A strategy for the suppression of tumorigenesis induced by biomaterials: Restoration of transformed phenotype of polyetherurethane-induced tumor cells by Cx43 transfection

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Abstract

Biomaterials such as polyetherurethans (PEUs) are the scaffolding, which is indispensable for the development of the bio-artificial organs. However, PEUs can induce tumors in subcutaneous implantation sites in rat. We have shown that the different inhibitory potential of gap junctional intercellular communication (GJIC) on the surface of the biomaterials, including PEUs, is a key step in determining the tumorigenic potential. Here we show that suppression of a gap junctional protein connexin 43 (Cx43) plays an important role in *in vivo* tumorigenesis induced by PEUs for the first time and that Cx43 transfection may be an effective strategy for preventing tumorigenesis induced by biomaterials. Rat tumor cell line U41 is derived from tumors in the subcutaneous implantation of PEU films. The GJIC and the expression of Cx43 were suppressed in U41. The restoration of normal phenotype, such as reduction of growth rate, recovery of contact inhibition and loss of colony formation ability in soft agar, was achieved by Cx43 transfection. These results strongly suggest that suppression of Cx43 expression plays an important role in the development of rat malignant fibrous histiocytoma (MFHC) caused by PEUs and that Cx43 transfection is effective for prevention of tumorigenesis induced by PEUs.

Introduction

Polyetherurethanes (PEUs) are used for implant applications because of their useful elastomeric properties and high tensile strength, lubricity, good abrasion resistance, and ease of handling. However, some kinds of PEUs are known to be unstable in body (Wu et al. 1992; Xi et al. 1994) can induce tumors in rats (Nakamura et al. 1992). We have reported the tumorigenic potentials of these PEUs *in vivo* and *in vitro* (Tsuchiya et al. 1995a, 1995b). For many years, foreign-body carcinogenesis in rodent species has been recognized as a classic model of multistage endogenous tumorigenesis that requires half to two-thirds of the life span for tumor development. A number of studies have demonstrated that physical,

not chemical, characteristics are responsible for this phenomenon and that a dose-dependent relationship is evident with respect to implant size and tumor frequency (Schoen 1987). We have examined the potency of tumor-initiating and tumor-promoting activities of the PEUs (Tsuchiya et al. 1996). The promoting activities were considered to be stronger than the initiating activities. Furthermore, inhibitory action of polyethylene's surface on gap junctional intercellular communication (GJIC) has been detected (Nakaoka et al. 1997). Thus, different inhibitory potential of GJIC on the surface of the biomaterials, including PEUs, may be likely a key step in determine the tumorigenic potentials (Tsuchiya and Nakamura 1995c). PEU-components had inhibited GJIC in cultures of Balb/c 3T3 A31-1-1 cells and Chinese ham-

ster V79 fibroblasts (Tsuchiya et al. 1996; Tsuchiya and Nakamura 1999a, 1999b).

GJIC has long been postulated to play an important role in the maintenance of cell homeostasis and in the control of cell growth (Loewenstein 1979). Therefore the loss of GJIC has been considered to cause aberrant development and tumor formation (Guthrie and Gilula 1989; Klaunig and Ruch 1990; Mesnil and Yamasaki 1993). Gap junctions are formed by juxtaposition of two hemichannels known as connexons, located on the surface of adjacent cells (Bennett et al. 1991). Each connexon consists of six connexin (Cx) molecules. Cx43 is the most widely expressed Cx type. Accumulating evidence indicates that loss or reduction of GJIC in malignant transformed cells or tumor cells closely correlates to transcriptional down-regulation of Cx genes and/or to aberrant localization of Cx proteins. Restoration of GJIC by transfection of Cx genes has been demonstrated to reverse the transformed phenotype of cancer cells (Huang et al. 1998; Zhang et al. 1998).

