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Connexin Expression and Intercellular Communication in Two- and Three-Dimensional *in Vitro* Cultures of Human Bladder Carcinoma

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The identification of gap-junctional proteins (connexins) and the preparation of related antibodies provides new tools to study patterns of intercellular communication in tumors. Focusing on the biology of human bladder carcinoma, we compared the expression of gap-junctional proteins (connexins Cx26, Cx32, and Cx43) with a dye-coupling assay for gap-junctional intercellular communication in three cell lines with different urothelial differentiation. The cell lines HCV-29, RT4, and J82 were initially grown as monolayers of different ages. Connexin expression was found mostly positive over the time of culture and found constantly negative only in J82 cells for Cx26 and HCV-29 cells for Cx32. In HCV-29 cells, Cx26 increased in positivity over the time of culture. Western blotting with the antibodies confirmed the findings. Comparisons of dye transfer using Lucifer Yellow showed an increase of coupling in the normal urothelial cell line HCV-29 in contrast to a decrease of coupling in the tumor cell lines. Data were extended by multicellular spheroid (MCS) co-cultures with the stromal fibroblast line N1. In three-dimensional cultures as MCSs, Cx26 was increased in proximity of RT4 tumor cells to fibroblasts, and positivity was maintained in J82 cells. E-cadherin expression in cell lines showed no change in dependence of growth state. The data suggest that Cx26 plays a role in negative growth control or differentiation of urothelial cells. Preliminary comparative data on normal and neoplastic urothelium show all three connexins in normal urothelium, in contrast to varying amounts of Cx43 and low amounts of Cx32 in tumors and evident loss of Cx26 in low-grade tumors. Discrepancies between monolayer and MCS cultures are most likely due to higher differentiation in MCSs, and the continuation of systematic work with heterologous MCSs is indicated for more information on the role of gap-junctional proteins in human tumors. (Am J Pathol 1996, 149:1321–1332)

Connexins are a family of transmembrane proteins that form intercellular channels known as gap junctions. Six connexins form a hemichannel (connexon) in the plasma membrane, and the connexons of adjacent cells are juxtaposed to allow the exchange of ions, metabolites, and signal factors up to a size of 1 kd.[1,2] This gap-junctional intercellular communication (GJIC) is implicated in electrical coupling and metabolic cooperation as well as in embryonic development, differentiation, and growth control of cells.[3–5] Since Loewenstein's hypothesis in 1979 it is widely assumed that a decrease or even complete loss of GJIC is an important feature of malignant cells that allows them to escape tissue-specific growth control signals.[6,7] A number of *in vitro* studies showed a lack of tumor cells in assembling functional gap junctions and membrane insertion of connexons, caused either by incorrect post-translational phosphorylation of connexins or by alterations in gene expression.[8–10] The connexin gene family has been postulated to belong to a class of tumor-suppressor genes that show decreased transcription rates in cancer cells.[11] For some mammary tumor and bladder cancer cell lines, Cx26 seems to be such a candidate, as the first studies showed significant down-regulation of Cx26 mRNA with the grade of malignancy.[12,13] In other types of tumors as in primary liver cancer, connexin expression of Cx32

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was unaltered in comparison with normal surrounding liver tissue; however, aberrant cellular distribution indicated a change in the functional state of gap junctions in this type of tumor.[14] Transfection of Cx43 into various animal tumor cell types (eg, mouse MCA-10 cells[15]) has resulted in a decrease in proliferation and loss of tumorigenicity.

These data indicate the organ specificity of connexin expression and led to the study presented, in which GJIC and expression of Cx26, Cx32, and Cx43 were investigated in *in vitro* models and tumor specimens of human bladder cancer. Bladder cancer presents in biologically different entities as the papillary, frequently recurrent tumor and the mostly more aggressive primarily invasive tumor type. The cell lines are derived from and represent normal urothelium, the noninvasive papillary tumor, and invasive bladder cancer. Both tumor types and normal urothelium could be mimicked in a co-culture model with fibroblasts, which has been included in this study.[16]

With the aims of defining the relation between GJIC and connexin expression for human bladder cancer and learning about the potential of connexin protein expression as a diagnostic aid, additional preliminary studies on patient urothelial lesions were carried out.

Material and Methods

Cell Culture

Three clonal urothelial cell lines of different differentiation were used; HCV-29 was derived from normal urothelium, whereas the tumor cell lines RT4 and J82 were originally derived from a well differentiated papillary bladder carcinoma (grade 1 to 2) and from a poorly differentiated invasive bladder carcinoma (grade 3), respectively.[17]

For co-cultures with fibroblasts, the fibroblast cell line N1, originally derived from adult skin, was used. The cell line was kindly provided by the Department of Clinical Chemistry, University of Regensburg.

Monolayer Culture

Urothelial cells were grown under standard conditions in an incubator with 5% or 8% CO_2 in RPMI 1640 (Biochrom, Berlin, Germany) and passaged weekly with split ratios of 1:7 to 1:10. Fibroblasts were cultured in Dulbecco's modified Eagle's medium (Sigma, Deisenhofen, Germany) and were passaged 1:5. Ten percent fetal calf serum (Sigma), 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 100 IU/ml penicillin, and 100 mg/ml streptomycin (all ingredients from GIBCO, Eggenstein, Germany) were added to the medium before use. Monolayers were seeded in concentrations of 1×10^4 cells/ml.

Multicellular Spheroid (MCS) Culture

For MCSs the liquid overlay culture technique was used. Single-cell suspensions obtained from exponentially growing monolayers were seeded in 96-well plates coated with solid agarose (Sigma). Either 3000 cells (tumor) or 6000 cells (fibroblasts) formed three-dimensional aggregates within 4 days of static incubation. At this age, average diameters of MCSs were between 300 and 400 μm. MCSs were cultured further for a maximum of 7 days. The medium was changed every other day.

Heterologous MCSs were obtained by transferring one tumor MCS (4 days) into one well containing a fibroblast MCS (4 days) and co-cultured for 7 days. As published previously,[16] MCS co-cultures of the three urothelial cell lines with N1 reflected the different levels of differentiation and simulated the tumor situation. During co-culture, poorly differentiated J82 MCSs infiltrated and intermingled with N1 cells as an invasive tumor, whereas more differentiated RT4 cells just migrated around the fibroblasts. Normal urothelium, represented by HCV-29 MCSs, merely attached to N1-MCSs.

