western blot analysis from protein extracts of normal human testicular tissue (*2*). *1* Control

Material and methods

Materials

Investigation was performed on biopsies of 12 patients (age: 26–59 years; mean: 36.2 years) with obstructive azoospermia and histologically normal spermatogenesis (score ≥3 8, according to Holstein and Schirren 1983) as well as on biopsies of 50 infertile patients (age: 19–48 years; mean: 39.8 years). In the latter cases, the seminiferous tubules showed spermatogenic defects such as hypospermatogenesis or arrest of spermatogenesis at the level of round spermatids, spermatocytes, or spermatogonia, or SCO syndrome. The testicular biopsy specimens were fixed by immersion in Bouin's fixative and embedded in paraffin using standard techniques. For extraction of protein, four testes from 2 patients (age: 52 and 66 years) with prostatic carcinoma were used. Histological analyses of these testes revealed normal spermatogenesis.

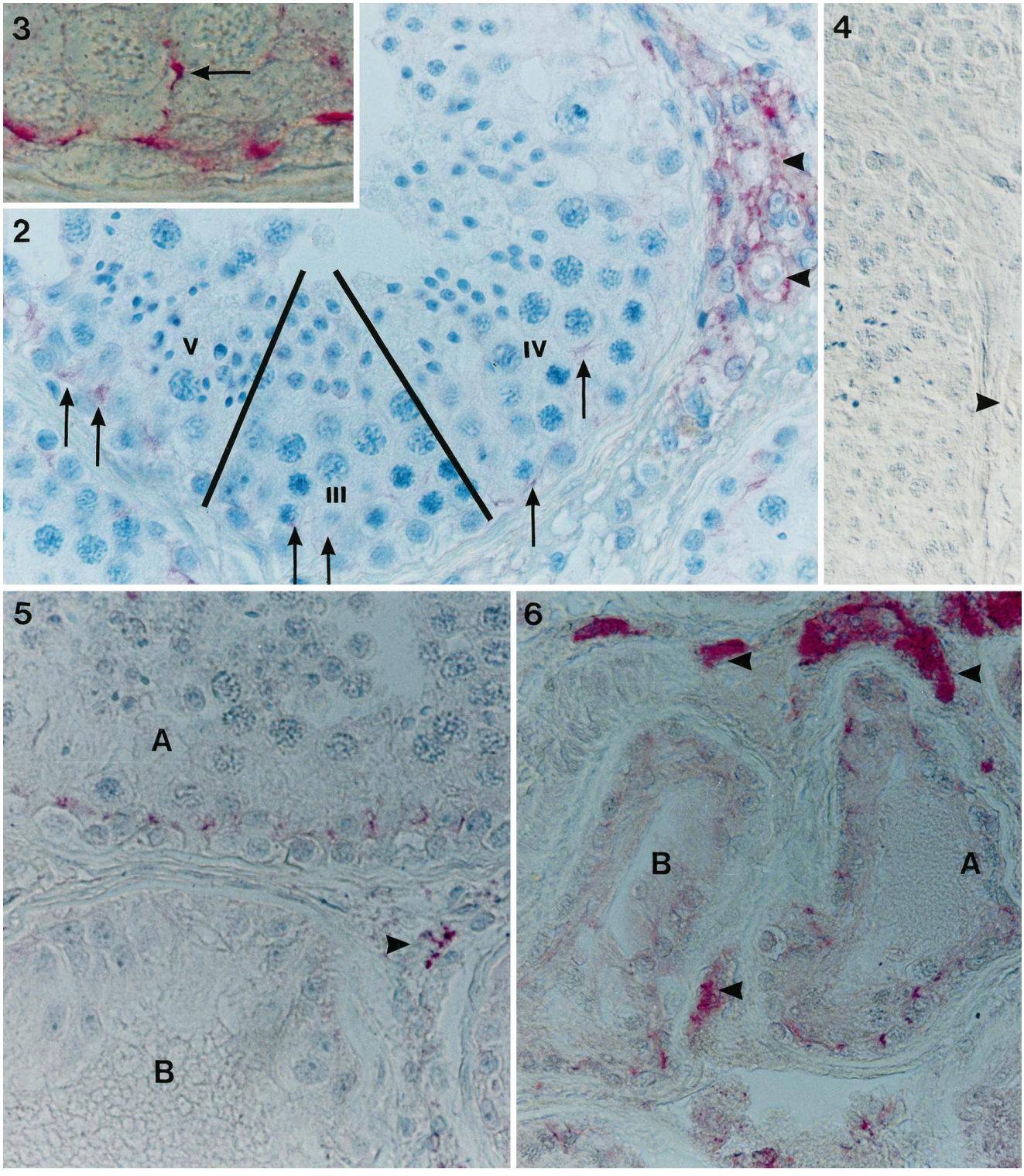

Fig. 2 Seminiferous tubule showing stages III, IV, and V of the seminiferous epithelial cycle (staging according to Clermont 1963). *Arrows* point to the Sertoli cell junctional complex. Cx43 immunostaining is nearly absent at stage III, but clearly demonstrable at stage IV and stage V. *Arrowheads* point to a group of Leydig cells. Here, cx43 protein is localized on the cellular membrane. Both nucleus and cytoplasm are immunonegative. Magnification ×750

Fig. 3 Cx43 immunostaining occurred apical to spermatogonia and basal to primary spermatocytes, as well as along the Sertoli cell junctional complex (*arrow*). Magnification ×1950

Fig. 4 Negative control. *Arrowhead* points to immunonegative Leydig cells. Magnification ×350

Fig. 5 Cx43 protein is present in tubule *A* showing spermatogenic impairment at the level of round spermatid, but is absent in tubule *B* showing Sertoli-cell-only syndrome. *Arrowhead* points to immunopositive Leydig cells. Magnification ×750

Fig. 6 Cx43 protein is present in tubule *A* showing spermatogenic impairment at the level of spermatocytes, but is absent in tubule *B* showing spermatogenic impairment at the level of spermatogonia. *Arrowheads* point to immunopositive Leydig cells. Magnification ×750

Protein extraction and western blot analysis (Fig. 1)

