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Original Paper

All-trans Retinoic Acid Enhances Gap Junctional Intercellular Communication Among Renal Epithelial Cells *In Vitro* Treated with Renal Carcinogens

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Epidemiological and clinical studies imply that retinoids have a chemopreventative action against cancer and can suppress the growth of cancer cells. The regulation of connexin (Cx) expression by retinoids varies among tissues and organs. In this study, we investigated whether all-trans retinoic acid (ATRA) upregulates gap junctional intercellular communication (GJIC) in renal epithelial cells exposed to renal carcinogens. Madin Darby canine kidney (MDCK) cells were incubated with ATRA for 3 days, then briefly exposed to 12-O-tetradecanoyl-phorbol-13-acetate (TPA) or renal carcinogens potassium bromate ($KBrO_3$) and dimethylnitrosamine (DMN). ATRA increased the expression of connexin 43 mRNA and protein without affecting Cx 43 phosphorylation and prevented inadequate Cx 43 localisation caused by TPA/$KBrO_3$ or DMN. Consequently, ATRA prevented the disruption of GJIC in MDCK cells. These data suggest that ATRA enhanced GJIC by upregulating Cx 43 expression and that ATRA might be useful for prevention of renal cell carcinoma. © **1999 Elsevier Science Ltd. All rights reserved.**

Key words: all-trans retinoic acid, gap junction, renal epithelial cells, renal carcinogens
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INTRODUCTION

GAP JUNCTIONS are small aqueous channels that connect neighbouring cells and allow the passage of low molecular weight metabolites (for review see [1]). These junctions are formed by hexameric proteins known as connexins (Cx), each of which has unique electrophysiological properties and permeability [2]. Cx 43 is the major connexin expressed in human renal tubular epithelial cells [3]. Gap junctional intercellular communication (GJIC) may play an important role in the maintenance of cell organisation, and regulate many normal biological processes, including tissue homeostasis, embryonic development, glandular secretion, cellular differentiation and growth [4]. Most tumour promoting agents inhibit intercellular communications, and reduced GJIC capacity has been identified frequently during carcinogenesis [5].

Retinoids, the natural derivatives of vitamin A, play a critical role in a variety of physiological events such as the visual cycle, embryonic development and cellular differentiation [6]. They can suppress malignant transformation in mammalian tissues, and have an antiproliferative effect in renal carcinoma cells [7, 8]. The biological effects of retinoids are mediated by retinoic acid receptors (RARs and RXRs). Cancer preventive agents, such as retinoids, cyclic AMP and β-carotene, can upregulate GJIC [9, 10]. Several studies have indicated that retinoids restore GJIC inhibited by some carcinogens and suppress the induction of morphological transformation [11–16]. However, there is no evidence of an effect of retinoids on the GJIC in renal epithelial cells.

Renal cell carcinoma (RCC) is serious, because there is no effective systemic therapy against metastasis. Clinical trials of combination with retinoids and interferon (IFN) to treat advanced renal cell carcinoma have been initiated [8]. IFNs are a class of cytokines that inhibit proliferation of tumour cells and stimulate tumour cell differentiation like retinoids. The molecular mechanism of the retinoids' effect in renal carcinogenesis has remained obscure. We have shown in a previous study that a panel of renal carcinogens could inhibit GJIC in the non-transformed renal tubular epithelial cell line, Mardin-Darby canine kidney (MDCK) cells [17]. In the present study, we examined the effect of all-trans retinoic

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acid (ATRA) on gap junctional permeability in MDCK cells treated with renal carcinogens, and investigated the localisation and phosphorylation of Cx 43 protein.

MATERIALS AND METHODS

Reagents

Tissue culture reagents, Dulbecco's modified Eagle's medium and fetal calf serum (FCS), were purchased from Life Tech Oriental Co. (Tokyo, Japan). ATRA, potassium bromate ($KBrO_3$), 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and Lucifer Yellow were obtained from Sigma Chemical Company, St Louis, Missouri, U.S.A. Dimethylnitrosamine (DMN) was from Wako Pure Chemical Industry, Osaka, Japan. ATRA was dissolved in dimethyl sulphoxide (DMSO) (Sigma) at a concentration of 1×10^{-3} M and stored at $-80°$C. ATRA was added from stock solutions in DMSO, to a final concentration not exceeding 0.1% DMSO.

Cell culture

MDCK cells were obtained from the Japanese Cancer Research Resource Bank. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, L-glutamine (0.2 mg/ml) and gentamicin (1 μg/ml) at 37°C in an atmosphere of 95% air and 5% CO_2.

