Enhanced Tumorigenicity of Rat Bladder Squamous Cell Carcinoma Cells after Abrogation of Gap Junctional Intercellular Communication

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We previously demonstrated a clear tendency for actively communicating rat bladder carcinoma cell lines with elevated expression of connexin 43 mRNA to possess strong tumorigenicity. In the present study, immunohistochemical analysis established that normal bladder epithelium did not express connexin 43 protein, but bladder carcinomas often expressed the protein, particularly on the membranes of cells within areas of squamous cell differentiation. To investigate the role of connexin 43 overexpression in rat bladder carcinoma cells, an anti-sense connexin 43 expression vector was transfected into BC31 cells having a high communication capacity. In the resultant transfectants, there was little or no communication capacity and connexin 43 expression. The growth rate *in vitro* was not changed compared to that of cells treated with the vector alone (without the anti-sense sequence), but tumorigenicity in nude mice was dramatically enhanced. The results indicate that connexin 43 overexpression in rat bladder carcinogenesis is related to squamous cell differentiation, and the protein can have tumor suppressor characteristics, as in other organs.

Key words: Gap junction — Connexin 43 — Bladder — Tumor — Rat

Direct transfer of nutrients, ions, nucleotides and other regulatory molecules between adjacent mammalian cells can occur through gap junctions, such gap junctional intercellular communication (GJIC) being important for regulation of cell proliferation, embryogenesis and differentiation.[1] Certain nongenotoxic carcinogens inhibit GJIC and this inhibition may be involved in the clonal expansion of initiated cells by releasing them from the suppressive control exerted by surrounding normal cells.[2, 3] In addition, many cancer cells have a decreased number of gap junctions and loss of GJIC has been correlated with the degree of malignancy of tumors.[4–7]

Recently, at least 12 cDNAs for connexin gap junction proteins in mammals have been cloned.[8] Of these, connexins 26, 32 and 43 are the best characterized, and their transfection into cell lines causes growth retardation or suppression of tumorigenicity.[9–12]

Rat bladder carcinogenesis has been well studied and several rat bladder cell lines are available. We previously showed that there is a tendency for these with greater communication capacity to have greater connexin 43 and connexin 26 mRNA expression and tumorigenicity.[13] Furthermore, while connexin 43 mRNA expression was found to be barely detectable in normal bladder tissue, we showed it to be abundant in rat bladder carcinomas.[13]

Since these observations for rat bladder carcinomas do not fit with the reported observations for gap junctions and carcinogenesis in other organs, we conducted the following experiments.

First, connexin 43 protein localization in normal and cancerous bladder tissue was investigated in order to confirm the results obtained for mRNA levels. In a preliminary study, we found that connexin 26 protein was not expressed in the cell lines despite abundant connexin 26 mRNA expression. We conclude that connexin 43 plays a key role in determining the gap junctional communication capacity of rat bladder carcinoma cells. Therefore we transfected an anti-sense connexin 43 expression vector into BC31 cells, which have good communication capacity, a high level of connexin 43 expression and consistent tumorigenicity in nude mice, to test the hypothesis that decreased connexin expression and GJIC might influence their growth.

MATERIALS AND METHODS

Induction of rat bladder tumors The design of the animal experiment and histopathological criteria for diagnosis were described in a previous report.[14] Urinary bladder tumors, induced in male F344 rats exposed to 0.2% N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide (FANFT) in the diet, were fixed in Bouin's solution and embedded in paraffin for immunohistochemical and histological analyses.
Bladder cell line The BC31 cell line was established

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from a bladder carcinoma arising in an ACI/N rat treated with N-butyl-N-(4-hydroxybutyl)nitrosamine.[15, 16] The cells were maintained in Dulbecco's minimal essential medium with 10% fetal bovine serum.

Connexin 43 anti-sense expression vector A 391 bp fragment of the connexin 43 gene, including the initiation codon, was amplified by polymerase chain reaction (PCR) using the primers CAGACATGGGTGACTGGAGT and TGCTTCAGGTGCATCTCCAC for Cx43-1 and Cx43-2 respectively. The fragment was subcloned into the pCR3 expression vector (Invitrogen Corp., San Diego, CA), which has a cytomegalovirus immediate-early promoter and a bovine growth hormone polyadenylation signal, in the anti-sense direction. This connexin anti-sense vector was then transfected into BC31 cells using the Transfectam reagent (Promega, Madison, WI) according to the manufacturer's instructions. Stable transfectants were selected with Geneticin (GIBCO BRL, Life Technologies, Gaitherburg, MD) (500 μg/ml) for more than 3 weeks. To examine tumorigenicity, aliquots of 10^6 cells were injected s.c. into male 6-week-old nude mice (CD-1-*nu/nu*; Charles River Japan Inc., Atsugi) and the resultant tumor weights were measured 25 days thereafter. Statistical analysis of the data was performed using ANOVA followed by the Fisher's protected least significant difference test (Stat View 4.0, Abacus Concepts, Inc., Berkeley, CA).