Protein extraction was carried out on orchiectomized testes following the protocol from Cyr et al. (1996). In brief, the material was homogenized in 0.1 M PBS containing 1% SDS, 2 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 2 mM EDTA, 50 mM sodium fluoride, and 0.5 mM sodium orthovanadate. Proteins were boiled for 5 min and then separated by a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, blotted onto a Poly-Screen PVDF transfer membrane (Dupont NEN, Bad Homburg, Germany), and probed with the monoclonal anti-cx43 antibody (1:500; Biotrend, Cologne, Germany). The treatment of the membrane followed the ECL protocol (Amersham, Braunschweig, Germany). As secondary antibody, a peroxidase-conjugated goat anti-mouse antibody (1:50,000; Dianova, Hamburg, Germany) was used. Finally, the membrane was treated with western blot chemiluminescence reagent for non-radioactive detection of proteins (Dupont NEN) and exposed to a Kodak X-OMAT AR film. Control western blots were carried out omitting the primary antibody.

Immunohistochemistry

Sections (5 µm) were mounted on slides coated with 3-aminopropyltriethoxysilane (Sigma, Deisenhofen, Germany). After deparaffinization and rehydration, sections were blocked with bovine serum albumin for 30 min and then incubated with the anti-cx43 primary antibody (1:100; Biotrend) overnight at 4°C. The immunoreaction was visualized using Dako-EnVision (goat anti-mouse conjugated to alkaline phosphatase; Dako, Hamburg, Germany) and HistoMark Red (KPL, Md., USA). Finally, sections were mounted in Dako-Glycergel. For each test, control incubations were performed by substituting the primary antibody by buffer.

Results

The monoclonal antibody against human cx43 revealed a single immunoreactive band at 43 kDa on western blots from extracts of normal human testicular material (Fig. 1). Immunohistochemistry revealed a focal to linear localization of the cx43 protein on the cellular membrane between neighboring Leydig cells (Fig. 2). In seminiferous tubules with normal spermatogenesis, cx43 immunoreactivity occurred apical to spermatogonia and basal to primary spermatocytes. This localization is similar to the Sertoli cell junctional complex constituting the anatomical basis of the blood–testis barrier. The immunopositive signal for cx43 was drastically reduced or absent during stages II and III of the seminiferous epithelial cycle (staging according to Clermont 1963) when primary spermatocytes cross from the basal to the adluminal compartment of the seminiferous epithelium (Figs. 2, 3, 4). While seminiferous tubules with hypospermatogenesis and spermatogenic arrest at the level of round spermatids or spermatocytes revealed a staining pattern which is similar to that in normal testis (Figs. 5, 6), seminiferous tubules with spermatogenic arrest at the level of spermatogonia and SCO characteristics were completely immunonegative for the anti-cx43 antibody (Figs. 5, 6).

