Aberrant Cytoplasmic Accumulation of Connexin 43 in Human Testicular Seminoma

V. Mauro[1], D. Chevallier[2], J. Gilleron[1], N. Defamie[3], D. Carette[1], J.M. Gasc[4], F. Senegas-Balas[1], D. Segretain[1] and G. Pointis*[,1]

[1]INSERM U 895, Team 5 « Physiopathologic control of germ cell proliferation: genomic and non genomic mechanisms», 151 route Saint-Antoine de Ginestière, BP 2 3194, 06204 Nice cedex 3, France and Université Paris Descartes, 45 rue des Saint-Pères, 75006, Paris, France; [2]Department of Urology, Pasteur Hospital, Nice, France; [3]Institut de Physiologie et Biologie Cellulaire, UMR-CNRS 6187, Université de Poitiers, France and [4]INSERM U833, Collège de France, Paris, France

Abstract: In the present study Cx43 mRNA and protein were analyzed in germ cells of men with normal spermatogenesis and in human testicular seminoma. In normal testis Cx43 mRNAs were basally located within seminiferous tubules and expressed in the most basally located germ cells (spermatogonia, early spermatocytes, and pachytene spermatocytes) and in Sertoli cells. Immunofluorescence analysis showed that Cx43 signal was mainly located in the basal compartment of seminiferous tubules and was stage-dependent. Cx43 mRNAs were also detected in human testicular seminoma. Transcripts were present within seminoma cells identified by PLAP staining. However, Cx43 protein exhibited an intracytoplasmic accumulation, within an intracellular compartment distinct from the Golgi apparatus and was undetectable at the plasma membrane level, suggesting post-translational rather than transcriptional abnormalities. This aberrant intracytoplasmic accumulation of Cx43 is due neither to a dysfunction of the protein trafficking machinery nor to a specific alteration of its major protein partner, ZO-1, since the tight junction associated protein was detected at the plasma membrane level and did not colocalize with Cx43.

INTRODUCTION

Gap junctions are clusters of plasma membrane channels, formed of proteins called connexin (Cx), which allow the intercellular exchange of small signaling molecules (< 1kD) between the cytoplasmic compartments of two adjacent cells [1]. Gap junctional intercellular communication (GJIC) is involved in the regulation of cellular growth, tissue differentiation, homeostasis and neoplasic transformation [2].

There is now strong evidence that mutated Cx genes could result in human genetic disorders [3]. Specifically, impaired GJIC and Cx dysfunction have been described as a typical feature of cancer *per se* and have been associated with the actions of carcinogens. Indeed, altered GJIC has been shown to correlate with neoplastic transformation in several human tissues [4]. In many cancer cases this alteration has been associated with aberrant intracytoplasmic localization of Cx proteins [5, 6].

In the rodent testis, the presence of gap junctions was morphologically shown and these results were supported by the identification of mRNAs for at least eleven Cxs [7]. Targeted disruption of Cx43 gene, the predominant Cx in the testis, resulted in a deficiency of germ cell development and initiation of spermatogenesis indicating that Cx43 play a major role in the developing process of germ cells [8-12]. Whether a similar role of Cx43 could be exerted in human was also hypothesized. Gap junctions were described in infertile azoospermic and oligospermic human testes [13] but were undetectable in patients with Sertoli-cell-only seminiferous tubules [14]. In addition, Cx43 protein was localized in both tubular and interstitial compartments of human testis [15] and affected Cx43 levels were found in testis with Sertoli-cell-only syndrome [16-18].

In human testis infiltrated with carcinoma-in-situ or seminoma, Cx43 was undetectable by immunohistochemical approaches [19] and reduced Cx43 mRNA levels were measured in neoplastic human testis [20, 21]. In a previous study using neoplastic cells originating from the JKT1 seminoma cell line we reported that overexpression of Cx43 by transfection of a Cx43-GFP vector not only restores GJIC but also blocks abnormal proliferation of these cells [22]. These observations raised the question of the role of endogenous Cx43 in these tumoral cells.

In the present study, we first identified by in situ hybridization (ISH) and indirect immunofluorescence the specific cells that express Cx43 within the seminiferous tubules of human testis. Second we compared the Cx43 levels between healthy and seminoma human testes. Lastly, to better address the role of Cx43 in the testicular tumoral process we analyzed Cx43 mRNA expression and Cx43 localization in human testis seminoma.