Tumor Specimens

Normal bladder tissue and neoplastic tissue from either papillary or invasive human bladder carcinomas were snap-frozen in liquid nitrogen and stored at −80°C. Samples chosen were the most recent samples from a bladder tumor bank, routinely gathered at the institute of pathology and diagnosed by a senior pathologist (R. Knuechel). Samples recently frozen were chosen, as *in vitro* samples had shown loss of antigenicity in older (more than 1 month old) samples. For immunohistochemical analysis, 5-μm cryostat sections from a total of 18 specimens were cut and fixed in ethanol.

Immunohistochemistry

At day 4 (subconfluent) and at day 10 (confluent) of culture, monolayers were fixed in ethanol at room temperature. MCSs and MCS co-cultures were washed briefly in cold phosphate buffer (PBS) and cryofixed in Jung embedding media (Leica,

Muenchen, Germany) at days 4 and 7 for MCSs and at days 1 and 7 for MCS co-cultures. The 5-μm sections of all three-dimensional cultures were cut on a cryostat (Reichert-Jung, Nussloch, Germany) and fixed in ethanol.

Detection of connexins was carried out using indirect immunofluorescence with affinity-purified polyclonal rabbit antibodies directed against the entire Cx26 and Cx32 and against the proteins of the Cx43 carboxy terminus. Specificity of the connexin antibodies have been described recently by Traub et al[18] and Hofer et al.[19] Antibodies were applied in dilutions of 1:50 (Cx26), 1:100 (Cx32), and 1:400 (Cx43). The second antibody (goat anti-rabbit; Sigma), conjugated with fluorescein isothiocyanate, was diluted 1:300 in PBS. Negative controls were performed by omitting the first antibody during the incubation protocol or by preabsorption with the peptide to which the antibodies were originally produced (Cx32 and Cx43).

Scoring of immunohistochemical detection of gap-junctional proteins was performed on nine randomly chosen high power fields (HPFs, ×40), and data were confirmed by controlling the findings with a 100× oil immersion objective. For semiquantitative scoring, three categories were defined: positive (++), a minimum of six HPFs with membrane-bound staining immunoreactivity; moderately positive (+), specific membrane-bound staining in less then six but a minimum of two HPFs or staining patterns with cytoplasmic paranuclear staining instead of membrane-bound staining; and negative (−), no specific staining.

For staining against E-cadherin, a murine monoclonal antibody (TaKaRa Biomedicals, Tokyo, Japan) was applied in a 1:1500 dilution, followed by incubation with a biotinylated goat anti-mouse antibody (Dakopatts) and detection with a peroxidase-based three-step reaction kit (StreptABC complex; Dakopatts, Hamburg, Germany). Diaminobenzidine was used as chromogenic substrate. All incubations and washing procedures were carried out with 1 mmol/L calcium in the buffer solutions to conserve epitopes. Sections were counterstained in hemalum before embedding in xylene.

Photodocumentation was done with a camera mounted onto a Leitz Axioplan microscope using 29 DIN slide film and 33 DIN film for black and white prints.

Western Blots

Cell lysates were obtained according to a protocol described by Goldberg and Lau.[20] Briefly, confluent monolayers of the three cell lines were covered for 2 minutes with lysis buffer (for ingredients, see Ref. 21) containing 0.1% sodium dodecyl sulfate (SDS), scraped, and suspended in lysis buffer. All procedures were done on ice. Cell suspensions were transferred in an Eppendorf cup and pelleted for 10 minutes at 13,000 rpm and 4°C. Protein concentration of the pellet and the supernatant was determined in a Bradford protein assay.

Data from immunocytochemistry were confirmed by Western blotting with the anti-Cx32 and anti-Cx43 antibodies. Protein suspensions of pellets or membrane lysates (20 mg/lane) were separated according to Laemmli's method[21] by SDS-polyacrylamide gel electrophoresis using 12% gels in a Mini Protean system (BioRad, Munich, Germany). Proteins were transferred overnight at 40 V onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Membranes were blocked for 1 to 2 hours in 5% skimmed milk/0.5% Tween/Tris-buffered saline. Incubation with the primary anti-Cx antibodies, diluted 1:1000 for Cx32 and 1:3000 for Cx43 in 0.1% Tween/Tris-buffered saline was followed by reaction with a biotinylated second antibody (1:5000) and StreptABC complex). Detection was carried out with an enhanced chemiluminescence system (ECL; Amersham, Braunschweig, Germany) on x-ray film.

Dye Transfer Assay

To correlate connexin expression with the functional state of the gap junctions in urothelial cells, monolayers were assayed for GJIC by microinjections with the fluorescent dye Lucifer Yellow (Sigma). Cultures were examined in HEPES buffer to avoid serum autofluorescence and to keep cells in an orderly physiological state during the experiments. Transfer of the dye into the culture was observed with an inverse fluorescence microscope (Zeiss, Munich, Germany) and documented with an attached camera with films as mentioned above. The numbers of injections with Lucifer Yellow spreading into neighboring cells are presented as percentages. At least five independent series of injections in monolayers were carried out with a minimum of 20 injections each.

Results

Connexin Expression in Monolayer Culture

Immunofluorescence reaction in tumor cell lines and primary bladder tissue was compared with that in positive controls (mouse heart for Cx43 and mouse

Table 1. *Connexin Expression in Monolayer Cultures at Different Growth States (4 and 10 Days)*

Cell line	Cx26	Cx32	Cx43
HCV-29			
4 days	+	−	+
10 days	++	−	+
RT4			
4 days	+	+	++
10 days	++	++	++
J82			
4 days	−	+	++
10 days	−	++	++
N1			
4 days	−	−	+
10 days	−	−	++

Semiquantitative assessment from a minimum of five independent stainings for which nine HPFs were scored and the following criteria for scoring were applied: ++, positive, a minimum of six HPFs with membrane-bound staining pattern; +, moderately positive, specific membrane-bound staining in less then six but a minimum of two HPFs or staining patterns with cytoplasmic paranuclear staining instead of membrane-bound staining; −, negative, no specific staining.

liver for Cx32). In case of distinct and reproducible distribution of the fluorescent spots, staining was considered to be positive and specific. Tables 1 to 3 summarizes the connexin expression patterns in monolayer culture.

Expression of Cx26 was found positive in the normal urothelial cell line HCV-29 cells and increased in intensity over the time of culture (Figure 1A; Table 1). Cx26 could be detected as well in RT4 cells and was found negative in J82 monolayers. All monolayers expressed Cx43 but with different patterns. Whereas HCV-29 cells were clearly marked along the plasma membranes with additional intracellular staining in subconfluent cells (Figure 1C), J82 cells showed a diffuse cytoplasmic staining in most cases (Figure 1D). In RT4 cultures, staining was found in the membrane as well as in the cytoplasm. Despite identical fixation procedures and standardized tissue culture sampling, Cx43 was detected variably in RT4 cells.

