



Molecular studies of human connexin 43 (Cx43) expression in isolated corporal tissue strips and cultured corporal smooth muscle cells

S Serels¹, NS Day¹, YP Wen¹, A Giralardi^{1,2}, SW Lee¹, A Melman¹ and GJ Christ¹

¹Department of Urology, Albert Einstein College of Medicine/Montefiore Medical Center, Bronx, New York, USA and ²Department of Medical Physiology, University of Copenhagen, Denmark

Intercellular communication plays an important role in erectile function. The goal of this study, therefore, was two-fold. Firstly, to determine if cultured corporal smooth muscle cells provide a valid model system for evaluating the role of junctional communication to erectile physiology, and secondly, to explore the possibility that there may be age-related alterations in Cx43 mRNA expression. Human corpus cavernosum tissue was obtained from 31 patients with a mean age of 58 (range 27–89), while cell cultures were developed from 21 distinct patients with a mean age of 57 (range 26–59). Northern blots revealed that mRNA for Cx43 was expressed at detectable levels in all samples examined. It migrated as a transcript with an apparent size of 3.1 Kb. Western blots revealed the presence of multiple bands of Cx43 protein in both tissues and cells. However, Cx43 protein in tissue predominantly migrated as a 45 kDa band, while the Cx43 from cultured cells predominantly migrated as 41 kDa band. Cx43 mRNA expression was similarly heterogeneous in both frozen tissues and cultured cells. An ~3–5-fold increase in Cx43 mRNA levels was observed in cultured cells relative to frozen tissue, but the expression of Cx43 mRNA was not further altered upon passaging (p1–5). When Cx43 mRNA levels were normalized, and expressed as a ratio of the Cx43/ β -tubulin mRNA, there was a significant negative correlation between patient age and Cx43 levels on frozen tissues, but not on cultured cells. We conclude that: (1) There is similar heterogeneity/variability in Cx43 mRNA levels in frozen tissues and cultured cells derived from human corpus cavernosum. (2) That the expression of Cx43 mRNA in cultured cells is sufficiently stable, and similar to, expression levels in tissue as to provide a valid and physiologically relevant model system for further studying the role(s) of Cx43 in the regulation of penile erection. (3) There is a statistically significant, albeit modest, negative correlation between the Cx43/ β -tubulin ratio and patient age in frozen corporal tissue strips, but not on cultured corporal smooth muscle cells. Such observations provide further evidence for the plasticity of intercellular communication in the erectile process. Moreover, the similarities in the apparent regulation of Cx43 mRNA levels and that of the putative 'housekeeping' gene β -tubulin, may suggest that Cx43 is constitutively synthesized in this tissue.

Keywords: connexin 43; corporal smooth muscle; molecular studies; erectile dysfunction; gap junctions

Introduction

Gap junctions formed by the connexin protein family are specialized plasma membrane structures that mediate the direct exchange of ions and small molecules resulting in metabolic and electrical coupling of adjacent cells in diverse tissues.¹ Such coupling clearly contributes to propagation of

intracellular calcium waves from one cell to another^{2,3} as well as to synchronized muscle contraction⁴ and has been suggested to play a role in maintenance of tissue homeostasis,⁵ embryonic development,^{6,7} and control of cell growth and neoplastic transformation.^{5,8} More than a dozen mammalian connexins, ranging in molecular weight from 26–56 kDa, have been reported in many species and diverse tissues.¹

With respect to erectile physiology, the real importance of gap junctions is best reflected by the integrative nature of the erectile process.^{9,10} More specifically, it is the coordination of neuronal input, namely, autonomic effector pathways, intracellular signal transduction mechanisms, namely, receptor

Correspondence: Dr GJ Christ, Associate Professor, Ben Marden Distinguished Scholar in Urology, Albert Einstein College of Medicine, Room 716S, Forchheimer Building, 1300 Morris Park Avenue, Bronx, New York 10461, USA.
Received 15 November 1997; revised 7 January 1998; accepted 21 January 1998

activated second messengers such as cAMP, cGMP and calcium, and junctional communication that is truly responsible for maintaining potency. In this scenario, gap junctions contribute to the regulation of corporal smooth muscle tone by providing the anatomic substrate for coordinated cell-to-cell interactions among the vast array of smooth muscle cells that comprise the corporal parenchyma.^{11–13} Intercellular communication, in turn, ensures the rapid and syncytial contraction and relaxation responses that are an absolute prerequisite to flaccidity and detumescence, respectively. Therefore, the interrelationship between innervation density, intracellular signal transduction and intercellular communication is critical to erectile capacity, and has been referred to as the ‘syncytial tissue triad’.^{9,10}

In spite of the wealth of evidence from both clinical and experimental studies supporting the supposition that intercellular communication through gap junctions provides an important mechanism for modulating erectile capacity, the effects of age or disease on the extent of intercellular communication have never been evaluated. While various biophysical aspects of junctional communication have been established *in vitro* in short term explant cell cultures, the precise physiological relevance of these observations to the situation *in vivo* is still unclear. As a first step in this direction, we evaluated Cx43 mRNA and protein levels in frozen tissues and short-term (namely, passages 1–5) explant cell cultures developed from excised human corporal smooth muscle. To this end, the goal of these studies was two-fold. Firstly, to determine if cultured corporal smooth muscle cells prove a valid and physiologically relevant model system for evaluating the role of junctional communication to erectile physiology and secondly, to explore the possibility that there may be age-related alterations in Cx43 mRNA expression.