To clarify a tumor promoting activity of PEU, we have established an U41 rat tumor cell line from malignant fibrous histiocytoma (MFHC) developed in the PEU film implanted sites. Function of GJIC in our cell line was assayed by the scrape-loading dye transfer method and the expression of Cx43 connexin was examined either by western blotting or by reverse transcription-PCR (RT-PCR). Since the introduction of Cx43 gene overcame the GJIC function, poor GJIC development in U41 cells might be caused by a suppressed connexin expression. The Cx43 up-regulated stable transfectant showed rather controlled cell growth on contact inhibition, and revealed poor anchorage independent growth in soft agar.

Materials and methods

Cell culture

The U41 and U51 cell lines were established from rat malignant fibrous histiocytoma (MFHC) induced by polyetherurethane (PEU) implantation. Normal rat control cell line was established from the same region of the implantation experiments from the control rat. These Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS). Cell cultures were maintained in a 37 °C incubator under a humidified 5% CO₂/95% atmos-

phere and were routinely subcultured by trypsinization.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cultured cells by TRIzol reagent (Life Technologies, Inc., Frederick, MD, USA) according to manufacturer's instruction. Five µg of total RNA was used for cDNA synthesis using SuperScript II RNase H⁻ reverse transcriptase (Life Technologies, Inc., Frederick, MD, USA). Cx43 mRNA was amplified using oligonucleotide primers Cx43RT1 (5'-ATCCAGTGGTACATCTATGG-3') and Cx43RT2 (5'-TTTCTGTGCGCCAGTAACCAG-3') for 30 cycles (94 °C for 30 s, 56 °C for 30 s, 72 °C for 90 s). Reaction products were resolved on 1.5% agarose gel and stained with SYBR Green I (FMC BioProducts, Rockland, ME, USA). The expected amplified fragment for rat Cx43 was 368 bp. As an internal control, GAPDH primers GAPDH-F (5'-CCCATCACCATCTTCCAGGAGCGAGA-3') and GAPDH-R (5'-TGGCCAAGGTCATCCATGACA-CTTTGG-3') were used to detect 577 bp of GAPDH product.

Plasmid construction and transfection

Full-length Cx43 DNA fragment was amplified by polymerase chain reaction (PCR) using primers Cx43F (5'-TTGACTCGAGCCTCCAAGGAGTTCACCAACT-3') and Cx43R (5'-CAACTCGAGTTAAATCTCCAGGTCATCAGGCC-3'). The *Xho* I site (CTCGAG, underlined) was inserted into these primers for subcloning into a mammalian expression vector pB45-neo (Karasuyama and Melchers 1988). The cloned 1217 bp PCR fragment was subcloned into pB45-neo. Cx43 expression vector pB45-Cx43-neo and control vector pB45-neo were then transfected into U41-22 cells using the SuperFect reagent (QIAGEN K.K., Tokyo, Japan) according to manufacturer's instruction. Stable transfectant clones were selected with 500 µg/ml G418 (Life Technologies, Inc., Frederick, MD, USA), and clones were isolated using cylinder trypsinization method followed by further expansion and storage at -85 °C.

Western blot

When cells reached confluence in 60-mm tissue culture dishes, all cells were lysed directly in 500 µl

of lysis buffer (50 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride). Protein concentrations of lysates were measured using D/C protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) according to manufacturer's instruction. Equal amounts of proteins dissolved in gel loading buffer (50 mM Tris-HCl, pH 6.8, 100 mM 2-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) were analyzed by 7.5% SDS-polyacrylamide gel electrophoresis, and proteins were transferred to Hybond-ECL nitrocellulose membranes (Amersham Pharmacia Biotech UK Limited, Buckinghamshire, UK). Cx43 protein was detected by anti-Cx43 polyclonal antibodies (ZYMED Laboratories, Inc., San Francisco, CA, USA) and ECL system (Amersham Pharmacia Biotech UK Limited, Buckinghamshire, UK).