Northern blotting Total RNA was extracted from the cell line using ISOGEN (Wako Pure Chemical Industries, Ltd., Tokyo), based on the acid guanidium thiocyanate-phenol-chloroform method. Ten microgram aliquots of total RNA were loaded onto 1% agarose gels, electrophoresed and transferred to nylon membranes (Nytran; Schleicher & Schuell, Keene, NH). The cDNA probe for connexin 43 was labeled with [^{32}P]dCTP using a random primer labeling kit (High Prime; Boehringer Mannheim, Tokyo), hybridized to the membranes and analyzed with an image analyzer (BAS 2000, Fuji Film, Tokyo)

Western blotting When cells reached confluence in T25 flasks, all cells were lysed directly in 100 μl of 2% sodium dodecyl sulfate (SDS)-gel loading buffer (50 m*M* Tris-HCl, pH 6.8, 100 m*M* dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol), and 20 μl aliquots of protein were loaded onto 10% SDS-polyacrylamide gel electrophoresis (PAGE) gels, electrophoresed and transferred to nitrocellulose membranes (Hybond-ECL, Amersham, Tokyo). The membranes were blocked with 5% non-fat dried milk in Tris-buffered saline and incubated with connexin 43 monoclonal antibody (Zymed Laboratories Inc., South San Francisco, CA) for 1 h, then signals were detected with an ECL kit (Amersham).

Immunostaining for connexin 43 For the BC31 cell line, cells were grown on glass chamber slides (Nunc, Inc., Naperville, IL) and fixed in 100% acetone at −20°C for 5 min. They were treated with 1% Triton-X100

(Sigma, Tokyo) in phosphate-buffered saline (PBS) for 30 min to permeabilize the cell membranes, and incubated with connexin 43 monoclonal antibody (Zymed Laboratories Inc.) for 2 h. For bladder tissue, we found that Bouin's solution-fixed, paraffin-embedded materials could be used for connexin 43 immunostaining as well as frozen samples. After deparaffinization and rehydration of the Bouin's solution-fixed, paraffin-embedded tissue, sections cut at 4 μm were incubated with the connexin 43 antibody, and biotinylated anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA), followed by FITC-conjugated avidin (Sigma), and examined under a fluorescence microscope. For negative controls, one of the two was omitted. For positive controls, squamous cells of normal rat forestomach, known to express connexin 43 in abundance (unpublished data), were stained.

Gap junctional communication capacity: scrape dye loading method GJIC activity was measured as follows[17]: after having been rinsed in PBS, monolayers of cells immersed in PBS containing 20 mg/ml Lucifer Yellow (Sigma) were scraped with a sharp blade. The preparations were incubated in the dark for 10 min at room temperature, then the Lucifer Yellow was removed and the cultures were washed several times in PBS. The scrape-loaded cultures were photographed under fluorescence illumination.

RESULTS

Immunofluorescence staining of connexin 43 While staining was clear in rat forestomach squamous cells, no connexin 43 protein was detected in normal rat bladder transitional cell epithelial (urothelial) tissue (Fig. 1a). However, in limited areas of three of 15 transitional cell carcinomas induced by FANFT, weak connexin 43 spots were observed (Fig. 1b). Such spots were more intense on the membranes of cells within areas where cancer cells were stratified and diagnosed as having squamous cell differentiation, but not on the side of attachment to the basement membrane of basal cells (Fig. 1c).