Growth experiments

To evaluate the effect of ATRA on growth, MDCK cells were seeded into 60 mm plastic wells at a density of 1×10^4/well. Twenty-four hours later, the medium was replaced with fresh DMEM containing 10% FCS and ATRA at concentrations between 10^{-10} M and 10^{-4} M. The final concentration of DMSO was adjusted to 0.1% (W/V) and cells without ATRA were cultured in medium containing 0.1% DMSO. Three days later, the cells were counted using a haemocytometer.

MDCK cell culture with renal carcinogens

MDCK cells were seeded into 10 cm dishes at a density of $1.0\times10^3/cm^2$. Twenty-four hours later, the medium was replaced with fresh DMEM containing 10% FCS and 0.1% DMSO or 10^{-6} M ATRA and the cells were cultured for 3 days. The cells were then exposed to 5 mM $KBrO_3$, 1% (W/V) DMN or 1.6 μ M TPA for 15 min, then GJIC assays and other studies proceeded as described below [17].

Gap junctional intercellular communication assay (GJIC assay)

We examined gap junction-mediated intercellular communication using a scrape loading dye transfer technique. Cells were rinsed with phosphate buffer saline (PBS) three times, then scraped with the sharp edge of a surgical blade in the presence of PBS containing 0.5 mg/ml Lucifer Yellow. Three minutes later, the dye was removed and the cells were washed four times with PBS. The degree of Lucifer Yellow transfer between MDCK cells, obtained from the scraped edge, was investigated by fluorescent microscopy. If the GJIC function is intact, the dye will spread more than five rows from the wounded cells.

Immunofluorescent staining

MDCK cells were cultured on plastic slides (Permanox Lab-Tec Chamber; Nunc. Inc., Naperville, Illinois, U.S.A.) and incubated with 0.1% DMSO or ATRA for 3 days. The cells were then incubated with TPA, DMN or $KBrO_3$ for 15 min. After extensive washing with PBS, the cells were fixed in ice-cold acetone for 3 min, rinsed in PBS and incubated with PBS containing 5% (W/V) skimmed milk, 0.2% (V/V) Tween-20 and 0.1% (W/V) sodium azide for 60 min. The cells were then incubated with monoclonal anti-rat connexin 43 (Cx 43) antibody (Zymed Laboratories, San Francisco, California, U.S.A.) diluted 100-fold for 60 min. The cells were washed with PBS and incubated with biotinylated F $(ab')_2$ fragment of goat anti-mouse IgG (H + L) (Zymed) diluted 500-fold for 60 min followed by fluorescent-conjugated streptavidin (DAKO Japan, Kyoto, Japan) for 30 min. After extensive washing, photographs were taken using a fluorescent microscope. All procedures were carried out at room temperature.

Western blotting

MDCK cells were cultured on 10-cm dishes with 0.1% DMSO or ATRA for 3 days, exposed to TPA, DMN or $KBrO_3$ for 15 min, then washed twice with ice-cold PBS containing 1 mM phenylmethylsulphonyl fluoride (PMSF). The cells were lysed on ice in lysis buffer (25 mM Tris–HCl, pH 7.4, 50 mM NaCl, 2% sodium dodecyl sulphate (SDS), 1 mM PMSF, 1 mM Na_3VO_4, 2 μg/ml aprotinin), sonicated for 30 sec on ice, then clarified by centrifugation at 10,000 ***g*** for 20 min at 4°C. The protein concentration was determined in the supernatant using the Bio Rad Detergent Compatible Protein Assay (Bio Rad Laboratories, Tokyo, Japan). Ten micrograms of proteins from lysates were separated by 12% SDS–polyacrylamide gel electrophoresis and transferred on to PVDF membranes (Millipore Japan, Tokyo, Japan). Proteins affixed to membranes were blocked with PBS containing 5% (W/V) skimmed milk, 0.2% Tween 20 and 0.1% sodium azide (blocking solution), then incubated with rabbit anti-Cx 43 polyclonal antibody (Zymed) diluted 2500-fold with blocking solution at room temperature for 2 h. After washing with PBS containing 0.2% Tween-20, the membranes were incubated with peroxidase-conjugated anti rabbit IgG (Amersham International, Bucks, U.K.) for 1 h at room temperature. Connexin proteins were visualised using the ECL-chemiluminescence detection kit (Amersham).

Northern blotting

Total RNA was isolated from cells incubated with TPA, DMN or $KBrO_3$ in the presence of 0.1% DMSO or ATRA using guanidine thiocyanate. Twenty micrograms of total RNA was electrophoresed in 1.2% agarose gels, transferred on to Hybond-N + nylon membranes (Amersham), and hybridised with 1.4 kb fragments of human Cx 43 cDNA (a gift from Chia-Cheng Chang and James E. Trosko, Michigan State University, East Lansing, U.S.A.), labelled with digoxygenin-H-dUTP (Boehringer Mannheim, Mannheim, Germany). Hybridised probes were visualised by the chemiluminescent reaction between anti-digoxygenin-alkaline phosphates Fab fragments (Boehringer Mannheim) and Lumi-Phos 530 (Boehringer Mannheim, Mannheim, Germany). The membranes were then sealed in plastic bags and exposed on to X-ray films (Amersham).