MATERIALS AND METHODOLOGY

Human Tissue Collection and Cell Line

Biopsies of normal testes were performed in young men (28-38 years) with excretory azoospermia due to deferent

*Address correspondence to this author at the Bâtiment Universitaire ARCHIMED, INSERM U 895, C3M, 151 route Saint Antoine de Ginestière, BP 2 3194, 06204 Nice cedex 3, France; Tel: (33) 04 89 06 42 66; Fax: (33) 04 89 06 42 66; E-mail: pointis@unice.fr

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duct agenesis, after informed and written consent, according to INSERM Ethical Guideline (n=8) and approval by the local ethic committee. Three frozen testicular tumors were classified as pure seminoma by placental like alkaline phosphatase (PLAP) positive staining, a specific seminoma marker [23]. The JKT1 cell line that originated from a pure human testicular seminoma [24] was maintained in DMEM supplemented with 10% FBS (Gibco Brl, Cergy Pontoise, France) as previously reported [22].

In Situ Hybridization (ISH)

Briefly, the radiolabelled Cx43 antisense and Cx43 sense probes were generated by *in vitro* transcription and incorporation of ^{35}S-uridine triphosphate (specific ratio activity, 1000 Ci/mmol, Amersham, UK) as previously described [25]. Testicular sections (5 µm) received the antisense RNA probe and the other received the RNA sense probe as background control. After washing, the slides were dipped in NTB2 emulsion (Kodak, Rochester, NY) and exposed at 4°C in lightness boxes for 15 days. Slides were developed, fixed, and counterstained with toluidine blue. Hybridization intensity was quantified using a computer-assisted image analysis system Visilog 4.15 (Noesis, Les Ulis, France) as reported [25]. The intensity of hybridization was expressed as percentage of pixels within a marked area occupied by silver grains that was above a set grey threshold level. Measurements were taken from 10 seminiferous tubules from 3 different sections for each testis. Data were expressed as means ± SEM. A one way analysis of variance ANOVA followed by a Student-Newman-Keuls post hoc test was used to test for differences between cell types. Values were considered statistically different when $p < 0.05$.

Immunofluorescence analysis

The integrity of spermatogenesis was tested by fixing a piece of each testis in aqueous Bouin, which was examined by classical histological procedures. Stages identification of the seminiferous epithelium were identified according to morphological criteria, including the depth and shape of elongate spermatid nuclei in Sertoli cells, the presence or absence of two generations of spermatids (round and elongate spermatids), and the size of the acrosome surmounting the round spermatid nucleus according to criteria previously described [26].

For immunofluorescence analyses, tissues were embedded in OCT embedding compound (Tissue Tek; Miles Inc., Naperville, ILL) and cryosectioned. Sections (7 µm) were then applied to 3-aminopropyltriethoxysilane-coated slides and fixed for 10 min with methyl alcohol at -20°C and treated as described above. Testis sections were incubated for 2 h with the rabbit anti-Cx43 antibody (1:100 Zymed Laboratories, San Francisco, CA) and mouse-anti-vimentin (Sigma-Aldrich, St. Quentin-Fallavier, France) and subsequently incubated with goat anti-rabbit FITC conjugated and goat anti-mouse TRITC conjugated antibodies (Jackson Immunoresearch Laboratories, Baltimore, U.K.). For studies on the JKT1 cell line, cells were fixed in cold methyl alcohol at -20°C for 5 min, washed with PBS and incubated with mouse anti-Cx43 and rabbit anti-ZO-1 antibodies (1:50, Zymed Laboratories) in PBS containing 3% BSA, 1% normal goat serum (Dako, Glostrup, Denmark), overnight at 4°C as previously reported [25]. Cells were rinsed with PBS and then incubated for 1 h with an Alexa fluor 488 conjugated goat anti-rabbit F(ab')$_2$ IgG (1:50, Molecular Probe, Eugene, OR) and a rhodamine Red^{TM}-X-labelled F(ab')$_2$ fragment of goat anti-mouse antibody (1:50, Jackson ImmunoResearch, West Grove, PA) in PBS containing 3% BSA, 1% normal goat serum. After washing with PBS, the slides were mounted in Vectashield medium supplemented by DAPI (Vector Laboratories, Burlingame, CA). For detection of the Golgi apparatus, tissue sections and cells were concomitantly incubated with Cx43 antibody and with a specific Golgi apparatus CTR433 antibody (1/10, a gift from M. Bornens, Institut Curie, Paris France). Slides were then examined with a confocal laser scanning microscope (Leica TCS SP).

High-resolution deconvolution microscopy analysis was performed with a wide field immunofluorescence deconvolution microscope (Nikon TE2000E, Service Commun de Microscopie, IFR Biomédicale des Saint-Pères, Paris, France) and acquired image stacks were deconvoluted using Autoquant image package algorithms as previously described [27].

For PLAP immunolocalization, tissue sections or cells were incubated with a monoclonal mouse anti-PLAP antibody (clone 8A9) purchased from Dako (1/100, Giostrup, Denmark). Slides were subsequently incubated with a Horseradish peroxidase-labelled F(ab')2 fragment of anti-mouse IgG (1:500) for 2 h. Staining was completed by incubation with 3, 3'diaminobenzidine. Slides were counterstained with hemalun and washed in tap water. Controls were performed by incubating slides with whole pre-immune mouse serum (1:100, Jackson ImmunoResearch, West Grove, PA), instead of the primary antisera, or PBS replacing the conjugated antibody.