Table 2. *Connexin Expression in Multicellular Spheroids of Different Ages of Culture (4 and 10 Days)*

Cell line	Cx26	Cx32	Cx43
HCV-29			
4 days	++	−	++
10 days	+	−	++
RT4			
4 days	++	−	++
10 days	++	−	++
J82			
4 days	++	++	+
10 days	+	+	+
N1			
4 days	−	−	++
10 days	−	−	++

Evaluation criteria are as in Table 1.

Table 3. *Connexin Expression in Tumor Cells in Three-Dimensional Co-Cultures with Fibroblasts at Different Times of Co-Culture (24 Hours and 7 Days)*

Cell line	Cx26	Cx32	Cx43
HCV-29			
	++	−	++
	+	−	++
RT4			
	++	+	−
	++	+	−
J82			
	++	+	−
	++	++	−

Evaluation criteria are as in Table 1.

The only connexin found in N1 cells was Cx43, showing a distinct intercellular pattern even in subconfluent cultures (Figure 1E). The Cx32 antibody showed no specific reaction in HCV-29 cultures. Both tumor cell lines, however, were Cx32 positive. Cx32 immunoreactivity was represented by large submembranous accumulations rather than by single spots along the plasma membrane (Figure 1F).

Connexin Expression in MCS Culture

Compared with monolayer results, expression of Cx43 became weaker both in RT4 and J82 MCSs and was not detectable in the corresponding MCS co-cultures (Figure 2A). All three-dimensional cultures constantly expressed Cx26, including J82 cells that were found to be Cx26 negative in monolayers. The pattern of Cx26 expression was more prominent in proximity to the fibroblast MCSs than in the periphery of the tumor spheroid as shown in RT4/N1 co-cultures (Figure 2B). In co-cultures of N1 and J82, the Cx26 immunopositivity was even enhanced in J82 cells and unaltered in N1 cells, which remained negative (Figure 2, C and D). Results for Cx32 resembled that in monolayers, as this connexin could be detected in J82 and RT4 MCSs and in MCS co-cultures of both tumor cell lines.

Characterization of Connexins by Western Blotting

The anti-Cx43 antibody recognized a band with corresponding mobility of 43 kd in all three cell lines (Figure 3A). In the lanes of RT4 and HCV-29 lysates, there was an additional marked band at approximately 48 kd, presumably representing a higher phosphorylated isoform of Cx43. This band could not be detected clearly in lysates from the poorly differentiated tumor cell line J82 (Figure 3A).

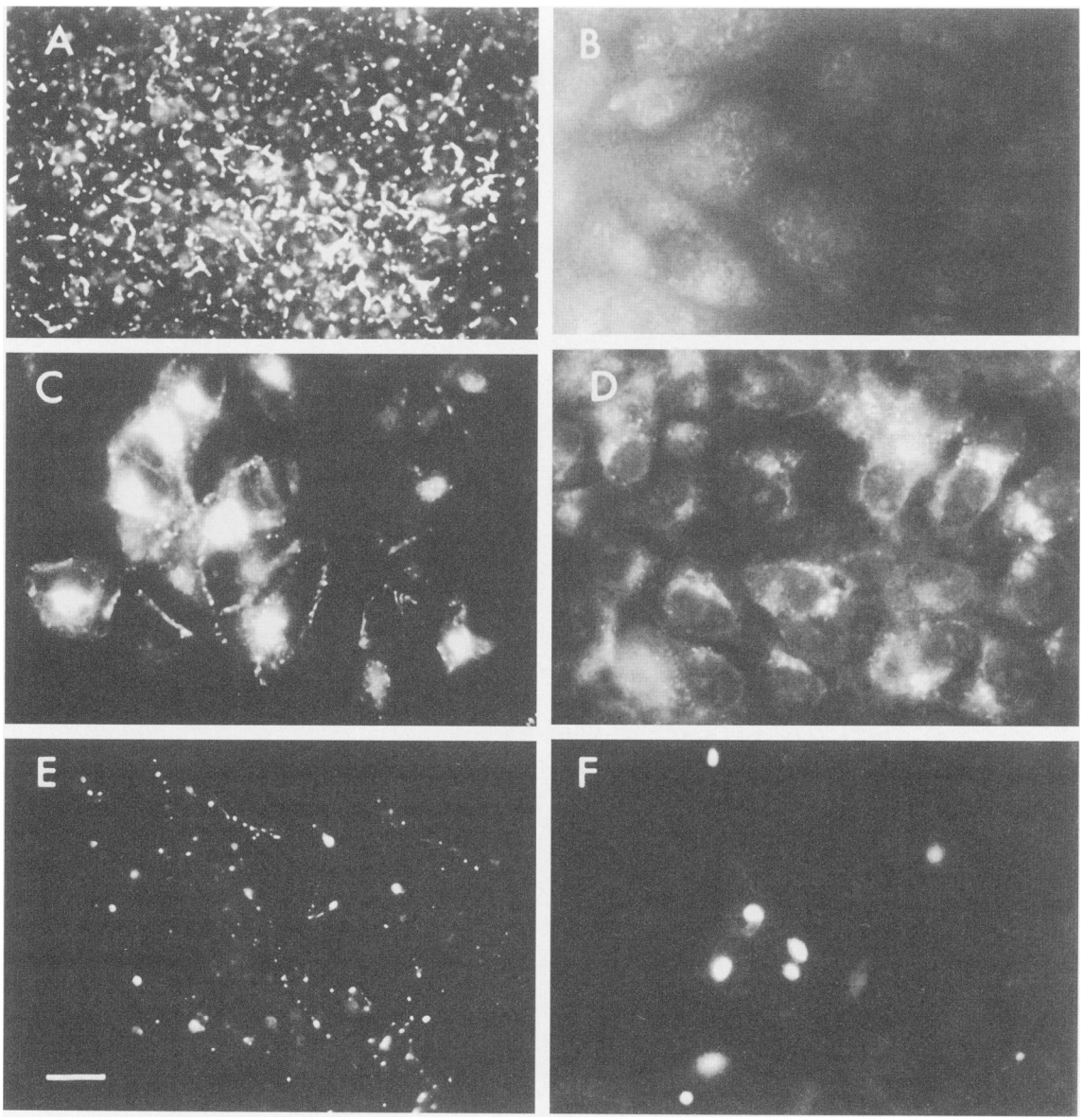

Figure 1. *Indirect immunofluorescence of monolayers using antibodies against connexins. Scale bar (SB) in* **E** *is representative for all pictures.* **A:** *Overview of confluent HCV-29 monolayer culture with strongly positive staining with anti-Cx26. SB, 400 μm.* **B:** *Staining with IgG control antibody does not show intercellular staining. SB, 50 μm.* **C:** *Anti-Cx43 staining in a subconfluent monolayer of HCV-29 cells, showing a mixture of diffuse cytoplasmic and distinct dotted intercellular staining. SB, 50 μm.* **D:** *Anti-Cx43 in confluent J82 cells, showing little intercellular and strong perinuclear staining. SB, 50 μm.* **E:** *Anti-Cx43 in subconfluent N1 cells, showing distinct intercellular dotted staining pattern. SB, 50 μm.* **F:** *Anti-Cx32 in subconfluent RT4 cells, showing a submembranous globular staining pattern. SB, 50 μm.*