Connexin 43 mRNA and protein expression in BC31 cells transfected with the anti-sense connexin 43 expression vector Three anti-sense connexin 43 transfectants (ASCX43-1, -2, and -3) selected with Geneticin (500 μg/ml) all exhibited similar mRNA expression levels to that of a control line (pCR3-2) transfected with the pCR3 vector lacking the anti-sense connexin 43 sequence (Fig. 2a). However, protein levels were much reduced in these transfectants, as revealed by both western blotting and immunostaining. ASCX-1 demonstrated slightly positive reactions on western blotting and immunohistochemistry, but ASCX-2 and -3 were totally negative (Figs. 2b, 3a and 3b). Growth rate *in vitro* was not changed compared to cells exposed to the vector alone.
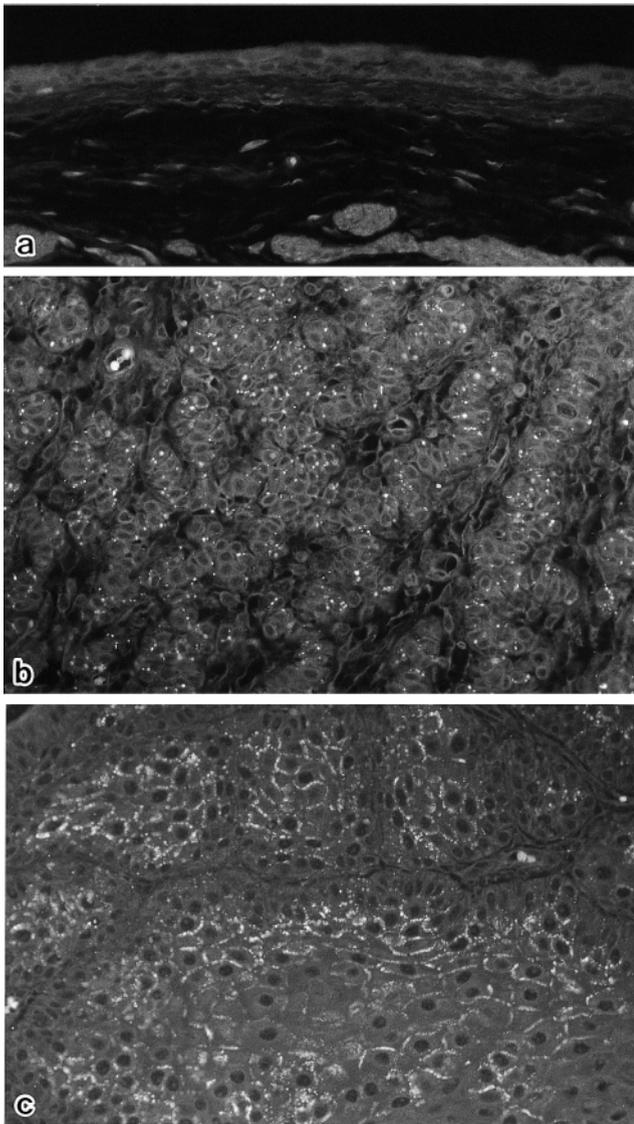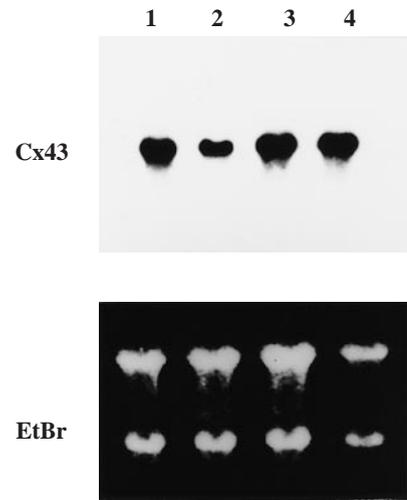
Fig. 1. Immunofluorescence staining of connexin 43. a, Normal rat bladder tissue; b, rat bladder transitional cell carcinoma; c, area of squamous cell differentiation in a transitional cell carcinoma. There is no staining for connexin 43 in the normal epithelium (a) but the transitional cell carcinoma is positive (b), and the protein is abundant in the squamous cell area where cancer cells are stratified (c). Original magnification, ×100.

Fig. 2. Connexin 43 mRNA (a) and protein expression (b) in BC31 cells transfected with the anti-sense connexin 43 expression vector. a: Lane 1, ASCX-1; lane 2, ASCX-2; lane 3, ASCX-3; lane 4, pCR3-2. b: Lane 1, lysate from mouse brain as a positive control; lane 2, ASCX-1; lane 3, ASCX-2; lane 4, ASCX-3; lane 5, pCR3-2. The phosphorylated form of connexin 43 (P) is also apparent.

Gap junctional intercellular communication assay: scrape dye loading assay Both BC31 parent cells and pCR3-transfected control cells demonstrated good communication. The three anti-sense transfectants, in contrast, had a much reduced communication capacity, especially ASCX-2 and -3, where no dye transfer was apparent (Fig. 4).