RESULTS

Analysis of Cx43 mRNA and Protein in Human Normal Germ Cells

In situ hybridization analysis of Cx43 was carried out on testicular biopsies of patients with excretory azoospermia. Histological analyses of these testes revealed normal spermatogenesis (data not shown). Darkfield images demonstrate a high accumulation of silver grains at the base of seminiferous tubules and in the interstitial compartment of human testis with normal spermatogenesis. In addition, the pattern of Cx43 mRNA distribution appeared variable within and between seminiferous tubules (Fig. **1A**). Control sections incubated with the sense probe revealed a low non specific hybridization signal (Fig. **1B**). The human seminiferous epithelium cycle is divided into six stages and at least three spermatogenic stages can be observed in the same seminiferous tubule sections [26]. Three spermatogenic stages have been analyzed here. At stage I, characterized by the presence of two generations of spermatids and pachytene spermatocytes a consistent ISH signal for Cx43 was detected in the basal compartment (Fig. **1C**). The number of silver grains was markedly reduced at stage II identified by the elongated spermatids moving to the luminal aspect of the seminiferous epithelium (Fig. **1D**). The seminiferous tubules corresponding to stage V, where one generation of elongating spermatids having typically pointed and deeply stained nuclei directed toward the limiting membrane, appeared also strongly