Expression of Cx32 in the tumor cell lines RT4 and J82 could be confirmed in Western blots with polyclonal rabbit and monoclonal mouse antibodies. Antibodies reacted with a protein band at approximately 30 kd (Figure 3, A and B). Although the rabbit antibody was able to detect Cx32 only in RT4 cells (Figure 3A), the mouse-derived antibody showed positive staining in both RT4 and J82 cells. HCV-29 cells proved negative for Cx32 (Figure 3B).

Dye Coupling

Data obtained from microinjections with Lucifer Yellow differed significantly for the three cell lines. Over the time of culture, dye coupling in HCV-29 monolayers and co-cultures increased up to 50% (Figures 4A and 5, A and B) but decreased both in J82 and RT4 monolayers (Figure 4B). Generally, coupling was weaker in J82 cultures than in better differenti-

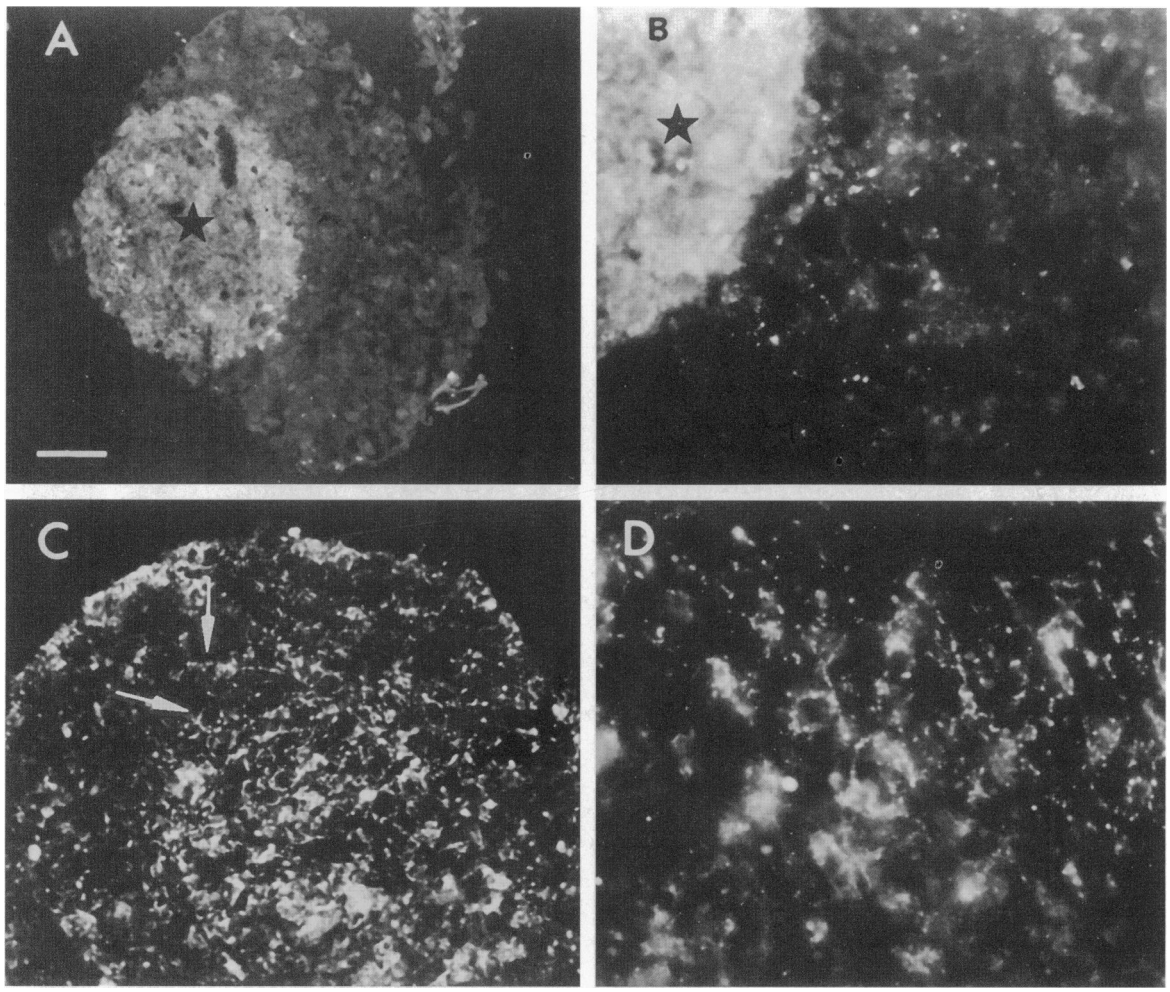

Figure 2. *Indirect immunofluorescence of MCS co-cultures using antibodies against connexins. SB in* **A** *is representative for all pictures.* **A:** *Anti-Cx43 staining of a 3-day-old co-culture of RT4-MCS and N1-MCS (★). N1 cells show a higher autofluorescence; positivity for Cx43 can still be seen. Tumor cells are completely negative. SB, 300 μm.* **B:** *Anti-Cx26 staining of a co-culture of RT4-MCS and N1-MCS (★). Positivity for Cx26 is much stronger in tumor cells in proximity to the fibroblast-MCS. SB, 50 μm.* **C:** *Anti-Cx26 staining of a co-culture of J82-MCS and N1-MCS. Fibroblast and tumor cells are mixed diffusely, and besides patchy autofluorescence of fibroblasts, distinct networks of intercellular Cx26 positivity can be recognized (as examples, see* **arrows**). *SB, 300 μm.* **D:** *Enlargement taken from* **C**. *SB, 50 μm.*

ated RT4 monolayers (Figure 4B). At day 4 of culture, 50% of the injected RT4 cells transferred Lucifer Yellow (Figures 4B and 5, C and D). In Figure 5, the early cluster formation in exponentially growing cultures is visible and leads to increasingly smaller cells in the center of the cluster over the time of cultures. In 12-day-old cultures, the average dye-coupling rate decreased to approximately 30% (Figure 4B). Control experiments with the normal fibroblast cell line N1 showed normal coupling rates that increased from 40% at day 4 of culture to 50% at day 12 (Figure 3A), which is comparable with the coupling incidence found in HCV-29 cells.