Discussion

Gap junctions have been demonstrated between Leydig cells (Kawa 1987; Risley et al. 1992; Perez-Armendariz et al. 1994; Varanda and de Carvalho 1994), between Sertoli cells (Dym and Fawcett 1970; Gilula et al. 1976; Pelletier and Friend 1983; Risley et al. 1992; Lablack et al. 1998), and between Sertoli cells and germ cells (Russell 1977; McGinley et al. 1979; Parvinen et al. 1986). So far, five connexins have been described in adult rodent testes: cx26, cx32, cx33, cx37, and cx43 (see Table 1). Multiple connexin expression may facilitate the establishment of communication pathways (Paul 1995). Cx26, cx32, cx37, and cx43 have been found to make functional channels either by themselves or as hybrid channels. However, cx33 seems to inhibit functional expression of other connexins. It is suggested that within the seminiferous epithelium such an inhibitory function of cx33 may cause both asynchronous maturation of germ cells and communication between Sertoli cells and germ cells (Chang et al. 1996). The expression of gap junctional cx43 was shown to be dependent on cell type, testis maturation, and stage of the seminiferous epithelial cycle (Risley et al. 1992; Pelletier 1995; Tan et al. 1996).

Leydig cells revealed a focal to linear localization of cx43 protein on the cellular membrane, in all sections studied, corresponding to the extensive gap junctions of Leydig cells known from electron microscopy and electrophysiological investigations (Perez-Armendariz et al. 1994; Varanda and de Carvalho 1994). Therefore, this cell type can serve as a positive control within the section.

Gap junctions between adjacent Sertoli cells or Sertoli cells and germ cells are thought to facilitate the coordination of Sertoli cell metabolism and promote the cell signaling between Sertoli cells and germ cells, respectively (Risley et al. 1992). There is evidence suggesting an interdependence of gap junction communication and cellular adhesion as well as a stage-dependent cx43 expression being associated with changes in Sertoli cell adhesion and reorganization of the basal Sertoli cell junctions in order to facilitate spermatocyte movement from the basal to the adluminal compartment. The immunoreaction of cx43 remains low well after spermatocyte translocation is complete (Risley et al. 1992; Tan et al. 1996).

During puberty, the Sertoli cell junctional barrier in rat (Bergmann and Dierichs 1983) and man (Landon and Pryor 1981) is not synchronously established along the length of the seminiferous tubules, but in accordance with the stage of meiosis in the associated germ cells. Sertoli cells form an effective barrier only in those parts of the seminiferous tubules where germ cells are in the leptotene and zygotene stage of meiosis (Russell 1977; Connell 1978, 1980; Dym and Cavicchia 1978; Bergmann and Dierichs 1983; Pelletier 1986, 1988; Cavicchia and Miranda 1988).

During spermatogenesis, the distribution pattern found in man coincided with the location of the

blood–testis barrier demonstrated in the guinea pig (Pelletier and Friend 1983). Cx43 immunoreactivity was demonstrated between adjacent Sertoli cells occurring as a ring which is localized apical to spermatogonia and basal to primary spermatocytes. The immunopositive signal for cx43 was drastically reduced or absent during stages II and III of the seminiferous epithelial cycle suggesting a stage-dependent Sertoli cell function. However, the presence of gap junctions between Sertoli cells and spermatogonia cannot be excluded, because the number of this type of gap junction may be too low (Russell and Peterson 1985) to show a positive immunoreactivity.

While testes with hypospermatogenesis and spermatogenic arrest at the level of round spermatids or spermatocytes revealed an immunostaining pattern similar to that of normal adult testis, the seminiferous tubules showing spermatogenic arrest at the level of spermatogonia and SCO characteristics were completely immunonegative. While in man Cavicchia et al. (1996) was unable to observe intact gap junctions in seminiferous tubules with SCO characteristics, Mayer et al. (1996) did. On the basis of ultrastructural examination, it has been shown that two kinds of Sertoli cell are present in SCO, undifferentiated and normal cells (Nistal et al. 1990; Terada and Hatekayama 1991). The seminiferous tubules showing spermatogenic arrest at the level of spermatogonia and SCO characteristics are completely immunonegative assuming that severe spermatogenic impairment is associated with a population of Sertoli cells exhibiting a stage of differentiation deficiency and confirms former studies investigating the distribution pattern of cytokeratin 18 and anti-Müllerian hormone as prepubertal differentiation markers (Bergmann and Kliesch 1994; Steger et al. 1996, 1998).

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