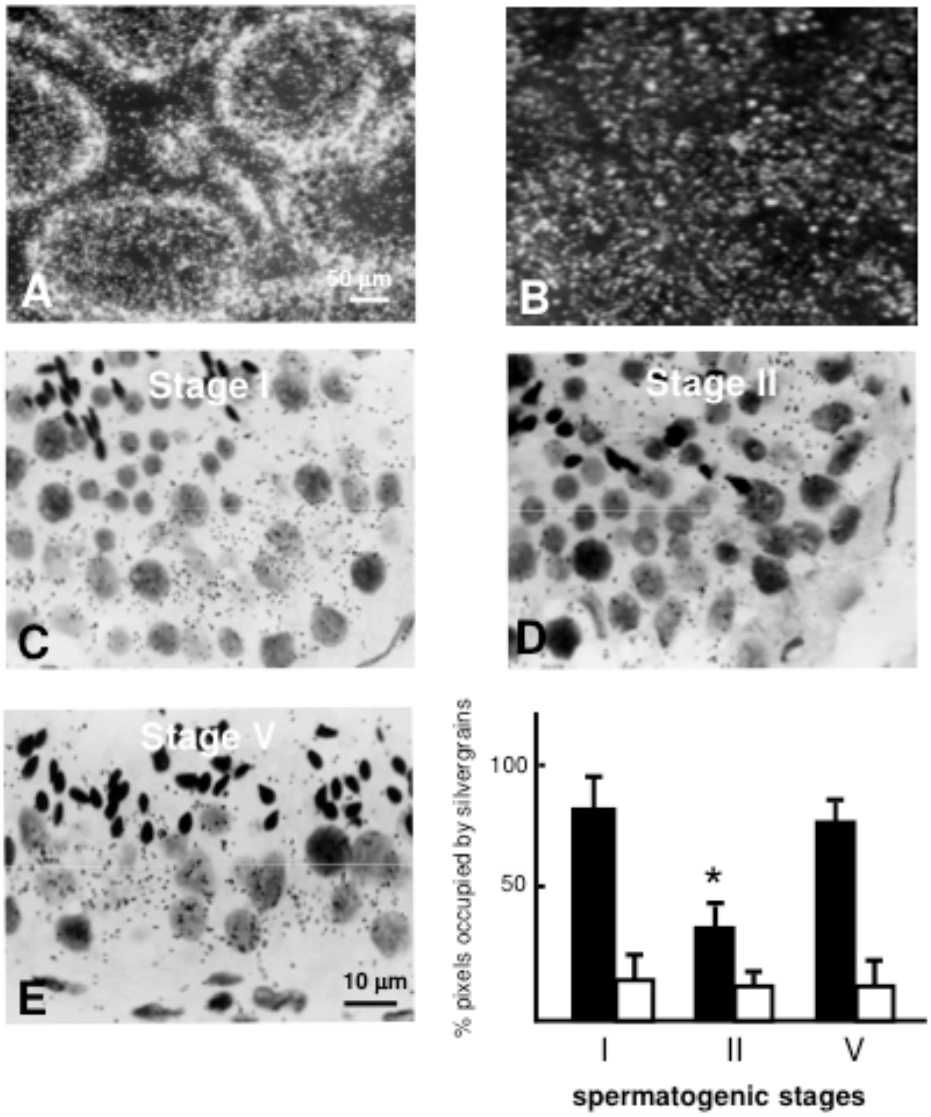

Fig. (1). Stage-dependence localization of Cx43 mRNA in seminiferous tubules of human testis. Darkfield micrographs of seminiferous tubules section hybridized with ^{35}S-labelled antisense (**A**) and sense (**B**) Cx43 RNA probes. **A** and **B** have the same magnification. High magnification of brightfield micrographs illustrates the variation of intensity of Cx43 mRNA distribution among seminiferous tubules. The hybridization signal is consistent at stages I and V of spermatogenesis (**C** and **E**) and relatively reduced at stage II (**D**). Cx43 mRNA are present mostly in the cytoplasms of Sertoli cells and in basally located germ cells. **C-E** have the same magnification. The lower right panel represents semi-quantitative analysis of the Cx43 ISH signals in seminiferous epithelium of human normal testis at stage I, II and V. Representative of three different experiments. The intensity of hybridization was quantified as percentage pixels occupied by silver grains. Black columns represent analysis of antisense hybridization. Open columns represent analysis of sense hybridization. Values are means ± SEM, n=20. $P<0.05$ as compared to stages I and V.

labelled (Fig. **1E**). Semi-quantitative analysis indicated that the intensity of the signal, measured with the antisense probe, was significantly lower ($P< 0.05$) at stage II compared to stages I and V.

High magnifications of the basal ISH signal revealed that silver grains were localized over and/or surrounded the nuclei of spermatogonia (Fig. **2A**), early spermatocytes (Fig. **2B**) and pachytene spermatocytes (Fig. **2C**). A slight ISH signal similar to that observed in control sections incubated with the sense probe (data not shown) was detected in round and elongated spermatids. Semi-quantitative analysis of Cx43 mRNA indicated that the levels of silver grains in spermatogonia and in pachytene spermatocytes were not significantly different from those quantified in the Sertoli cells (right lower panel) suggesting that gap junction communication occurs between the somatic cells and germ cells.

The existence of Cx43 based gap junctions between Sertoli cells and germ cells was verified by the presence of Cx43 immunosignals between a vimentin-positive cell, identified as Sertoli cells, and a vimentin-negative cell corresponding to spermatogonia as suggested by their small cytoplasmic surfaces, the shape of their nuclei and their basal localization within the seminiferous tubule (Fig. **3A**), and between vimentin-positive Sertoli cells (Fig. **3B**).