Immunohistochemistry of E-Cadherin

Characterization of E-cadherin expression in the urothelial cell lines was performed parallel to connexin analysis. Poorly differentiated invasive J82 cells were E-cadherin negative, whereas RT4 cells showed strong expression and HCV-29 cells showed moderate expression of this cell adhesion molecule. The expression pattern showed no changes from two- to three-dimensional cell cultures. No difference in staining intensity or distribution pattern was seen between monolayers and MCS sections (data not shown).

Immunohistochemistry of Connexins in Vivo

A number of cases showing normal urothelium, one squamous metaplasia, and papillary partly superficially invasive as well as muscle-invasive tumors have been screened repeatedly with the three anti-connexin antibodies, and resulting data are summa-

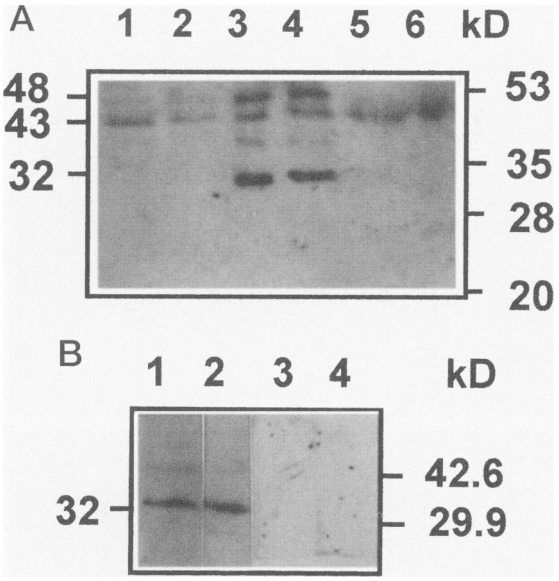

Figure 3. *Western blotting with rabbit and mouse antibodies against connexins.* A: *Double blotting of proteins from cell lysates with rabbit antibodies against Cx43 and Cx32.* Lanes 1 *and* 2, *HCV-29;* lanes 3 *and* 4, *RT4;* lanes 5 *and* 6, *J82. Numbers on the right indicate molecular weight of protein standard in kilodaltons. Cx43 can be detected in all* lanes *and shows an additional band at 48 kd in RT4 and HCV-29 cells. With the rabbit antibody, Cx32 is detected only in RT4 cells.* B: *Blot of proteins from cell lysates with a mouse antibody against Cx32.* Lane 1, *RT4;* lane 2, *J82;* lanes 3 *and* 4, *HCV-29. Numbers on the right indicate molecular weight of protein standard in kilodaltons. This antibody detects Cx32.*

rized in Table 4. Overall, all three types of connexins could be found in normal urothelium. Cx26 was present throughout all biopsies (see also Figure 6A), whereas Cx32 was found in only one case and expressed very weakly. Cx43 was found variably and was negative in some cases (Figure 6B). A squamous metaplasia of the bladder was found weakly positive for Cx26 in the upper layers (Figure 6C) but was found strongly positive for Cx43 in the basal and intermediate cell layers (Figure 6D), a pattern not observed in urothelium. Stromal components such as connective tissue were found to be Cx43 positive, and endothelial cells of capillaries showed distinct Cx43 patterns (Figure 6A).

Cx26 was expressed stronger in most papillary tumors (eg, Figure 7A) in comparison with normal urothelium and found negative only once in a papillary tumor with superficial invasion and medium grade. As in normal urothelium, the positivity for Cx43 was very variable. In contrast to papillary tumors, Cx26 was never found in invasive tumors (Figure 7C and Table 4). Overall expression of the three proteins was low in tumor cells of muscle-invasive tumors, whereas only Cx43 was detectable in one case (Table 4).

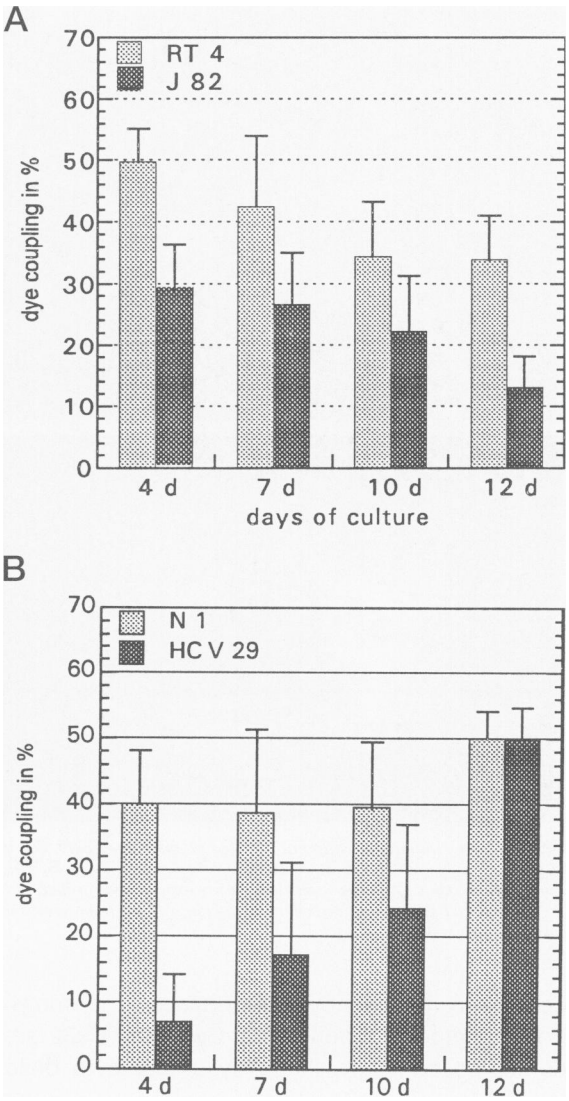

Figure 4. *Dye transfer after Lucifer Yellow injection in monolayers of normal non-urothelial (N1) and urothelial cells (HCV-29)* (A) *and malignant urothelial cells (RT4 and J82)* (B). *Standard deviation bars are indicated only in the positive direction. Dye transfer was checked in first-order cells (cells in direct contact with the injected cells).*