Scrape-loading and dye transfer (SLDT) assay for detection of GJIC

The scrape-loading and dye transfer (SLDT) technique was adapted after the method of El-Fouly et al. (1987). Confluent monolayer cells in 35-mm culture dishes were used. After rinsing with Ca^{2+} Mg^{2+} phosphate-buffered saline (PBS (+)), cell dishes were loaded with 0.05% Lucifer Yellow (Molecular Probes, Eugene, OR, USA)/PBS (+) solution and were scraped immediately with a sharp blade. After incubation for 5 min at 37 °C, cells were washed with PBS (+) and monitored using fluorescence microscope. The distance the dye migrated was measured from the cell layer at the scrape to the edge of the dye front that was visually detectable (Upham et al. 1995).

Cell proliferation assay

Cells (10,000/well) were seeded into a 24-well tissue culture plate and allowed to grow for various length of time. The trypsinized cells were counted in duplicate for each cell line using a hemocytometer. The cell number were then plotted against culture time.

Soft agar assay

Approximately 500 cells from each clone were seeded in 1 ml of 0.3% soft agar in culture medium on a solidified basal layer per well in 12 well tissue culture plates. The plates were cultured for 2–3

weeks and then stained with *p*-iodotetrazolium violet overnight before counting. Colonies with sizes $>19.625 \mu\text{m}^2$ were scored as positive.

Results

Analysis of Cx43 expression and GJIC in PEU-induced tumor cell lines

To assess functional GJIC, scrape-loading and dye transfer (SLDT) assay was employed. Lucifer yellow does not diffuse through intact plasma membranes and its low molecular weight permits its transmission from one cell to another, presumably across patent gap junctions (Stewart 1979, 1981; Lo and Gilula 1979). Therefore, the SLDT activities reflect the functional detection of GJIC. Migration of Lucifer yellow fluorescence was forwarded to decrease in U41 and U51 cells (Figure 1A). These results indicated that functional GJIC were lost in U41 and U51 cells. Then we examined the Cx43 protein expression in U41, U51, and normal rat control cells. Normal rat control cells expressed large amounts of Cx43 protein, whereas U41 and U51 cells expressed small amounts of Cx43 protein (Figure 1B). To investigate whether this down-regulation of Cx43 expression was mediated by transcriptional down-regulation, mRNA levels were assayed by semi-quantitative RT-PCR analysis. As shown in Figure 1C, the level of Cx43 mRNA expressions were decreased in U41 and U51 cells compared to normal rat control cells. These results suggest that the expression of Cx43 is down-regulated in PEU-induced rat tumor cell lines, and decreased Cx43 expression occurs mainly at the transcriptional level.

We obtained 12 subclones of U41 cells and analyzed the Cx43 expression levels of these clones by Western blot. Though differing between subclones, Cx43 expressions were lower than control cells (data not shown). This difference of Cx43 expressions in each clone was correlated with the GJIC levels detected by the SLDT method (data not shown). In the following transfection experiments, we used the U41-22 clone to represent lower Cx43 expression and GJIC level.

Establishment of stable U41-22 clones expressing Cx43 and GJIC

U41-22 cells were transfected with a full-length rat

Figure 1. Suppression of Cx43 expression is responsible for dysfunction of GJIC in PEU-induced tumor cells. (A) SLDT assay; Transmission of Lucifer yellow into contiguous cells detected 5 min after scrape-loading in rat control, U41 and U51 cells. Results were expressed as the mean $\pm$ SEM (n=27). *Significantly different from control (rat control cells) at $P<0.01$ by student's *t*-test. (B) Western blot analysis of Cx43 protein expression; The lysates of rat control, U41 and U51 cells were applied to SDS-PAGE. Fractionated proteins in gel were transferred onto nitro cellulose membrane and immunoblotted with the anti-Cx43 polyclonal antibody as described in "Materials and methods". (C) RT-PCR analysis for mRNA expression of the Cx43 in rat control, U41 and U51 cells. Total RNA was extracted from rat control, U41 and U51 cells. After reverse transcription was performed, PCR amplifications carried out 30 cycles (94 °C for 30 s, 56 °C for 30 s, 72 °C for 90 s) for Cx43 and GAPDH.