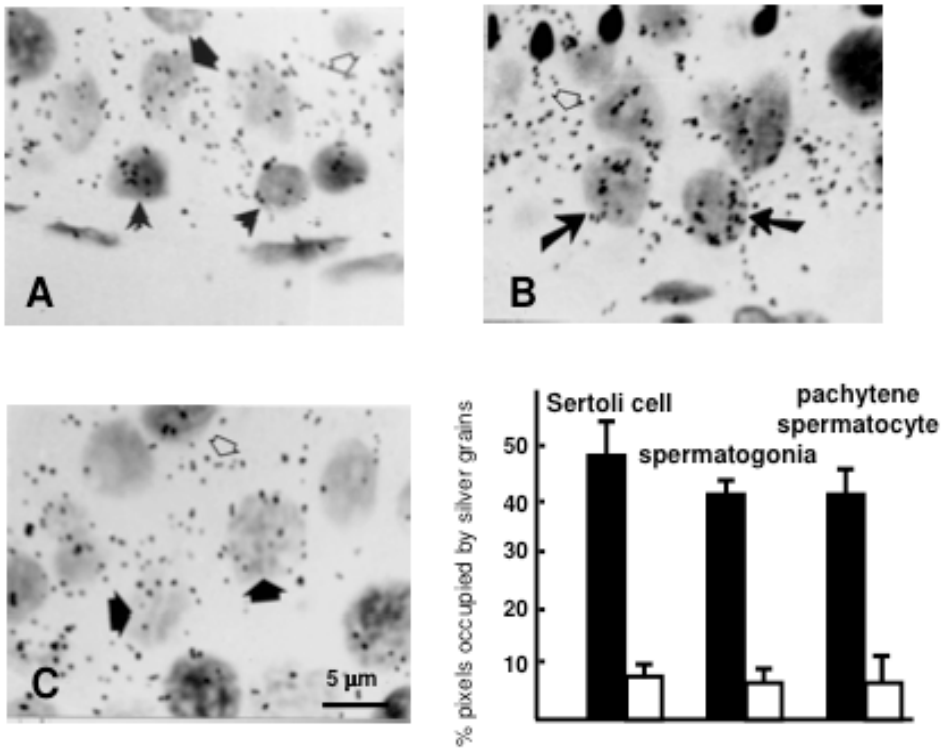

Fig. (2). Localization of Cx43 mRNA in seminiferous tubules of human testis. High magnification of the basally located ISH signal shows that silver grains are associated with spermatogonia (**A**, arrowheads), early spermatocytes (**B**, small arrows) and pachytene spermatocytes (**C**, large arrows). ISH signal was also detected over the intercellular germ cell spaces which represent the Sertoli cell cytoplasm (open arrows in **A**, **B** and **C**). **A-C** have the same magnification. Representative of three different experiments. The lower right panel represents semi-quantitative analysis of Cx43 ISH signal in seminiferous epithelium of human normal testis. The intensity of hybridization in Sertoli cells, spermatogonia and pachytene spermatocytes was quantified as percentage pixels occupied by silver grains. Black columns represent analysis of antisense hybridization. Open columns represent analysis of sense hybridization. Values are means ± SEM, n=30.

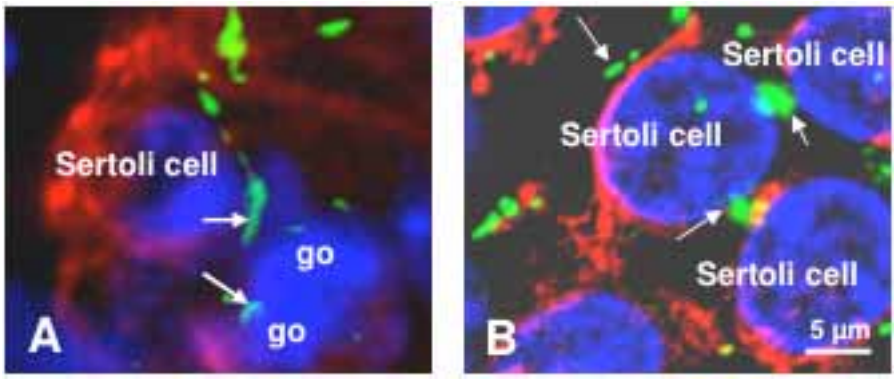

Fig. (3). Immunolocalization of Cx43 within the seminiferous epithelium of human testis. (**A**) Localization of Cx43 (green fluorescence, arrows) between a vimentin-positive cell identified as a Sertoli cell (red fluorescence) and spermatogonia (go) identified by the shape of their nuclei and their localization within the seminiferous tubule. Cell nuclei are identified by Dapi staining. (**B**) Localization of Cx43 (green fluorescence, arrows) between vimentin-positive Sertoli cells (red fluorescence). Representative of three different experiments. **A** and **B** have the same magnification.

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Cx43 mRNAs were detected in the three human testicular seminoma analyzed. As shown in Fig. **4A**, darkfield micrographs of human seminoma hybridized with labelled antisense probe revealed a high accumulation of silver grains that were distributed on all the surface of the seminoma section. High magnifications of brightfield micrographs showed that Cx43 mRNAs were mainly located in the cytoplasms of seminoma cells (Fig. **4B**, arrows). No IHS signal was detected in lymphocytes (double arrowhead) or in red blood cells present in the tumor. Control sections incubated with the sense probe revealed a slight unspecific signal (Fig. **4B**, inset).

As expected seminoma cells were PLAP-positive (Fig. **4C**). Immunofluorescence analysis revealed strong Cx43