Discussion

Connexin Expression in Different States of in Vitro *Culture*

Looking at monolayer cultures of various cell lines in different growth states, all three connexins were detected, although expressed variably in the different cell lines. This stresses the notion that different but homologous proteins are localized in the areas of cell-to-cell channels[22] and seem to form organ-specific patterns. The selection of the antibodies has been made according to data in the literature relating either loss of Cx43 (rat glioma cells[23]) or loss of Cx26 (human breast cancer

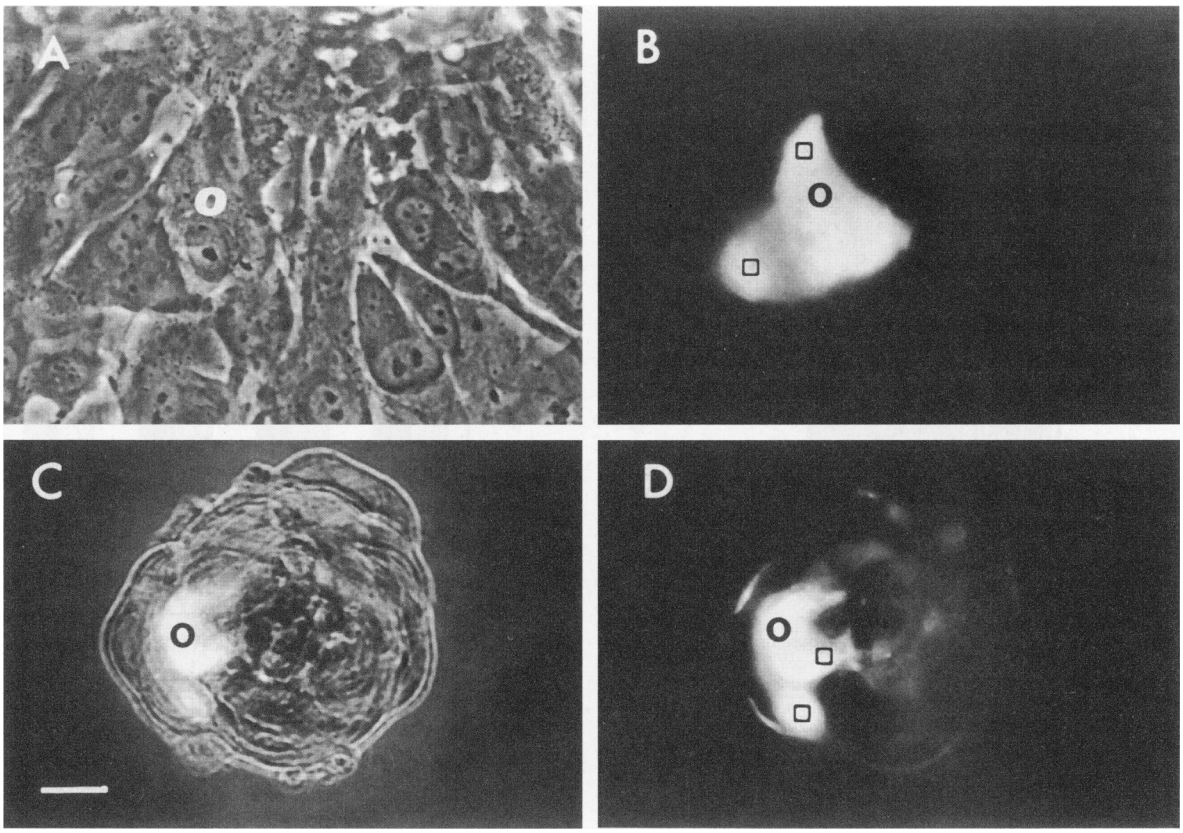

Figure 5. *Dye coupling in different states of monolayer growth. SB in* C *is representative for all pictures.* A: *Phase contrast of confluent monolayer of HCV-29 cells identical to* B; ○, *Lucifer-Yellow-injected cell.* B: *Fluorescence of Lucifer Yellow indicating dye transfer from injected cell* (○) *into adjacent cells* (★). C: *Phase contrast of early exponential growth of RT4 monolayer cells as small cluster, with double exposure after dye injection;* ○, *injected cell.* D: *Mere fluorescence of Lucifer Yellow shows even more distinctly, that initial dye injection* (○) *leads to spread into a minimum of two adjacent cells* (★).

cells[12]) to malignancy. Furthermore, it is known from hepatocyte plaques that Cx26 and Cx32 are co-expressed[24] and that they are able to build heterologous gap junctions,[25] and indications of phenotypic switching within the family of connexins as seen between Cx32 and Cx26 *versus* Cx43[26] seem an interesting finding to be assessed in normal *versus* malignant tissue. The only work on human bladder cancer except older preliminary data on connexin distribution in various tissues[27] dealt with mRNA of Cx43 and Cx26.[13] Both connexins were found in normal urothelial cells cultured from human ureters and to a lesser extent in human bladder carcinoma cell lines. In addition to these findings, it is important to also look at connexins on the protein level and to include information on cellular localization, as three control mechanisms of gap junctions are proposed: fast control by gating responses (in milliseconds), intermediate control by vesicular insertion or withdrawal of connexins in or out of the plasma membrane (in minutes), and long-term control for which transcription of Cx mRNAs is adjusted accordingly.[7] Data and hypotheses for the intermediate control mostly result from immunohistochemistry, for

Table 4. *Screening of* in Vivo *Urothelium and Urothelial Neoplasias*

Diagnosis	Cx26	Cx32	Cx43
Normal urothelium	++	+	++
Normal urothelium	++	−	−
Normal urothelium	+	−	−
Normal urothelium	+	−	−
Squamous metaplasia	+	−	++
pTa G1	++	+	−
pTa G2	++	+	−
pTa G2	+	−	+
pTa G2	++	+	−
pT1 G2	++	+	−
pT1 G2	++	++	++
pT1 G2	+	−	+
pT1 G2	−	+	+
pT2 G3	−	−	−
pT2 G2	−	−	−
>pT2 G3	−	−	−
>pT2 G3	−	−	+
pT3G3	−	−	−

Evaluation criteria are as in Table 1.