Materials and methods

Patient population

Frozen corporal tissue specimens were obtained from 27 men undergoing implantation of penile prostheses to correct erectile dysfunction. Eighteen of the patients were nondiabetic (that is primarily vasculogenic or post-radical prostatectomy), nine were diabetic (either type I or type II diabetics). Frozen tissue was obtained from four additional patients whom were undergoing a gender change operation for treatment of gender dysphoria. The median age of the patient population was 58, with a range of 27–89. Cell culture material was obtained from our frozen cell culture library, and consisted of a mutually exclusive group of 21 patients with a median age of 57, and a range of 26–59. The

Table 1 Demographics of patient population

Age	Tissue sample patients	Cell cultured patients ^a
≤ 40	4	5 (6)
41–50	5	3 (3)
51–60	9	4 (10)
61–70	5	6 (10)
≥ 71	8	3 (3)

^aNumbers in parentheses add up to more than 21, as some experiments were conducted on serially passaged cells from the same patient, in order to evaluate the possibility of passage-dependent alterations in Cx43 expression (see Methods section).

demographic profile of the patient population is displayed in Table 1. All tissues were obtained from patients undergoing surgery, according to a protocol approved by the Internal Review Board of the Montefiore Medical Center/Albert Einstein College of Medicine.

Frozen tissue preparation

Excised tissues were flash frozen in liquid nitrogen in the operating room, and thereafter, transferred to a deep freezer (– 80°C) until RNA preparation.

Explant cell cultures

As previously described^{3,14,15} explant cell cultures of corporal vascular smooth muscle cells were prepared, and cellular homogeneity was verified by immunofluorescence staining using a monoclonal antibody specific for human smooth muscle α -actin. Briefly, sections of corporal tissue were placed in Dulbecco’s medium (DME, Gibco, Grand Island, New York) containing antibiotics (penicillin 100 u/ml and streptomycin 100 μ g/ml). Tissue was washed and cut into 1- to 2-mm pieces and placed in tissue culture dishes with sufficient nutrient medium to prevent drying. After the explants attached to the substrate, usually within 1–2 d, more medium was added. When the cells had migrated from the explant and undergone division, they were detached using 0.05% trypsin and 0.02% EDTA at 37°C for 5 min. Cells were subsequently grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 2 mM glutamine, and antibiotics. Only passages 1–5 were used for this study.

RNA preparation

Total RNA was extracted from tissue and cell culture material by the TRIzol method. Briefly, tissue was homogenized in TRIzol reagent by a

Polytron homogenizer (Brinkman, NY), and cultured cells were directly lysed in culture dishes by adding TRIzol reagent. The tissue lysate and cell lysate were transferred to a polypropylene, 10x1.8 cm round bottom tube (Falcon, Becton Dickinson, New Jersey), and then incubated for 5 min at room temperature. RNA was extracted in the aqueous phase from the TRIzol and chloroform mixture and precipitated from the aqueous phase by mixing with isopropyl alcohol. RNA was stored in 0.08M sodium acetate and 2.5 volumes of ethanol.

Northern blot analysis

Twenty µg of total RNA from each sample was electrophoresed in 1% agarose-formaldehyde gels. Gels were capillary blotted in 10x standard saline citrate (SSC, 1xSSC consists of 0.15 M NaCl and 0.015 M NaCitrate) onto nylon membranes and fixed by heating. Membranes were prehybridized in 5xSSC, 7% SDS, 10x Denhardt's solution, 20 mM sodium phosphate pH 7.4, and 10% dextran sulfate with 100 g/ml denatured salmon sperm DNA at 55°C and then hybridized in the same solution with the addition of a radiolabeled oligonucleotide probe (5'GAAGATGGTTTTCTCCGTGGG3') complementary to the nucleotides of human connexin 43 cDNA and probe complementary to the nucleotides of human β-tubulin cDNA (5'GATGGTGGAATGGCAGCA3'). After hybridization, the filters were washed with 3xSSC, 20 mM sodium phosphate buffer and 5% SDS at 55°C and subjected to autoradiography at - 80°C prior to Phosphor Image analysis.

Phosphor image analysis

The quantitative evaluation of the autoradiographic signals associated with the connexin43 transcripts and β-tubulin transcripts was performed using a computer-aided Molecular Dynamics imaging program. Each filter was exposed to the phosphor screen. Integrated densities (optical density per area) were evaluated and normalized to β-tubulin.

Protein preparation

After removing the aqueous phase containing RNA from tissue or cell lysate, the remaining material comprising the interface and organic phase was stored at -20°C until protein analysis. The DNA was precipitated from the remaining part with ethanol, while the protein was precipitated from the phenol-ethanol supernate with isopropyl alcohol. The protein pellet was washed in a solution containing

0.3 M guanidine hydrochloride in 95% ethanol, and was then dissolved in 1% SDS. The concentration of protein in 1% SDS was determined according to the Lowry method with BSA used as a standard.