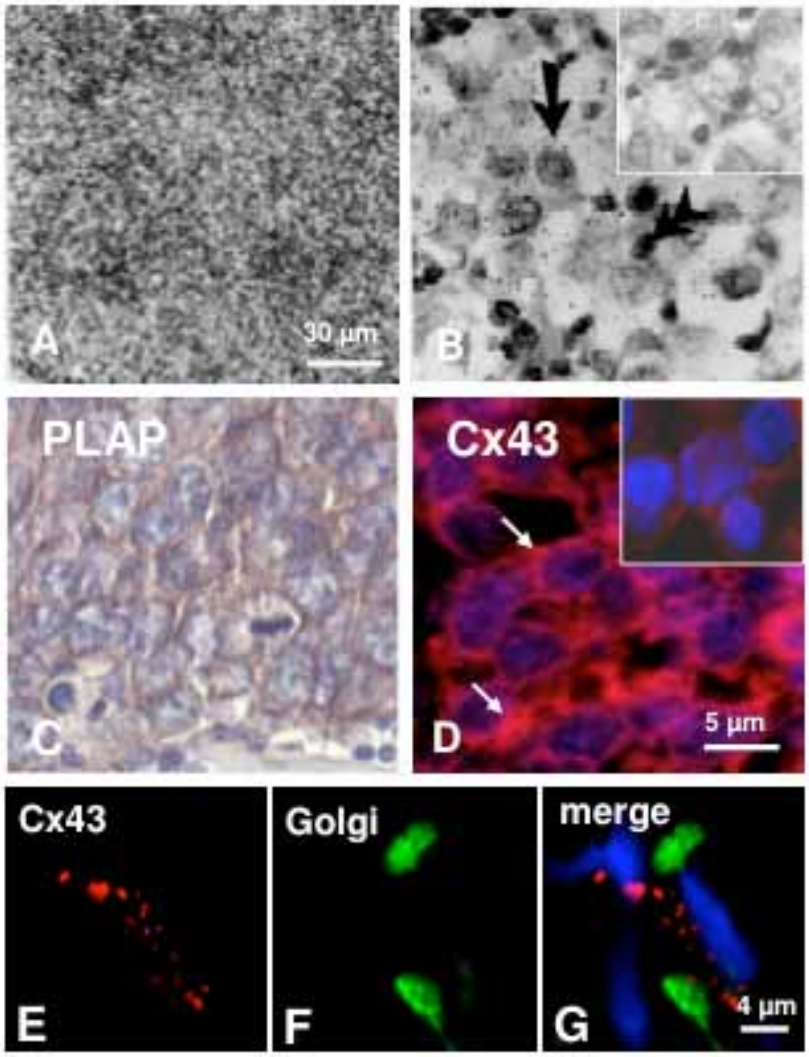

Fig. (4). Cx43 mRNA and Cx43 protein in pure human testicular seminoma. (**A**) Darkfield micrographs of human seminoma hybridized with ^{35}S-labelled antisense Cx43 mRNA probe. (**B**) High magnification of brightfield micrographs shows that silver grains were mainly located in the cytoplasm of seminoma cells (arrow) whereas they were absent in lymphocytes (double arrowhead). Control sections incubated with the sense probe revealed a slight but unspecific signal (inset). (**C**) All seminoma cells appear PLAP-positive. A signal for Cx43 protein was detected in the majority of seminoma cells with perinuclear localization (**D**, arrows). No specific Cx43 specific staining was detected in control cells (**D**, inset). (**E-G**) Immunodetection of Cx43 (red fluorescence) and of Golgi apparatus (green fluorescence) by high-resolution deconvolution microscopy. Cell nuclei are identified by Dapi staining. Representative of three different experiments. **B-D** and **E-G** have the same magnification respectively.

signals within the cytoplasms of these cells (Fig. **4D**) and their total absence at the cell–cell contacts as classically described in normal communicating cells. No distinct Cx43 immunoreactive signal was detected in control cells (Fig. **4D**, inset). The Cx43-antibody mainly labelled a perinuclear region that might be identified as Golgi-like structures (Fig. **4D**). To verify this hypothesis high-resolution deconvolution microscopy was applied on the same testicular sections immunolabelled with Cx43 and a specific marker of the Golgi apparatus (Figs. **4E-G**). In no cases, Cx43 colocalized with the Golgi apparatus. As observed in testicular tumoral tissues, seminoma JKT1 (Fig. **5A**) that expressed PLAP (Fig. **5A**, inset) exhibited a perinuclear intracytoplasmic localization of Cx43 (Figs. **5C**,**D**,**G**). This localization contrast with the Cx43 membranous level observed in the 42GPA9 Sertoli cell line used as a control (Fig. **5D**, inset). Application of high-resolution deconvolution microscopy demonstrated that this intracytoplasmic Cx43 labeling in most cases did not colocalized with the specific Golgi apparatus marker (Figs. **5E**,**F**). Dual immunofluorescence analysis with antibodies directed against Cx43 and ZO-1 also demonstrated that the two proteins did not colocalize within the same cellular compartment (Figs. **5G-I**). In contrast to Cx43, ZO-1 was never detected within the cytoplasm of JKT1 cells but appeared localized as small dots at the zone of contact between adjacent cells (Fig. **5H**).

DISCUSSION

Although there is compelling evidence that Cx43 gap junctions occurred, between Sertoli cells, the presence of Cx43 between the somatic cells and germ cells are less evidenced. The current in situ hybridization study clearly demonstrates that Cx43 transcripts are present in both Sertoli cells and basally located germ cells: spermatogonia, early

