Connexin 43 transfection in basaloid squamous cell carcinoma cells

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Abstract. To investigate the relationship between the expression of connexin in basaloid squamous cell carcinomas (BSCC) and their rapid proliferation and invasive potential, we examined the effect of overexpression of connexin 43 (Cx43) in a BSCC-derived cell line (BSC-OF). BSC-OF was transfected with Cx43 to obtain 15 clones with a stable expression of Cx43. In these cells, although Cx43 was distributed throughout the cytoplasm, it did not form connexon plaque. In almost all of the clones, cell proliferation was clearly suppressed. Furthermore, we investigated cell migration and invasion in three clones that showed a remarkable down-regulation in cell growth, and found that Cx43 transfection showed no significant effect on either. These results suggest that Cx43 plays a role as a tumor suppressor in the cytoplasm of Cx43-transfected BSC-OF cells. However, no definite correlation was found between Cx43 and cell migration and invasion.

Introduction

Gap junctions are an intercellular connection system, forming channels between cells and mediating the transfer of molecules smaller than 1000 daltons, ions and various second messengers. Gap junctions are formed by the docking of two connexons, each oligomer consisting of six connexin (Cx) molecules (1-3). Cx is a transmembranous protein, forming a multi-gene family classified by their molecular weight. There are more than 12 kinds of connexin in mammalian tissue. In most cells, connexons are made up of more than two types of connexin, with each pair of different Cxs forming a heteromeric connexon (1,2). Cx26 and 43 are distributed variably in the keratinocytes of squamous epithelium (3,4).

Disruption of connexins has been frequently reported in malignant tumor cells such as hepatocellular carcinoma (5) and breast carcinoma (6), and in carcinogenesis of the cervix (7) and endometrium (8). Saitoh *et al* demonstrated changes in the expression of Cx26 and 43 in hamster tongue epithelium during wound healing and carcinogenesis (9). However, few studies have investigated changes in the expression of Cx43 in oral squamous cell carcinoma (SCC) (10). Therefore, we investigated the effect of overexpression of Cx43 in oral carcinoma cells.

Basaloid squamous cell carcinoma (BSCC) is a relatively rare subtype of oral SCC that reveals rapid growth and aggressive local invasive potential, and its prognosis is much poorer than that of other SCCs (11,12). We hypothesized that the rapid proliferation and invasive potential of BSCC would have some relationship with expression of Cx43, and used BSC-OF cells derived from BSCC of the floor of the mouth. BSC-OF has shown more aggressive proliferation, invasion, and motility than other oral SCC cell lines (13). In this study, we investigated whether overexpression of Cx43 in BSC-OF cells affected their malignant characteristics, proliferation, phagokinetics or invasive potential.

Materials and methods

Cell culture and confirmation of expression of Cx43. BSC-OF cells were grown in D-MEM/F12 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS, 1% penicillin-streptomycin and 0.1% gentamycin (Invitrogen) solution at 37˚C in 5% CO_2 atmosphere. Saos-2 cells derived from osteosarcoma were cultured in D-MEM (Invitrogen) for use as a positive control. Total-RNA was extracted from the cells at confluence by AGPC method with TRIzol® (Invitrogen). To confirm expression of Cx43, conventional RT-PCR was performed. The sequence of the forward primer was 5'-gaattctggttatcatgt cggggaa-3', and the reverse primer was 5'-taccatgcgaccagtggt gcgct-3' (14).

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Vector construction and transfection. cDNA from the human Cx43 coding region was amplified by PCR with the following primers: forward 5'-cacaattgagtggaatcttgatg-3'; reverse 5'-caa catgggtgactggagc-3'. PCR product was purified and inserted into the mammalian expression vector (pTARGET, Promega, Madison, WI, USA). Expression vector was transfected into BSC-OF cells by the lipofection method using Lipofectamin™ Reagent (Invitrogen). Subclones were selected by ampicillin and 400 μg/ml antibiotic G418 sulfate (Calbiochem, San Diego, CA, USA). Cells of the subclones were diluted and seeded to obtain further clones.