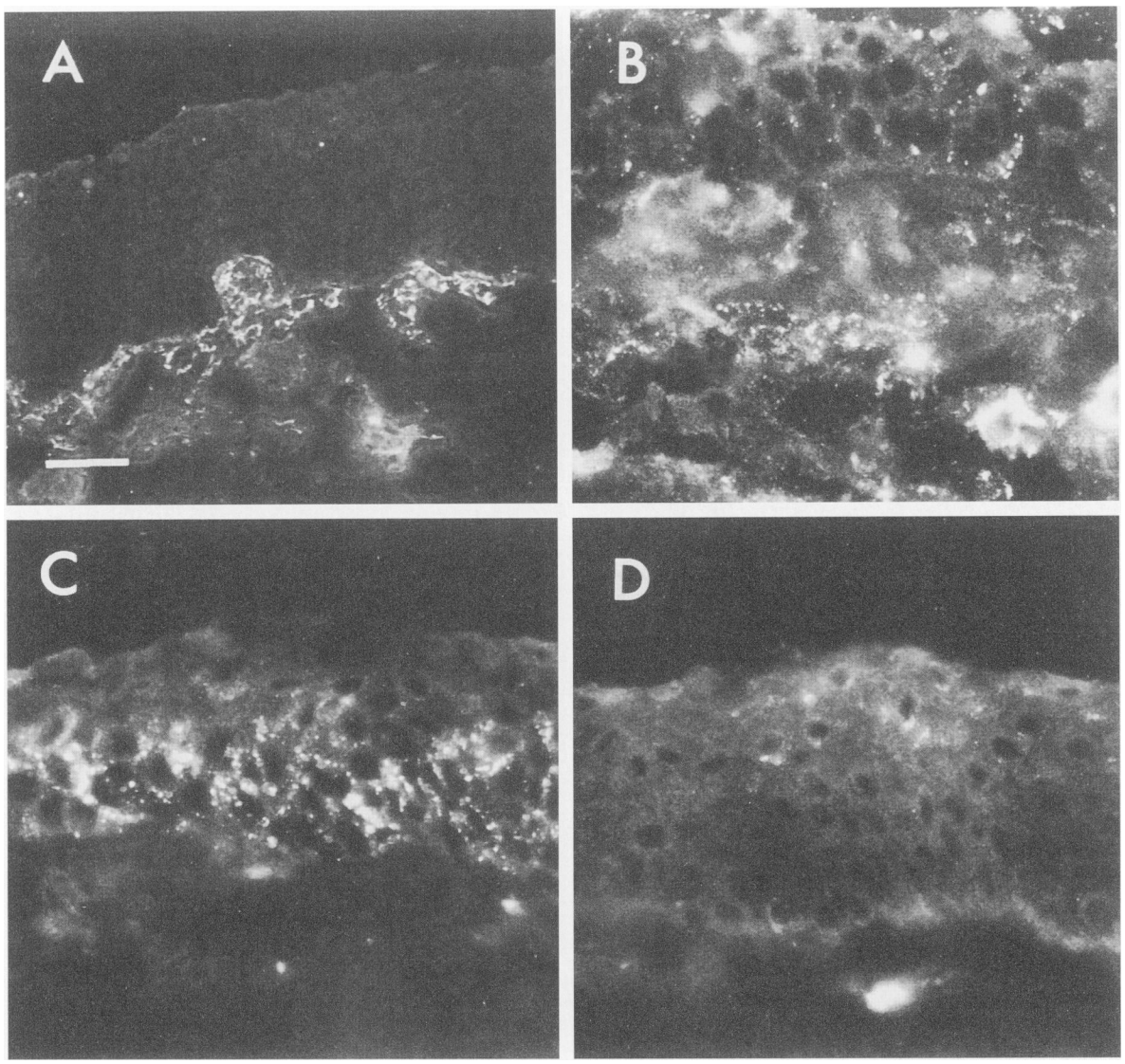

Figure 6. *Examples of indirect immunofluorescence staining of connexins in normal urothelium* (A: *Cx26;* B: *Cx43*) *and metaplastic squamous epithelium* (C: *Cx26;* D: *Cx43*). *The pattern of normal urothelium seems to be organ specific and changes in squamous metaplasia to a marked basal expression of Cx43. The SB in* C *is representative for all pictures (200 μm).*

which intracellular, submembranous pools are found, indicating a connexin pool, as seen in our experiments for Cx32, and in a less distinct but more diffuse intracellular staining for Cx26 and Cx43. The latter has also been described in tumor cells after transfection of Cx26 and Cx43.[28] It is suggested that Cx43 is not phosphorylated in this pool and that phosphorylation of the connexin happens only when connexins are membrane inserted.[9,29] Even under standard tissue culture conditions and defined staining protocols, Cx43 was expressed variably within the same type of culture. As the antibody does not differ in the degree of phosphorylation of the antigen, additional Western blots were carried out to check whether different bands would indicate differences in phosphorylation. Differences in phosphorylation of Cx43 were considered a reason for its variable detection, using an antibody against a cytoplasmic domain. However, Western blotting did not reveal a clear second band in the molecular weight range of Cx43 between the cell lines and may hint at differences in phosphorylation in this molecule in J82 cells.

Overall, the pattern of connexins in MCSs was more strongly intercellular, indicating that a different cell-cell interaction allowed for maturation of intercellular communication, as also seen in confluent monolayers. Interestingly, Cx26 appeared much stronger in RT4 MCSs and appeared *de novo*