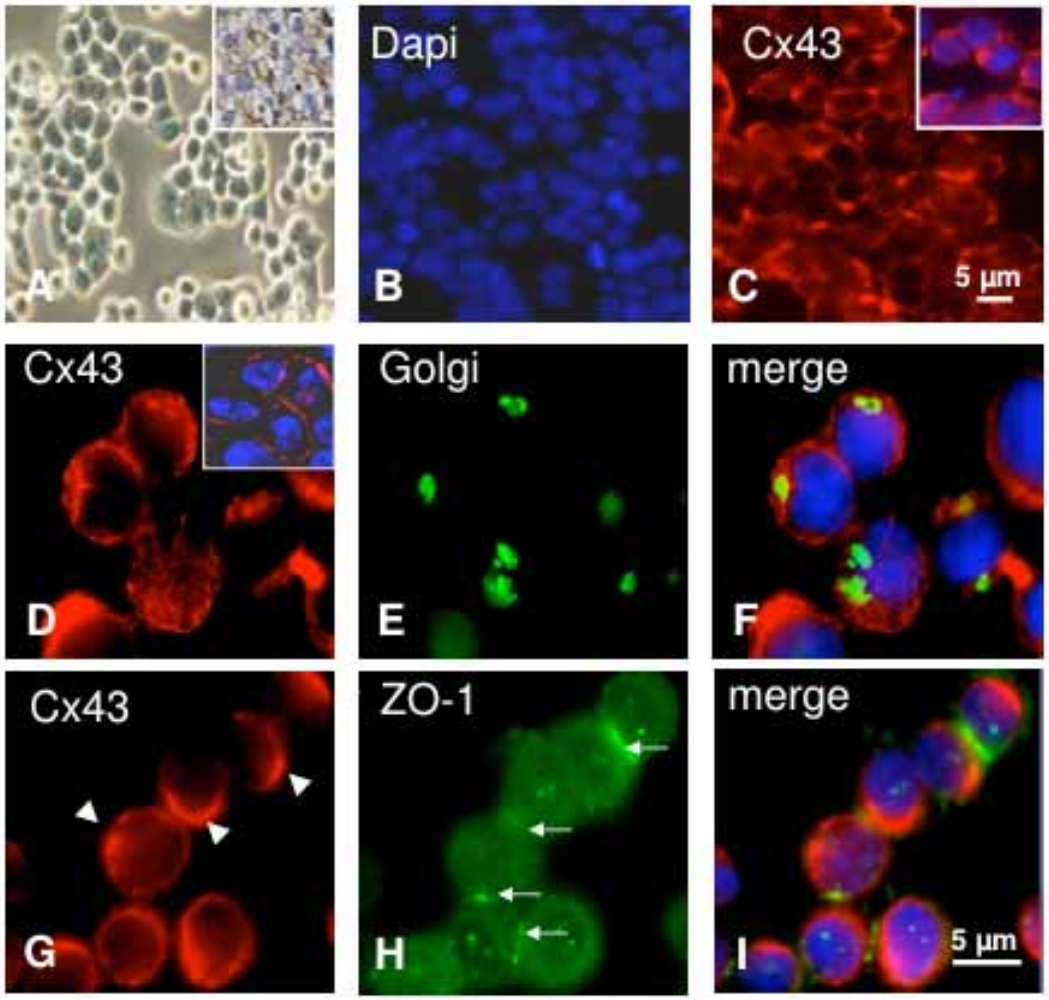

Fig. (5). Immunolocalization of PLAP, Cx43 and ZO-1 in the JKT1 cell line. (**A**) Phase contrast microscopy of cultured JKT1 cells and analysis of the expression of PLAP (inset). (**C**) Immunodetection of Cx43 in JKT1 cells. Cell nuclei are identified by Dapi staining (**B**) and inset in C reveals both labeling. (**D-F**) and (**G-I**) Dual immunofluorescence of Cx43 (red fluorescence in **D** and **G**) and the Golgi apparatus (green fluorescence in **E**) or ZO-1 (green fluorescence in **H**) in JKT1 cells analyzed by high-resolution deconvolution microscopy. The inset in D represents a positive control for Cx43 membranous localization observed in a Sertoli cell line. Note that Cx43 does not colocalize either with the Golgi apparatus or with ZO-1 (**F** and **I**). Representative of three different experiments. **D-I** have the same magnification.

spermatocytes and pachytene spermatocytes supporting recent findings [21]. In addition the present observation that Cx43 transcripts are equally distributed in the somatic and germinal cells leads to the assumption that channels formed by Cx43 occur between these two cell types. These data do not preclude that other Cxs, detected within the seminiferous epithelium as Cx26 and Cx32 that are able to form channels by them or in association with the two other Cxs, participate in this gap junctional cell-to-cell communication [28]. This hypothesis is, however, not in agreement with previous observations in rodents, which show that Cx43 protein is mainly found in the basal compartment whereas Cx26 and Cx32 are specifically detected in the apical compartment of the seminiferous epithelium [29]. From altogether these data and in agreement with our findings that Cx43 is closed to gap junction channels [30] it is likely that gap junctions, which occur basally between Sertoli and germ cells, are mainly formed by Cx43.