Western blotting. Total cell lysate was obtained from the BSC-OF and 15 clones with RIPA buffer (pH 7.2) which included the protease inhibitors, aprotinin, leupeptin, PMSF, Na_3VO_4 and MG132. SDS-PAGE (12%) was performed with 50 μg protein of each sample, and transferred to PVDF membrane (Bio-Rad, Hercules, CA, USA) at 4˚C for 1 h. After blocking the non-specific reaction, anti-Cx43 polyclonal antibody (1:1000, Zymed, South San Francisco, CA, USA) was applied and incubated at 4˚C overnight. After washing in TBS with 0.1% Triton-X, horseradish peroxidase conjugated anti-rabbit IgG (Amersham, Buckinghamshire, UK) was applied for 1 h. To detect expression of Cx43, ECL™ Western Blotting Detection Reagents (Amersham Bioscience) was used.

Immunofluorescence analysis. Cells were seeded on cover slips at the bottom of the culture dishes until they became subconfluent and then fixed with acetone and methanol for 20 min. To block non-specific reaction, goat normal serum was applied for 30 min, and then anti-Cx43 monoclonal antibody (1:300, Chemicon, Temecula, CA, USA) was applied at room temperature for 1 h. After washing, anti-mouse IgG, Alexa fluor® 488 (1:500, Molecular Probes, Eugene, OR, USA), and To-PRO®-3 iodide (1:500, Molecular Probes) for nuclear staining were applied at room temperature for 1 h. Observation was performed by fluorescence microscopy with the Axiophot2. (Carl Zeiss, München-Hallbergmoos, Germany).

Cell proliferation assay (screening). A $1x10^4$ aliquot of cells of BSC-OF and all clones were seeded onto the cell culture dishes. After one to seven days, cells were detrypsinized and the total number counted in triplicate using an erithrocytometer.

Migration assay. Phagokinetic assay was performed by modified Albrecht-Buehler's method (15). Three clones (A4, 9, 13) that showed remarkable down-regulation of cell proliferation were selected. A $1x10^4$ aliquot of cells of BSC-OF and the three clones were then plated onto the cover slips coated with colloidal gold salts (145 mM $AuCl_4H$), and cultured in DMEM/F12 containing 1% FBS for 24 h respectively. Then, more than 30 phagokinetic tracks per cell were measured by computer-assisted image.

Invasion assay. A $1x10^5$ aliquot of cells of BSC-OF and the three clones (A4, 9, 13) were plated onto a 6-well BioCoat™ Matrigel™ Invasion Chamber (BD Biosciences, San Jose, CA, USA) and cultured for 24 h, respectively. Cells digested the matrigel, enabling them to pass through the micro-pores in the gel, after which, they were fixed and stained. The numbers of invasion cells were counted per 1 mm^2 at the central area of the matrigel.

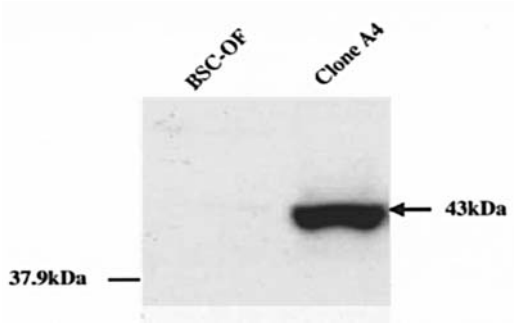

Figure 1. Expression of transfected Cx43 confirmed by 12% SDS-PAGE and Western blotting. Fifteen clones expressing transfected Cx43 were obtained.

Statistical analysis. Phagokinetic tracks and invasion indexes were analyzed by one-way factorial ANOVA and multiple comparison tests.

Results

Confirmation of transfection. Weak expression of mRNA of Cx43 was identified in BSC-OF parent cells by comparison with Saos-2. A slight increase in Cx43 expression was observed in all clones (A1-15) by RT-PCR, and Western blotting (Fig. 1). However, no quantitative differences in expression of Cx43 were found among the clones.