Western blots

Ten to 20 µg/ml of protein from each extract were subjected to electrophoresis on 0.75 mm 10% acrylamide-SDS gels. Prestained molecular weight standards (BioRad) were included in each gel. Proteins were then electrically transferred to nitrocellulose. Nitrocellulose blots were blocked by incubation for 30 min in TBS-T buffer (20 mM Tris base, 137 mM Sodium chloride, 0.1% Tween-20) containing 5% dry milk and 1.0% bovine serum albumin (BSA). Blots were then incubated for 2 h at room temperature in TBS-T containing 5% dried milk, and 1:1000 dilution of rabbit anti-connexin43 serum (prepared against residues 346-360 of rat connexin43, and generously supplied by Dr Hertzberg).^{16,17} Blots were washed three times in TBS-T and then incubated for 2 h with TBS-T, 5% dried milk containing 1:1000 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP). After two washes with TBS-T and three washes TBS, ECL reagents detected Cx43 immunoreactive bands.

Statistical analysis

Because the data were not normally distributed, Mann Whitney Rank Sum tests were used to compare groups. The Spearman correlation and descriptive statistics were also used where applicable. $P < 0.05$ was considered significant in all cases.

Results

Comparison of Cx43 mRNA expression in frozen tissues and cultured corporal smooth muscle cells

The 3.1 Kb Cx43 mRNA was detected by Northern blot analysis of frozen corporal tissue and cultured corporal smooth muscle cells following hybridization with a Cx43 oligonucleotide (see Methods). As depicted in Figure 1, significant variation in the apparent density of the detected Cx43 bands was observed for both the frozen tissue and cell culture samples, despite equivalent loading conditions. Quantitative densitometry analysis revealed that the Cx43 transcript values had a median equal to 26 222 (range 1502-557784) and 426 406 (range

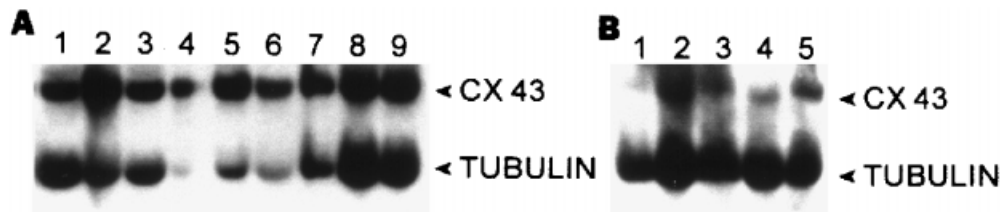


Figure 1 Comparison Cx43 mRNA levels on frozen tissues and cultured corporal smooth muscle cells. Expression of Cx43 mRNAs by Northern blot hybridization analysis in cultured cells (a) and frozen tissue (b) excised from human corporal smooth muscle. In all cases, twenty μg of total RNA per lane was hybridized with a radio-labeled oligonucleotide probe designed to specifically recognize human Cx43, prior to rehybridization of the same blot with a β -tubulin-specific probe as an internal control (see Methods section for sequences). Note the significant heterogeneity in the mRNA bands for both probes among different patients; for both cultured cells and frozen tissue. In both panel A and panel B, each lane represents a distinct observation from a different patient.

12 308–1 279 899) on frozen tissues and cultured cells, respectively (Figure 2a). As illustrated in Figure 2a, the median values for the Cx43 bands in frozen tissues and cultured cells were significantly different at the $P < 0.001$ level.

Similar findings were made for the putative ‘housekeeping’ gene, β -tubulin. Densitometry analysis yielded median values of 38 516 (range: 6246 996 632) and 139 406 (range: 9587–806 750) for frozen tissues and cultured cells, respectively.