Cx43 gene expression was down-regulated in human testis infiltrated with carcinoma-in-situ or in testicular seminoma [19-21]. For the latter authors the proliferation of neoplasic germ cells in the tumoral testis could result from a loss of Sertoli cell control on germ cells due to altered Sertoli cell Cx43 expression. However, no information was given on Cx43 expression in neoplasic germ cells. The current data, in seminoma testis section and in the JKT1 seminoma cell line, demonstrate that the main characteristic of seminoma cells is an aberrant accumulation of the Cx43 protein within the cytoplasm rather than a reduced Cx43 expression since ISH and immunohistochemical analyses demonstrate the maintenance of high levels of Cx43 mRNA and protein in the pathological tissue. This does not preclude that altered Cx43 gene expression might occur later during the tumoral development. The present findings support our previous data [22] and are in agreement with observations reported in other human tumoral tissues, in which an aberrant localization of Cxs has been observed, such as human liver tumors [31], breast tumors [32], prostate cancers [33] and bladder cancers [34] and more recently in human hepatoma cells [35]. However the role that intracytoplasmic localized Cx could play is

questionable. In addition to be involved in the classical gap junction communication, Cx proteins have been suggested to play additional roles when they are present within the cytoplasm [35, 36] or in the nucleus [37] mainly in tumoral tissues or tumoral cell lines. Thus it appeared interesting to specify the intracytoplasmic nature of Cx43 in seminoma tissue and cell line.

By using conventional imaging technology we previously reported in the JKT1 cell line that Cx43 accumulated in a cellular compartment identified as the Golgi apparatus [22]. Such localization has been also recently reported in hepatocellular carcinoma [35]. By using high-resolution deconvolution microscopy we demonstrated in the present work that Cx43 does not accumulated in the Golgi apparatus in seminoma tissue sections and in the JKT1 seminoma cell line. The precise localization of Cx43 remains to be determined. Since the Cx43 immunolabeling appears randomly distributed within all the cytoplasm, the possibility of impaired trafficking of Cx43 has been hypothesized. Altered global mechanisms implicated in the trafficking of proteins in the cytoplasm are unlikely since transfection of JKT1 cells with a tagged Cx43-GFP vector allows accumulation of the green fluorescent Cx43 protein to the plasma membrane level and concomitantly inhibit abnormal proliferation of these tumoral cells [22]. This hypothesis is also supported by the observation that the tight junction associated protein ZO-1 was detected at the plasma membrane level. Abnormality in Sertoli cell functions during testicular tumor progression has been suggested [38, 39]. Thus it is possible that the aberrant Cx43 localization result from a loss of normal Sertoli cells as regulators of Cx43 trafficking and assembly at the plasma membrane of seminoma cells [21]. If it is the case, it would be interesting to characterize the paracrine effectors from the Sertoli cells, which could be able to control cytoplasmic Cx43 trafficking. Cxs are also capable of interacting with a large number of proteins that control not only their ability to form functional channels but also their turnover to the membrane, formation, stability and internalization of gap junction plaques [40]. Thus it may be hypothesized that one of these partners abnormally associates with Cx43 and sequesters the latter within the intracytoplasmic compartment. To date the nature of this protein is unknown. Among the large number of Cx partners characterized [41], Zonula Occludens 1 (ZO-1) has been reported to be the major Cx interacting protein and an essential role of this protein in the turnover of gap junction formation and degradation has been postulated [42-45]. The current data clearly show that Cx43 and ZO-1 did not colocalize suggesting that ZO-1 is not the partner of Cx43 responsible for this specific cytoplasmic accumulation. In contrast ZO-1 was mainly present at the plasma membrane level between adjacent cells confirming that the general process involved in the trafficking of membranous proteins to the membrane was probably not affected. Whether other interacting proteins could play this role remains to be investigated. There is also evidence that the phosphorylation status of the Cx43 C-terminus could play a role in regulating Cx43 trafficking, assembly/disassembly and internalization and is under the control of various kinases [46]. Among them the Src tyrosine kinase that has been implicated in the progression of a wide variety of cancers, although no direct relationship between Src activation and testicular seminoma has been reported [47]. Thus it is possible that alteration of conformation of the C terminal region of Cx43 by specific phosphorylation sites controls its exit from the Golgi apparatus to the plasma membrane as recently suggested [48].

CONCLUSION

In conclusion the present results show that altered Cx43 gene expression in human testicular seminoma mainly occurs at the protein level suggesting post-translational rather than transcriptional abnormalities. They show that aberrant cytoplasmic accumulation of Cx43 probably associated with altered Cx43 trafficking to the plasma membrane is a typical feature of testis seminoma cells and is a potential process for uncontrolled germ cell proliferation in this cancer. Whether intracytoplasmic Cx *per se* can play favorable effects for tumor progression as suggested in other tumoral tissues [35] is presently questionable.

ABBREVIATIONS

Cx	=	Connexin
GJIC	=	Gap junctional intercellular communication
PLAP	=	Placental like alkaline phosphatase
ZO-1	=	Zonula Occludens 1
GFP	=	Green fluorescent protein
ISH	=	In situ hybridization
AEC	=	Amino ethyl carbazol

CONFLICT OF INTEREST

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