Localization of Cx43. Immunofluoresence analysis revealed a more strongly defined immunopositive signal for Cx43 in all clones than in the BSC-OF parent cells. However, although Cx43 was distributed evenly throughout the cytoplasm, no signal was observed in the intercellular areas of any of the clones. In the positive control, Saos-2, Cx43 displayed punctuated signals in the intercellular area (Fig. 2).

Growth characteristics. In most of the Cx43-transfected clones, cell proliferation showed greater down-regulation than in the BSC-OF parent and mock cells. Remarkable down-regulation of cell proliferation was distinct in three clones, A4, 9 and 13 (Fig. 3).

Migration assay. The averaged phagokinetic tracks revealed a slight increase in A9 and a decrease in A4 compared to in BSC-OF (Table I). However, there were no significant differences between BSC-OF and the other three clones.

Invasion assay. The number of cells that invaded the matrigel is indicated by the invasion index (Table II). The cell invasion index showed an increase in clone A9 and a decrease in clone A13 compared to in BSC-OF, although there were no significant differences. A9 and A13, however, showed a significant difference in invaded cell number.

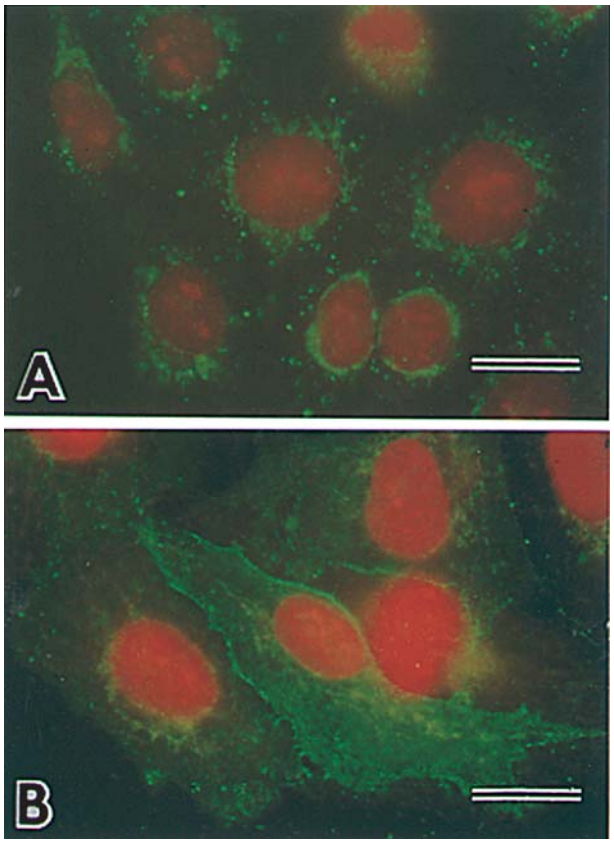

Figure 2. (A), Dot-shaped Cx43 distributed not at intercellular region but in cytoplasm of clone A4. (Bar=20 μm). (B), In Saos-2 (positive control) endogenous Cx43 distinct in intercellular region. (Bar=20 μm).

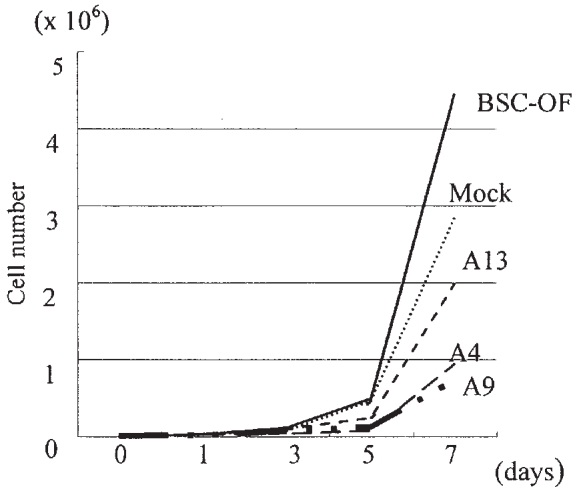

Figure 3. Effect of transfected Cx43 on proliferation of BSC-OF. Proliferation curve shows cell growth of three clones markedly suppressed.