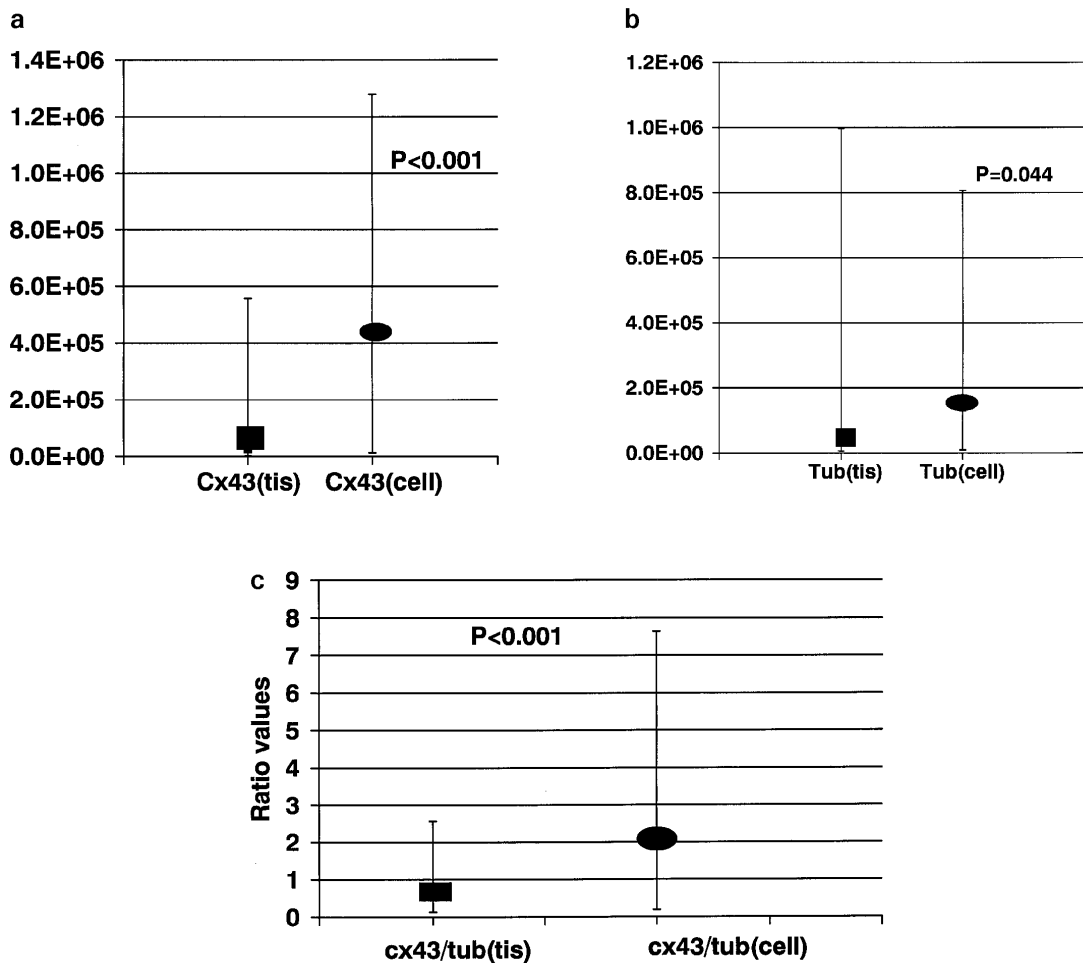


Figure 2 Plot summarizing Northern blot data obtained on all patients. Quantitative densitometry analyses depicting the median value and range of values for mRNA from frozen tissue and cultured corporal smooth muscle cells for Cx43 (a), β -tubulin (b), and the Cx43/ β -tubulin ratio (c). Note that nonparametric analyses revealed that the median value for Cx43 mRNA and β -tubulin mRNA, as well as the normalized Cx43 value, that is, Cx43/ β -tubulin ratio, were all significantly elevated in the cultured smooth muscle cells relative to the values determined on frozen tissue.

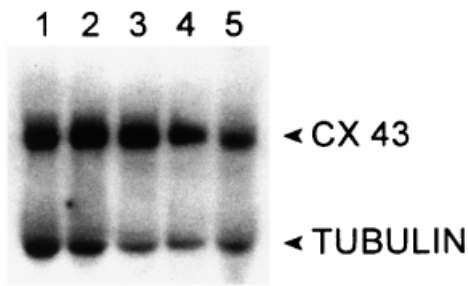


Figure 3 Representative Northern blot for comparison of the Cx43 mRNA and tubulin expression on different passages (1–5) of cultured corporal smooth muscle cells from the same patient. Twenty μg of total RNA per lane was hybridized with a human specific Cx43 oligonucleotide, with a β -tubulin oligonucleotide used as an internal control. Consistent with the data summarized in Table 2, this Northern blot reveals no consistent changes in Cx43 mRNA expression between cells of passages 1–5.

As illustrated in Figure 2b, the median values for β -tubulin also differed significantly between frozen tissues and cultured cells ($P = 0.044$) (Figure 2b).

In an attempt to obtain more quantitative information about alterations in Cx43, we expressed the Cx43 transcript densities as a ratio of the β -tubulin transcript densities. The ratio of the median values of Cx43/ β -tubulin was 0.515 (range: 0.13–2.57) and 1.99 (Range: 0.19–7.64), for β -tubulin on frozen tissue and cultured cells, respectively. As illustrated in Figure 2c, these two ratio values between frozen tissues and cultured cells were significantly different at the $P < 0.001$ level.

C43 mRNA expression is unaltered in different passages of explant cultured corporal smooth muscle cells

Cx43 mRNA levels were also evaluated on explant cultured corporal smooth muscle cells of different passages. As shown in the representative example illustrated in Figure 3, there was no obvious or consistent passage-dependent alteration detected in serially passaged smooth muscle cells obtained from

Table 2 Effects of cell passage number on the normalized Cx43 expression levels

Passage no.	N ^a	Median ratio value	25%	75%
1	9	1.9	1.2	2.4
2	11	3.3	2	4.5
3	9	1.8	1.4	2.8
4	2	1.8	1.6	1.9
5	1	1.3	1.3	1.3

^a N-denotes the number of observations. A Kruskal–Wallis One Way Anova on ranks revealed that there was no significant variation in the median value for the Cx43/ β -tubulin ratio among the different cell passages.

a given patient. Similar observations were made on cell cultures from other patients, and these data were pooled and summarized in Table 2. These findings document a lack of detectable passage-related changes in the expression of the ‘normalized’ Cx43 mRNA levels under these conditions.

Cx43 mRNA expression as a function of age

In order to evaluate potential age-related alterations in Cx43 levels, the Cx43/ β -tubulin ratios were determined in frozen tissue samples excised from individual patients and were plotted as a function of age, with age treated as a continuous variable. As illustrated in Figure 4a, there was a statistically significant negative correlation observed between the age of the patient, and the Cx43/ β -tubulin ratio (correlation coefficient $R = 0.36$, $P < 0.05$). A similar analysis was performed for Cx43/ β -tubulin ratios obtained on explant cell cultures derived from a distinct patient population. As illustrated in Figure 4b, there was no detectable relationship observed between patient age and the Cx43/ β -tubulin ratio among cell cultures obtained from distinct patients (correlation coefficient $R = 0.01$, $P = 0.94$).