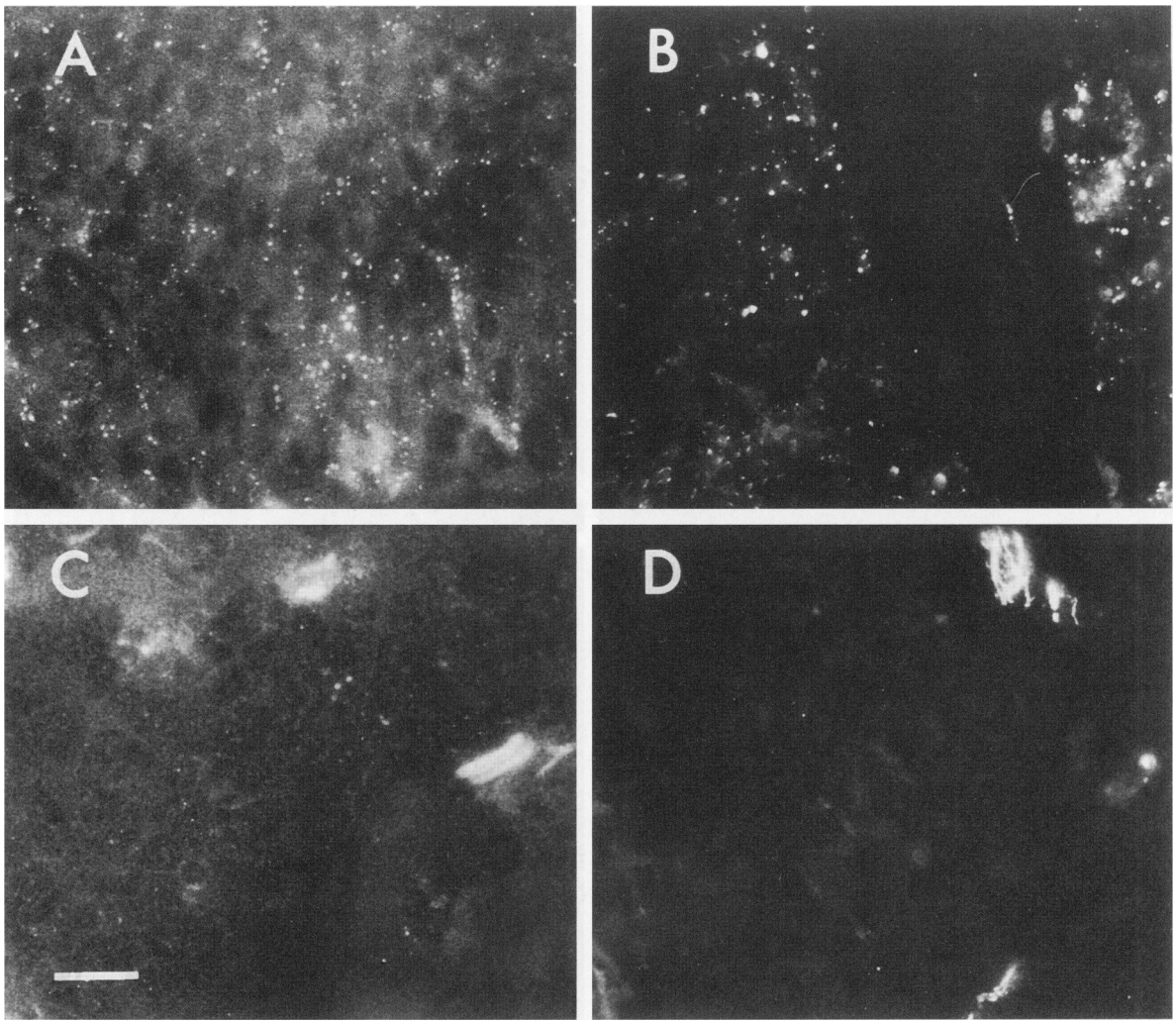

Figure 7. *Examples of indirect immunofluorescence staining of connexins in papillary noninvasive bladder carcinoma* (**A**: *Cx26;* **B**: *Cx32*) *and invasive bladder cancer* (**C**: *Cx26;* **D**: *Cx43*). *The SB in* **C** *is representative for all pictures* (*200 μm*). *Cx26 is found more abundantly in papillary tumors than in normal urothelium and is hardly found in invasive carcinomas.*

in MCSs of J82 cells, although it was not detected in these monolayers independent of the density of culture. This observation not only matches the observations on mRNA levels made by Grossmann et al,[13] suggesting that the transcription of Cx26 is higher in differentiated urothelial cells, but also reinforces a number of findings that cellular differentiation is higher in MCSs[30,31] and that physiological conditions are found to be more similar to the *in vivo* situation in comparison with monolayers.[32] The change in Cx26 expression was seen in heterologous co-cultures as well, being stronger in proximity to fibroblasts (Figure 1H). N1 cells are known to cause a reduction in proliferation of tumor cells of RT4/N1 co-cultures[16] and also show more collagen IV and laminin deposition. The controlled model also will allow systematic studies on heterologous cellular contacts as we have seen in monolayer co-cultures of fibroblasts and bladder cancer cell lines (data not shown) and as it is described *in vitro* between tumor cells and chicken embryo heart cells as a model to study invasion[33] and between keratinocytes and fibroblasts in monolayer cultures.[34] Heterologous cell interaction has been demonstrated by Tomasetto et al,[28] who systematically transfected various normal epithelial, stromal, and tumor breast cells, showing that heterologous communication can be asymmetrical and can be very selective, thus providing an interesting control system for cellular interaction. In contrast, a selective lack of gap-junctional communication was found in rat liver preneoplastic foci[35] and in human liver tumors in contact with the surrounding connective tissue capsule.[36,14] However, additional techniques (double staining) and higher resolution (confocal laser scanning micros-

copy) are required for our model system to obtain unequivocal data in tissue sections.

Connexin Expression Versus *Dye Coupling*

Monolayer data of Lucifer Yellow injections showed clear-cut differences between normal cells (N1 and HCV-29) and tumor cells (RT4 and J82), the latter showing a decrease in coupling with an increase in confluency. The increase of dye coupling in monolayers of normal urothelial cells correlates with Cx26 expression, which increased from exponential to plateau monolayers (Table 1). From immunohistochemical data we assume that Cx32 takes longer to be membrane inserted in monolayers, as the staining is mainly submembranous and globular.

Measurements made so far with MCSs[33] and that have been carried out by us as well (data not shown) are limited to the outer rim of MCSs and are insufficient to match immunohistochemical data, which show marked differences especially in the center of MCSs. Thus, the method of Lucifer Yellow injections of tissue sections,[36] although described as difficult, seems worthwhile to correlate immunohistochemical data in additional studies.

Connexin Expression in Vivo

Essentially, investigations *in vitro* are reflected by *in vivo* findings. The three connexins that could be observed *in vitro* occurred *in vivo* as well. This is in contrast with earlier investigations in which only Cx26 was found in urothelial neoplasias[27] but matches the mRNA data of Grossmann et al,[13] who used cultures of normal urothelium. It was interesting to see that highly differentiated urothelial tumors sometimes stained more intensively than normal urothelium, although experiments with different tissue samples of the same bladder are not yet included. The finding cannot simply be proliferation related, as the amount of cycling cells in pTa G1 tumors is mostly very low. The inconsistent findings of Cx43 detection *in vitro* also could be found *in vivo* and makes an important role of this connexin in bladder carcinogenesis unlikely. A parallel occurrence of Cx32 and Cx26 was not a constant finding for bladder cancer and normal tissues. The significance of these connexins will still have to be defined for bladder cancer.

Concluding Remarks

Systematic prospective studies including more tumors in prognostically inhomogeneous groups, such as superficial G2 tumors and flat urothelial lesions, using connexin antibodies will be one interesting aspect of additional work. Our data emphasize that Cx26 is a relevant molecule for the biology of bladder cancer with a possible tumor-suppressive role, but other newly found connexins should be included to complete the pattern of expression.

To further understand the regulation of connexins in terms of organ-specific homologous and heterologous cell interaction, the three-dimensional tissue culture model provides a dynamic, complex, and standardized model for investigations of humoral influences such as growth factors on GJIC or change of cell-cell interaction after transfection of connexins.

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