Tumorigenicity of cells transfected with the connexin 43 anti-sense expression vector The pCR3-transfected control cells gave rise to small tumors (mean weight, 0.122 ± 0.078 g) within 25 days after injection of 10^6 cells into 2 sites in groups of 5 nude mice. All three anti-sense connexin 43 transfectants produced much larger tumors under these conditions. Mean weights for ASCX-1, -2, and -3 were 0.722 ± 1.155 g, 1.986 ± 1.012 g, and 1.326 ± 0.817 g, respectively (Fig. 5). The differences in tumor weights between ASCX-2 or ASCX-3 and pCR3 were statistically significant ($P < 0.0001$ and $P < 0.005$, respectively). The histological features of the parent BC31 cells were typical of squamous cell carcinomas, and the transfection did not change the morphology.

DISCUSSION

One fundamental assumption regarding the role of GJIC in neoplasia is that it is present in normal cells with few exceptions (stem cells or mature blood cells), and it
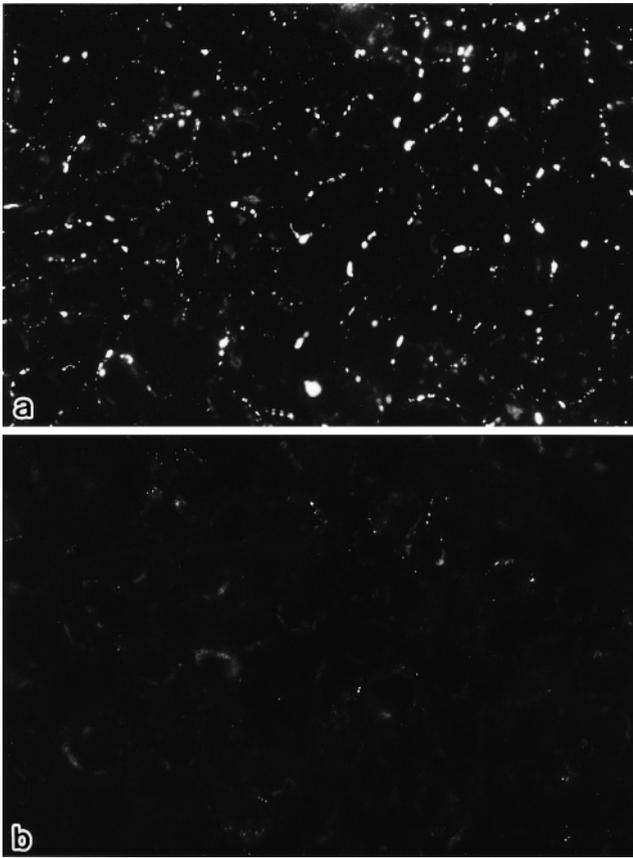
Fig. 3. Immunofluorescence staining of connexin 43 in anti-sense connexin 43 expression vector transfectants. a, BC31 parental cells; b, ASCX-2; connexin 43 anti-sense expression vector-transfected cells. The transfectants have a much reduced level (b) of connexin 43 protein, as compared to the control cells (a). Original magnification, ×400.
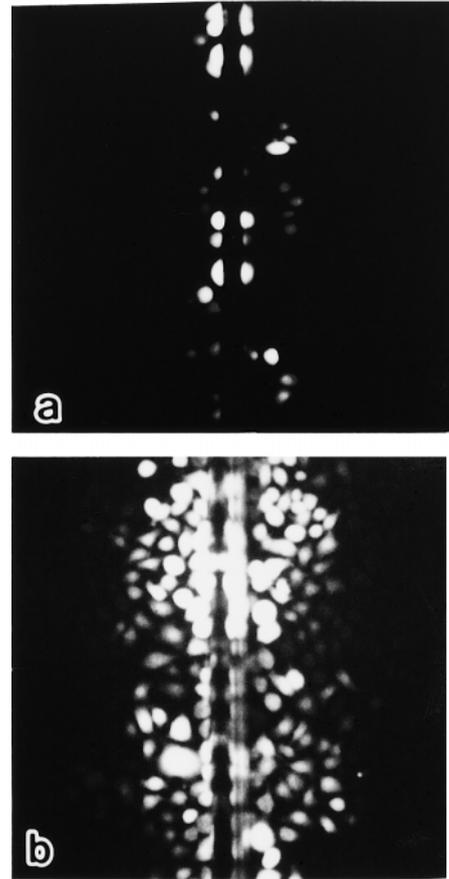