Comparison of Cx43 protein expression in frozen tissue and cultured corporal smooth muscle cells

In order to gain additional insight into the potential significance of our findings at the transcriptional level, Western blots were performed to examine Cx43 protein expression. As illustrated in Figure 5, three protein bands were observed in preparations from both frozen corporal tissues and cultured corporal smooth muscle cells. More specifically, a doublet was detected at 45 kDa and a single band at 41 kDa. Densitometry analysis revealed that the 45 kDa bands were ≈ 3 -fold more prevalent than the 41 kDa band in frozen tissue specimens. In contrast, cultured cells contained predominantly the 41 kDa form of Cx43, which was ≈ 5 fold more abundant than the 45 kDa band.

Discussion

Erectile dysfunction significantly affects the quality of life of many men and their sexual partners.^{18,19} In fact, the prevalence of impotence is known to increase with age, and has recently been noted to be $\approx 52\%$ among American men between the ages of 40–70 y. This corresponds to ≈ 18 million men,¹⁸ and indicates that age is an important risk factor for erectile dysfunction. In clinical and experimental

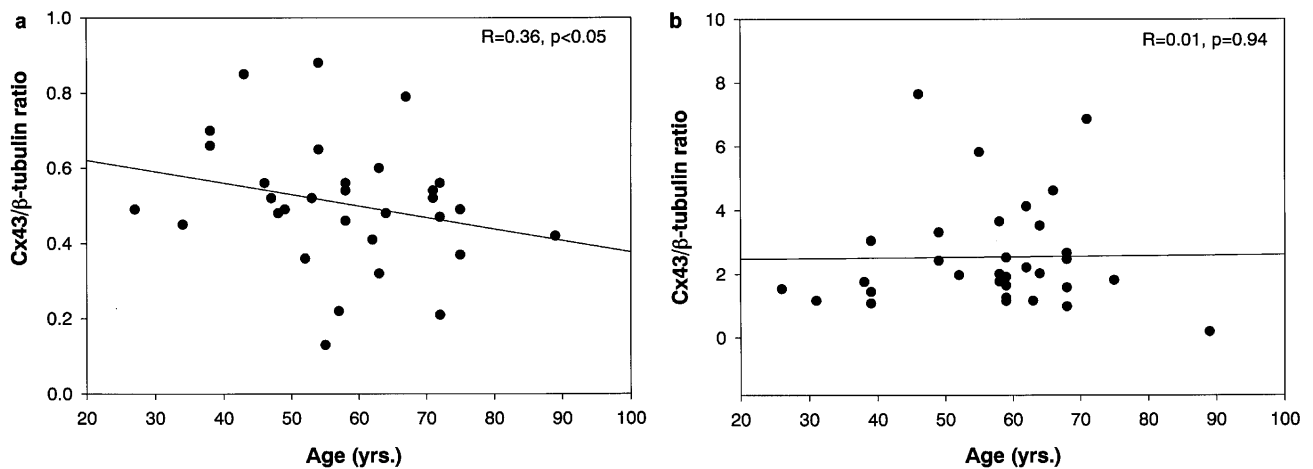


Figure 4 Relationship between patient age the expression of Cx43 mRNA in frozen corporal tissue (a) and cultured corporal smooth muscle cells (b). In both panels, the Cx43 mRNA levels are expressed as a ratio of Cx43/ β -tubulin, and plotted as a function of the age of the patient from which the tissue or cell culture was derived. Simple linear regression analysis was performed in both panels to provide a correlation coefficient and P value. As illustrated, there was a significant negative correlation between patient age and the Cx43/ β -tubulin ratio on frozen tissues, but no detectable relationship was found on cultured cells.

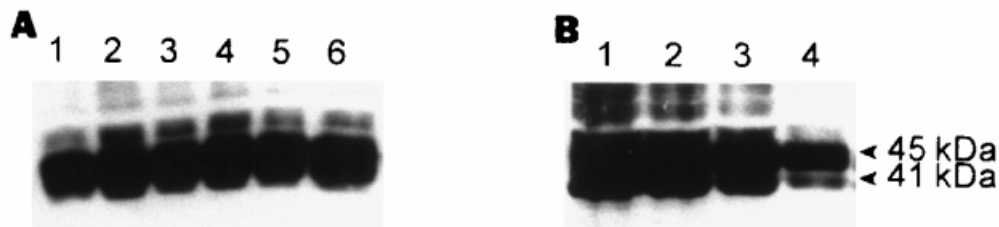


Figure 5 Western blot analysis of Cx43 protein expression in cultured corporal smooth muscle cells (a) and frozen corporal tissue (b). For these studies, thirty μ g of protein was loaded per lane, in a 10% SDS-PAGE gel for Western blotting. The 41 kDa (that is, largely unphosphorylated, and 45 kDa (that is, largely phosphorylated) Cx43 protein species are indicated to the right of the blots. Note that consistent with the observations of others, on distinct tissue/cellular preparations, the amount of the unphosphorylated Cx43 species is predominant in cultured cells relative to frozen tissue. In both panel A and panel B, each lane represents a distinct observation from a different patient.