Table I. Migration index.

Cell	Phagokinetic track (x10^3 mm^2)
BSC-OF	2.45±0.87
Clone A4	2.40±0.54
Clone A9	2.94±1.17
Clone A13	2.42±0.80

Table II. Cell invasion index.

Cell	Cell no. (/mm^2)	
BSC-OF	1.285±0.747	
Clone A4	1.187±0.772	
Clone A9	2.887±0.263	*
Clone A13	0.434±0.139	*

*p<0.05.

Discussion

In squamous epithelium, Cx43 is mainly expressed in the basal layer, forming gap junctions as an intercellular communication system (4). Generally, Cx decreases in malignant tumors in which intercellular communication may be lost (5-8). As a result, the nest of tumors expands and shows invasive growth into neighboring or distant tissues.

We investigated the effect of Cx43 transfection in BSC-OF oral carcinoma cells. All of the monoclonal Cx43 transfectants revealed increased expression of Cx43. However, although Cx43 was distributed throughout the cytoplasm, it did not form gap junctions in any of the clones. This result was supported by the scrape loading method (data not shown).

All the clones we obtained showed suppressed cell proliferation in comparison to the parent and mock cells. It has been suggested that transfection-induced abundance of Cx43 suppresses tumor proliferation, particularly in clone A9. Zhang *et al* have demonstrated that, in osteosarcoma cell line U2OS, the C-terminal of Cx43 binds the S-phase kinase-associated protein (Skp2), which inhibits ubiquitination of p27, the cell cycle regulator in the G1-S phase (16). Cx43 has been shown to cause an increase in p27 without forming gap junctions, leading to a reduction in the cell cycle (17,18). In oral SCC, it is unclear whether Cx43 inhibits p27 degradation, and we found no clear evidence of an interaction between Cx43 and Skp-2. However, our findings do suggest that abundant Cx43 not forming gap junctions in the cytoplasm indicates involvement in cell cycle regulation.

Generally, oral carcinoma cells frequently destroy the basement membrane, infiltrating deeply and metastasizing to the lymph nodes of the neck region (19). Loss of intercellular communication causes each individual cell to migrate. Graeber *et al* noted that, in HeLa cells, Cx transfection, especially with Cx43, induced invasive properties (20). In their investigation, Cx43-transfected HeLa cells formed gap junctions and became involved in intercellular communication. In our results, however, transfection with Cx43 showed no effect on cell motility or invasion, although the transfected Cx43 was

distributed throughout the cytoplasm, it did not form gap junctions, and, therefore, did not influence intercellular communication. For cell migration to take place, a change in cell skeletal protein is necessary. Cx43 in the cytoplasm may not interact with actin, tublin, or various filaments (21).

Our results suggest that one of the important factors is whether transfected Cx43 forms gap junctions or not. Zhu *et al* also investigated the effect of Cx43 transfection on C6 glioma cells, obtaining clones that showed various amounts of Cx43 displayed both at areas of intercellular contact and in the cytoplasm (22). The precise mechanism behind the occasional high expression of gap junctions in the clones remains to be determined. Momiyama *et al* observed the same phenomenon, finding Cx26 transfection in breast cancer cells produced clones both with and without gap junctions (23). In forming gap junctions in the process of intracellular transportation, the assembly, detachment, and degradation of Cxs is correlated with their phosphorylation (2,21). Cx43 phosphorylation is highly regulated during the cell cycle (24). The rise of intracellular cyclic AMP also promotes the assembly of connexons, which results in an increase in gap junctions (21). Depending on the clone, there may be differences in the level of cyclic AMP or phosphorylation. We surmise that phosphorylation of Cx43 may occur only rarely in BSC-OF, making it difficult for Cx to present itself for the construction of gap junctions.

In conclusion, transfected Cx43 in BSC-OF was found in the cytoplasm, clearly inducing tumor suppression. In terms of cell invasion and motility, there were significant differences between the parent cells and the Cx43 transfectant. Proliferation in clone A9 was suppressed remarkably, although both cell migration and motility were promoted.

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