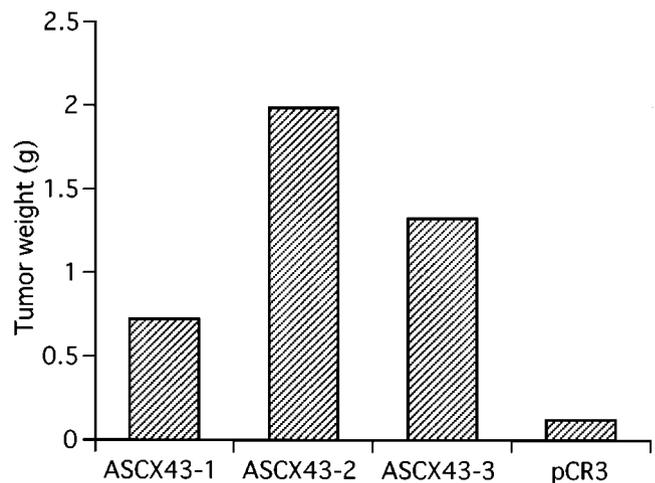Fig. 4. Scrape dye loading assay results for ASCX-2 (a) and pCR3-2 (b).

Fig. 5. Weights of tumors derived from connexin 43 anti-sense expression vector transfectants. The increases in tumor weights of ASCX43-2 and ASCX43-3, compared to pCR3 were statistically significant.

becomes decreased or lost during carcinogenesis.[1, 18] Several experiments have revealed that connexin genes have characteristics of tumor suppressors.[9, 11, 12, 19] However, we previously showed that the normal bladder expresses little connexin 43, whereas it is abundant in bladder carcinomas, and furthermore, GJIC was positively correlated with tumorigenicity of rat bladder cell lines.[13] In this study, we also found connexin 43 to be expressed in rat bladder transitional cell carcinomas, particularly in areas of squamous cell differentiation, but not in normal bladder tissue.

In several experimental systems, normal control of cell growth in GJIC-deficient and tumorigenic cells can be restored by transfection with connexin genes.[9, 11, 12] Therefore, negative signals for cell proliferation or tumorigenicity would appear to pass through gap junctions. However, our previous results suggested that the opposite might also be the case, i.e., that positive signals can be transferred.

To test this hypothesis we therefore introduced an anti-sense connexin 43 expression vector into tumorigenic rat bladder carcinoma cells BC31 to abrogate GJIC. Stable transfection was achieved, and the result was greatly enhanced tumorigenicity. Thus, as with other cell types, reduced GJIC capacity is positively associated with transitional cell neoplasia. Apparently there was a dose-dependent relation between the level of connexin 43 protein and the degree of tumor growth suppression in the transfectants. The level of communication observed in parental BC31 cells is obviously not sufficient for complete suppression of tumor growth, but its enhancement by transfection resulted in cessation of growth in nude mice (V. Krutovskikh, manuscript in preparation).

The reasons why rat bladder carcinomas are exceptional with regard to GJIC and connexin 43 expression are unclear. However, the present immunohistological analysis may provide a clue since the connexin 43 protein was detected most prominently in squamous cell areas, which tend to be increased during tumor progression in these tumors.[20] At this stage, several genes may be activated, some encoding positive and others negative factors for tumor progression, but in sum their effects must lead to growth. The increased connexin 43 expression may also be related to squamous differentiation, in contrast to transitional cell (urothelial) differentiation. Normal transitional cell epithelium in the bladder shows no expression of connexin 43 and relatively few gap junctions.[21, 22] In contrast, normal squamous epithelium shows considerable expression of connexin 43 with numerous gap junctions.

It would be interesting to establish whether connexin expression changes in squamous metaplasia in other organs. Although connexin 43 expression is increased in these tumors compared to normal transitional cells, the expression is primarily in areas of squamous differentiation. The level of expression in these areas is comparable to or lower than in normal squamous cells[23–25], with which comparison is more appropriate. To clarify whether this is indeed the case, studies of the mechanisms of transcriptional activation of the connexin 43 gene are necessary, although change of connexin 43 expression alone was not enough to alter the morphology of the cells. An expression vector for anti-sense connexin 43 may be a powerful tool to investigate the role of connexin 43 in various cells.

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