studies, erectile rigidity and sexual function gradually declines with age in male animals and humans.^{20,21} Many factors apparently contribute to this age-related decline, but decreases in testosterone,²² and morphological changes in the arterial bed of the penis, in particular, have been implicated in the onset of impotence.²² It is clear that no single factor will predominate in such a multifactorial disease process. Gap junctions, however, are known to play a crucial role in coordinating the erectile response. As such, it is conceivable that dynamic regulation of connexin synthesis and degradation could play a major role in the remodeling of cellular connections, and thus, affect erectile capacity. As a first step in this direction, the goal of these studies was to assess the possibility that age-related alterations in Cx43 mRNA expression may play a role in some aspects of erectile dysfunction, and moreover, to evaluate the physiological relevance of our corporal smooth muscle cell culture system.

Our current observations indicate that there is a similar variability/heterogeneity in Cx43 mRNA expression in frozen human corporal tissues and cultured cells, as evidenced by the ~ 1.5 – 2.5 orders

of magnitude difference between the highest and lowest Cx43 mRNA levels detected among entirely distinct patient populations (see Figures 1 and 2). The variable expression of Cx43 mRNA levels is most likely a reflection of the diversity of the patient population, as well as the plasticity of Cx43 mRNA expression (see text below). Interestingly, we found a similar variability in the expression of mRNA for the putative 'housekeeping' gene β -tubulin, which is thought to be constitutively synthesized.

Following placement in culture, a statistically significant increase in Cx43 mRNA expression, as well as a qualitatively similar, albeit more modest increase in β -tubulin mRNA expression was observed. Furthermore, when the Cx43 mRNA levels were normalized by expressing them as a fraction of the 'constitutive' β -tubulin mRNA signal, we found a ≈ 3 – 5 fold increase in Cx43 mRNA expression, similar to that reported for cultured smooth muscle cells derived from other vascular tissues.^{24,25} After the initial 3–5 fold increase in Cx43 mRNA expression in passage 1 cells, however, there was no consistent variation in the normalized Cx43 mRNA levels upon serial passaging of the cells; at least for

cultures of up to the 5th passage (see Figure 3 and Table 2). Thus, despite the problems and concerns associated with the quantitative interpretation of autoradiographic data for evaluation of gene expression (even when one 'normalizes' the data by using a common 'housekeeping' or constitutively expressed transcript as a control),^{26,27} these findings suggest that once altered by the culture conditions, Cx43 mRNA expression is not further altered in these cultured human corporal smooth muscle cells, and under these conditions. Such observations are entirely consistent with our previous work, which has shown no detectable passage-dependent variation in the macroscopic or single channel currents recorded in the dual whole cell patch mode for Cx43-mediated intercellular communication in cultured corporal smooth muscle cells of up to the 5th passage.

Interestingly, variability in Cx43 mRNA expression in cultured smooth muscle cells (see Figure 2) is similar to that observed in frozen tissue, and this is true for both the 'raw' (Figure 2a) and the 'normalized' Cx43 mRNA data (Figure 2c). If the range of values, (that is, highest to lowest), of Cx43 mRNA expression is somewhat indicative of the level of functional intercellular coupling, then the cultured cell system provides us with a valuable experimental tool. That is, one could explore the effects of significant differences in functional coupling on the intercellular spread of physiologically relevant second messenger molecules/ions in smooth muscle cell cultures derived from distinct patients. For example, a previous theoretical analysis indicated that physiologically relevant alterations in intercellular communication would require ≈ 10 – 100 fold changes in the expression/function of gap junctions between adjacent cells. Such a range of values is clearly present in the smooth muscle cell cultures derived from this patient population. Therefore, these cultured smooth muscle cells would appear to provide a valid model for further investigating the physiological/biophysical boundary conditions for altered junctional communication, and its subsequent impact on erectile function.

With respect to Cx43 mRNA levels on frozen tissue, nonparametric analysis of 'normalized' Cx43 mRNA levels, that is, Cx43/ β -tubulin ratio, revealed a significant negative correlation between Cx43 mRNA levels and patient age (Figure 4a). Extrapolating from the linear regression analysis depicted in Figure 4a, the observed decline in the 'normalized' Cx43 mRNA levels would correspond to an ≈ 2 – 3 fold decrease in Cx43 mRNA levels between a 20 y old man and a 100 y old man. Again, assuming that the amount of Cx43 mRNA bears some relationship to the amount of functional gap junctional protein (see below), this might imply a similar reduction in the extent of intercellular coupling. In light of the fact that theoretical analyses predict that physiologically relevant alterations in intercellular coupling would require such alterations to be on at least 1–2 orders of

magnitude, the observed 2–3-fold difference in the level of intercellular communication is, nominally, likely to be of modest physiological significance. Moreover, it should be emphasized that these modest changes in Cx43 mRNA expression occur over virtually the entire adult human life span. In short, such observations are arguably more indicative of the relative plasticity/adaptability of intercellular communication, than of any possible age-related alteration. Furthermore, as pointed out elsewhere,^{26,27} the very nature of the evaluation of quantitative variations in gene expression suggests that such a decrease in Cx43 mRNA levels with age, albeit a statistically significant one does not necessarily imply any precise physiological significance for these alterations. A similar relationship between age and Cx43 mRNA levels was not observed in cultured smooth muscle cells (Figure 4b).