Cx43 cDNA. Colonies resistant to G418 were isolated by the cylinder trypsinization method and screened by Western blot analysis. Among Cx43 transfectant clones, U41-22/Cx43-7 clone has the highest level of Cx43 protein (Figure 2A) as well as high capacity of GJIC (Figure 2B). To eliminate possible effects of vector plasmid DNA on GJIC characteristics and Cx43 expression level of transfectant clones, U41-22 clone, transfected with blank plasmid was also isolated. The transfectant clone U41-22/pB45 has the same levels of GJIC capacity and Cx43 protein expression as those of U41-22 (Figures 2A and B).

In vitro growth capacity of Cx43 transfectant clones

The significant suppression of cell growth rate in U41-22/Cx43-7 clone is shown in Figure 3A. In contrast to U41-22/Cx43-7, the transfectant clone U41-22/pB45 proliferated as well as the parental U41-22 cells. In addition to cell counting, the morphology of U41-22/Cx43-7 clone is significantly different from U41-22 cells (Figure 3B). The U41-22/Cx43-7 cells spread well in subconfluent culture and generally to form good cell-cell contacts. Contact inhibition was observed in confluent cultures of U41-22/Cx43-7 cells. Control transfectants (U41-

22/pB45) and parental clones (U41-22) were maintained in rounded shape and allowed for multilayer growth and weak attachment to the substratum.

Anchorage-independent growth capacity of Cx43 transfectant clones

Soft agar colony forming ability is one of several common phenotypes to assess cellular transformation. Soft agar assay was carried out by seeding 500 cells/well in 12-well tissue culture plates, at six wells per each clone. After 14 days of seeding, visible colonies were observed with control U41-22/pB45 and parental U41-22 clones, whereas U41-22/Cx43-7 clone grew poorly with no colony formation in soft agar (Figures 4A and B). These data suggests that the malignant properties of U41-22 cells were clearly suppressed by transfection with Cx43 cDNA.

Discussion

The mechanism of tumorigenesis induced by biomaterials has long been our major focus in the research for safe and efficient medical devices made of biomaterials. This paper discusses the tumorigenic potential of polyetherurethane (PEU) by examining the relationship between its tumorigenicity and gap

Figure 2. Forced expression of Cx43 restores the GJIC in PEU-induced tumor cell clone, U41-22. (A) Western blot analysis of Cx43 protein expression; The lysates of U41-22, U41-22/pB45 and U41/Cx43-7 cells were applied to SDS-PAGE. Fractionated proteins in gel were transferred onto nitro cellophane membrane and immunoblotted with the anti-Cx43 polyclonal antibody as described in “Materials and methods”. (B) SLDT assay; Transmission of Lucifer yellow into contiguous cells detected 5 min after scrape-loading in rat control, U41-22, U41-22/pB45 and U41-22/Cx43-7 cells.

junctional intercellular communication (GJIC). It has been shown that PEUs has tumorigenic potential by *in vivo* implantation experiment of PEU film in rats (Nakamura et al. 1992) and *in vitro* transformation assay using Balb/c 3T3 A31-1-1 cells (Tsuchiya et al. 1996). However, the mechanism of tumorigenesis induced by PEUs is not clear, as we have yet to study the PEU-induced tumor cells at molecular level

so far. Many factors which caused tumorigenesis were known, we especially paid attention to the inhibition of the GJIC in the PEU-induced tumor cells. The results from the PEU-induced tumor cells U41 and U51 indicated that the function of GJIC declined remarkably and the expression of Cx43 protein, a component of the gap junction, was inhibited. These results are consistent with other

Figure 3. Cx43 transfected clone shows significant retardation of cell growth rate and recovery of contact inhibition. (A) Growth curves of U41-22, U41-22/pB45 and U41-22/Cx43-7 cells. Cells (10,000/well) were seeded into a 24-well tissue culture plate and allowed to grow for various length of time. The trypsinized cells were counted in duplicate for each cell line using a hemocytometer. The cell number were then plotted against culture time. (B) Phase contrast micrographs of U41-22 and U41-22/Cx43-7 cells on the day 7 (original magnification, x100).