In order to gain additional insight into the physiological status of our corporal smooth muscle cell cultures, we performed Western blots on frozen corporal tissue and cultured corporal smooth muscle cells. Western blots revealed the presence of a differential migration of Cx43 protein in frozen tissue, relative to that observed on cultured smooth muscle cells. As illustrated in Figure 5, the 45 kDa-doublet, which represents the phosphorylated Cx43 isoform and the 41 kDa-band, which represents the unphosphorylated Cx43 isoform apparently differ between the two groups, that is, frozen tissues and cultured cells. The higher proportion of the phosphorylated isoform relative to the nonphosphorylated isoform in frozen tissue is consistent with the supposition that the localization and insertion of functional Cx43 protein in the junctional plaque requires phosphorylation.^{28–31} Conversely, the greater preponderance of the nonphosphorylated isoform in cultured corporal smooth muscle cells is also consistent with this hypothesis, as well as our previous reports of more pronounced punctate intracytoplasmic staining for Cx43 protein.¹¹

Dynamic regulation of junctional communication has been reported under certain circumstances. For example, the dramatic up regulation of Cx43 expression observed in myometrial smooth muscle just prior to parturition, or the increased synthesis and remodeling of gap junctions observed in the regenerating liver and post-infarcted heart, respectively.^{32–35} In this regard, the relative similarity in the range of values for β -tubulin and Cx43 mRNA levels, as well as the qualitatively similar effects of our cell culture conditions on expression of both transcripts, that is, increased mRNA levels, is noteworthy. As such, it is interesting to speculate that Cx43 may represent a 'constitutively' synthesized transcript in this tissue. In fact Cx43 mRNA and protein expression was detected in every cell culture and tissue sample we analyzed. Moreover, a relatively modest 2-fold decrease in Cx43 mRNA expression was observed over virtually the entire

adult male life span. Both of these observations are consistent with the supposition that Cx43 expression is 'constitutive', or at least relatively constant, in corporal smooth muscle cells. Given that intercellular communication is an important mechanism for coordinating the rapid and syncytial contraction and relaxation responses characteristic of detumescence and erection, respectively, such a possibility clearly has important implications to the integrity of penile erection, and thus, the survival and propagation of the species.

Taken together, these data confirm and extend our previous observations to indicate that cultured corporal smooth muscle cells of up to the 5th passage appear to provide a valid and physiologically relevant system for evaluating the role of intercellular communication in coordinating corporal smooth muscle tone. Future studies utilizing these cultured cells should assist in defining the physiological and biophysical boundary conditions for gap junctions in normal erectile function and impotence. Moreover, these observations are consistent with the recently advanced model of integrative erectile biology, in which the 'syncytial tissue triad' is used to describe normal erectile function and failure.^{9,10} In this model, gap junctions play a pivotal role in coordinating the activity of the autonomic nervous system, and ensuring that there is an adequate intercellular pathway for the flow of receptor mediated intracellular second messenger molecules from directly activated corporal smooth muscle cells to adjacent smooth muscle cells. In fact, as discussed in detail elsewhere³⁵, this type of recruitment of indirectly activated corporal smooth muscle cells is a crucial aspect of normal erectile function.

Conclusion

In this scenario, these observations would appear to suggest that transcriptional control of Cx43, at the tissue level, is not by itself a major regulatory pathway responsible for the impairments commonly observed during age-related changes in erectile capacity. This hypothesis is consistent with supposition that gap junctions provide a certain level of plasticity/adaptability in the penis, thus ensuring normal organ function under a wide range of physiological conditions. As such, it seems that in the penis, as already demonstrated in the heart, gap junctions provide a certain 'safety factor' for normal erectile capacity.

Acknowledgements

The authors are grateful to Dr Elliot Hertzberg for providing Cx43 antibody for the Western blots.