Figure 4. Cx43 expression reduces the soft agar colony-forming ability of PEU-induced tumor cell clone, U41-22. Approximately 500 cells from each clone were seeded in 1 ml of 0.3% soft agar in culture medium on a solidified basal layer per well in 12 well tissue culture plates. The plates were cultured for 2–3 weeks and then stained with *p*-iodotetrazolium violet overnight before counting. Colonies with sizes $>19.625 \mu\text{m}^2$ were scored as positive. (A) Total colonies of U41-22, U41-22/pB45 and U41-22/Cx43-7 cells (original magnification, x5). (B) Representative individual colony (original magnification, x100).

studies biomaterials suggest that the inhibition of the GJIC is involved in the regulation of tumorigenesis in tumor cells (Tsai et al. 1996; Wilgenbus et al. 1992; Lee et al. 1992; Tomasetto et al. 1993; Grossman et al. 1994; Tsuda et al. 1995). The down-regulation of Cx43 expression in our U41 and U51 cells are likely to take place at the transcriptional level. Such transcriptional down-regulation of Cx43 expression is also observed in human prostate, brain and lung cancer cells (Tsai et al. 1996; Wilgenbus et al. 1992; Huang et al. 1998; Zhang et al. 1998). The mechanism responsible for the down-regulation of Cx43 expression is currently unknown, but may be attributed to one or more of the following reasons, (i) deletion of the Cx43 promoter, (ii) point mutation in the promoter, (iii) methylation of the promoter, (iv) loss of transactivation factor, (v) presence of inhibitory factor. The identification of mechanisms responsible for the loss of Cx43 expression in our U41 and U51 cells may provide a molecular basis for the development of suitable biomaterials to activate Cx43 expression in cells at the transplantation sites.

Asamoto *et al.* reported that tumorigenicity is enhanced when the Cx43 expression was obstructed by the anti-sense RNA of Cx43 (Asamoto et al. 1998). Their report is the direct proof that the GJIC controls the tumorigenicity of tumor cells, and suggests the Cx43 protein can have tumor suppressor characteristics. To examine the tumor suppressor effect of connexin genes, many researchers had tried

to reverse the tumorigenic phenotype by forced expression of connexin genes in tumor cells. Actually, in several experimental systems, cell growth in tumor cells can be suppressed by transfection with connexin genes (Huang et al. 1998; Zhang et al. 1998; Eghbali et al. 1991; Zhu et al. 1991; Naus et al. 1992; Mesnil et al. 1995). Therefore, it is thought to be that negative signals for cell proliferation or tumorigenicity would appear to pass through gap junctions. However, the possibility of simple enhancement of adhesive capacity could not be entirely ruled out, because Huang et al. reported that the tumor suppression effects are not associated with the establishment of GJIC in Cx43 transfected cells (Huang et al. 1998). In our U41-22 cells, GJIC capacity was overcome by Cx43 gene transfection. Therefore, the up-regulation or expression of Cx43 inhibits the tumor promotion in due to the suppression of cell proliferation by making functional gap junction between the adjacent cells.

These results strongly suggest that cell proliferation in normal tissues should be controlled precisely by functional GJIC composed by Cx43 proteins. The GJIC of the cells attached to the PEU films is disturbed by the hyper-phosphorylation of connexin proteins (Tsuchiya et al. 1998). The cells lacking the GJIC become easy to react to the proliferative stimulus by growth factors. As a result of the continuous proliferative stimulation by growth factors, abnormal cell proliferation causes tumor at the implanted site. This context suggests a possible

strategy of gene therapy that involves a newly developed techniques (Klugherz et al. 2000). That is, biomaterials releasing the Cx43 gene in case of the tissue such as muscle, nervous, bone etc. will control the tumorigenesis and retain important function of differentiated cells (Tsuchiya 2000).

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