References

- 1 Bennett MVL *et al.* Gap junctions: new tools, new answers, new questions. *Neuron* 1991; **6**: 305–320.
- 2 Enkvist MOK, McCarthy KD. Activation of protein kinase C blocks astroglial gap junction communication and inhibits the spread of calcium waves. *J Neurochem* 1992; **59**: 519–526.
- 3 Christ GJ, Moreno AP, Melman A, Spray DC. Gap junction-mediated intercellular diffusion of Ca²⁺ in cultured human corporal smooth muscle cells. *Am J Physiol* 1992; **263**: C373–C383.
- 4 Garfield RE, Ganon MS, Daniel EE. Gap junction formation in myometrium: control by estrogens, progesterone and prostaglandins. *Am J Physiol* 1980; **238**: C81–C91.
- 5 Loewenstein WR. Junctional intercellular communication: the cell-to-cell membrane channel. *Physiol Rev* 1981; **61**: 829–913.
- 6 Caveney S. The role of gap junctions in development. *Annu Rev Physiol* 1985; **47**: 19–35.
- 7 Lee S, Gilula NB, Warner AE. Gap junctional communication and compaction during preimplantation stages of mouse development. *Cell* 1987; **51**: 851–860.
- 8 Hossain MZ *et al.* Enhancements of gap junctional communication by retinoids correlates with their ability to inhibit neoplastic transformation. *Carcinogenesis* 1989; **10**: 1743–1748.
- 9 Christ GJ. The 'syncytial tissue triad': A model for understanding how gap junctions participate in the local control of penile erection. *World J Urol* 1997; **13**: 36–44.
- 10 Christ GJ, Richards S, Winkler A. Integrative erectile biology: The role of signal transduction and cell-to-cell communication in coordinating corporal smooth muscle tone and penile erection. *Int J Impot Res* 1997; **9**: 1–16.
- 11 Campos AC *et al.* Gap junctions formed of connexin43 are found between smooth muscle cells of human corpus cavernosum. *J Urol* 1993; **149**: 1568–1575.
- 12 Moreno AP *et al.* Gap junctions between human corpus cavernosum smooth muscle cells: gating properties and unitary conductance. *Am J Physiol* 1993; **264**: C80–C92.
- 13 Christ GJ *et al.* Intercellular communication through gap junctions: a potential role in pharamechanical coupling and syncytial tissue contraction in vascular smooth muscle isolated from the human corpus cavernosum. *Life Sci* 1991; **49**: PL-195–200.
- 14 Brink PR, Ramanan SV, Christ GJ. Human connexin 43 gap junction channel gating: evidence for mode shifts and/or heterogeneity. *Am J Physiol* 1996; **271**: C321–331.
- 15 Palmer L *et al.* Characterization of cAMP accumulation in cultured human corpus cavernosum smooth muscle cells. *J Urol* 1994; **152**: 1308–1314.
- 16 Yamamoto T, Ochalski A, Hertzberg EL, Nagy JI. EM immunolocalization of the gap junctional protein connexin43 in rat brain. *Brain Res* 1990; **508**: 313–319.
- 17 Yamamoto T, Ochalski A, Hertzberg EL, Nagy JI. On the organization of astrocytic gap junctions in rat brain as suggested by LM and EM immunohistochemistry of connexin43 expression. *J Comp Neuro* 1990; **302**: 853–883.
- 18 Feldman HA *et al.* Impotence and its medical and psychosocial correlates: Results of the massachusetts aging study. *J Urol* 1994; **151**: 54–61.
- 19 National Institutes of Health: Consensus development conference statement on impotence. *Int J Impot Res* 1993; **5**: 181.
- 20 Bishop MW. Aging and reproduction in the male. *J Reprod Fertil* 1970; **12** (Suppl): 65–87.
- 21 Kinsey AC, Pomeroy WB, Martin CE. *Sexual Behavior in the Human Male*. Saunders: Philadelphia, 1948.
- 22 Davidson JM *et al.* Hormonal changes and sexual function in aging men. *J Clin Endocrinol Metab* 1983; **57**: 71–77.
- 23 Conti G, Virag R. Human penis erection and organic impotence: Normal histology and histopathology. *Urol Int* 1989; **44**: 303–308.
- 24 Lash JA, Crister ES, Pressler ML. Cloning of a gap junctional protein from vascular smooth muscle and expression in two-cell mouse embryos. *J Biol Chem* 1990; **265**: 13113–13117.

- 25 Rennick RE *et al.* Expression of connexin-43 gap junctions between cultured vascular smooth muscle cells is dependent upon phenotype. *Cell & Tissue Res* 1993; **271**: 323–332.
- 26 Spanakis E. Problems related to the interpretation of autoradiographic data on gene expression using common constitutive transcripts as controls. *Nucleic Acids Res* 1993; **21**: 3809–3819.
- 27 Spanakis E, Brouty-Boye D. Evaluation of quantitative variation in gene expression. *Nucleic Acids Res* 1994; **22**: 799–806.
- 28 Musil LS, Beyer EC, Goodenough DA. Expression of the gap junction protein connexin43 in embryonic chick lens: Molecular cloning, ultrastructural localization, and post-translational phosphorylation. *J Mem Biol* 1990; **116**: 163–175.
- 29 Musil LS, Goodenough DA. Biochemical analysis of connexin43 intracellular transport, phosphorylation, and assembly into gap junctional plaques. *J Cell Biol* 1991; **115**: 1357–1374.
- 30 Musil LS, Cunningham BA, Edelman GM, Goodenough DA. Differential phosphorylation of the gap junction protein connexin43 in junctional communication-competent and deficient cell lines. *J Cell Biol* 1990; **111**: 2077–2088.
- 31 Musil LS, Goodenough DA. Multiunit assembly of an integral plasma membrane channel protein, gap junction connexin43, occurs after exit from the ER. *Cell* 1993; **74**: 1065–1077.
- 32 Andersen *et al.* Expression of connexin-43 in human myometrium and leiomyoma. *Am J Obstet Gyn* 1993; **169**: 1266–1276.
- 33 Risek B, Guthrie S, Kumar N, Gilula NB. Modulation of gap junction transcripts and protein expression during pregnancy in the rat. *J Cell Biol* 1990; **110**: 269–282.
- 34 Garfield RE. Cell-to-cell communication in smooth muscle. In: Grover AK, Daniel EE, eds. *Calcium and Contractility-Smooth Muscle*. Humana: Clifton, New Jersey, 1985, pp 143–173.
- 35 Christ GJ. The penis as a vascular organ: The importance of corporal smooth muscle tone in the control of erection. *Urol Clin North America* 1995; **22**: 727–745.