RESULTS

Effect of ATRA on MDCK cell proliferation

GJIC is established after cell–cell contact, indicating dependence upon cell density. We determined the concentration of

ATRA for MDCK cell growth. Figure 1 shows that growth was moderately inhibited by 10^{-4} M ATRA but not by concentrations from 10^{-5} to 10^{-10} M. Therefore, 10^{-6} M ATRA was used in subsequent experiments in line with other reports [11, 12, 15].

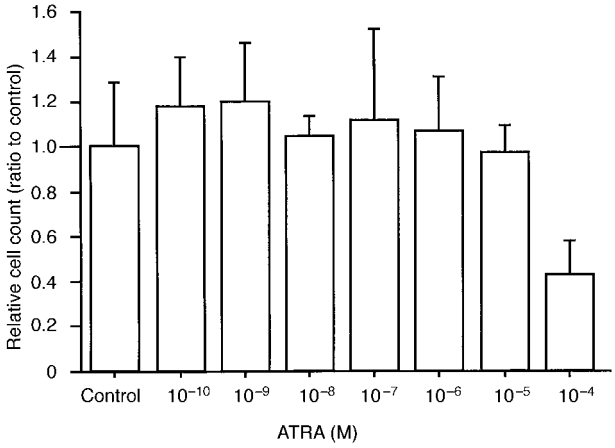

Figure 1. Effect of ATRA on the proliferation of MDCK cells. MDCK cells were grown in the presence of various concentrations of ATRA for 3 days. Control cells were cultured in medium containing 0.1% DMSO for 3 days. The cell number was counted using a haemocytometer. Columns represent the means of three observations ± S.D. (standard deviation) (bars) for triplicate experiments.

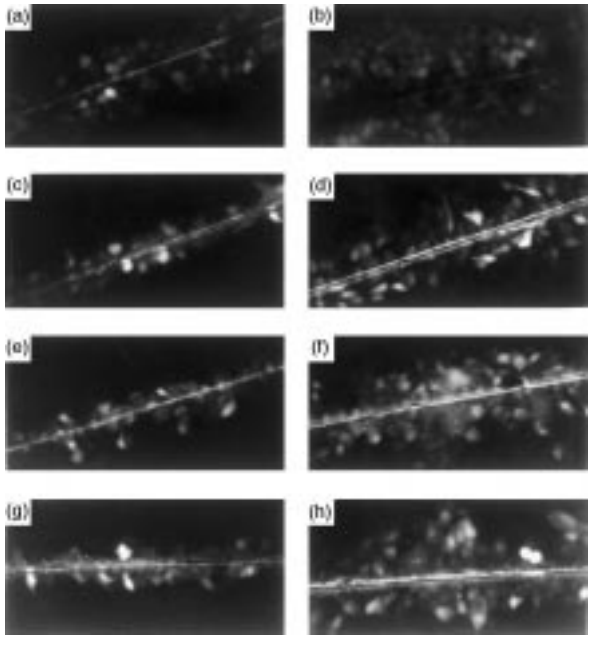

Figure 2. ATRA-enhanced GJIC of MDCK cells inhibited by TPA, DMN or $KBrO_3$. The GJIC function was determined by using the scraped-loading dye transfer technique, as described in Materials and Methods. A 3-day exposure to 10^{-6} M ATRA enhanced GJIC of MDCK cells inhibited by each carcinogen. (a) MDCK cells treated with 0.1% DMSO for 3 days; (b) MDCK cells treated with 10^{-6} M ATRA for 3 days; (c) MDCK cells exposed to 1.6 μM TPA for 15 min; (d) ATRA+TPA; (e) MDCK cells exposed to 1% (W/V) DMN for 15 min; (f) ATRA+DMN; (g) MDCK cells exposed to 5 mM $KBrO_3$ for 15 min; (h) ATRA+$KBrO_3$.

ATRA enhances the GJIC function of MDCK cells inhibited by TPA and renal carcinogens

Figure 2(a), (control, 0.1% DMSO treatment) shows that dye spread over more than five rows of cells from the scraped edge. Pretreatment with ATRA did not remarkably alter GJIC function in MDCK cells (Figure 2b). GJIC of MDCK cells was inhibited by the 15-min exposure to TPA (1.6 μM; Figure 2c), DMN (1%; Figure 2e) or $KBrO_3$ (5 mM; Figure 2g), since dye spread within three rows of cells. Pretreatment with ATRA for 3 days effectively protected against GJIC inhibition in MDCK cells exposed to TPA, DMN and $KBrO_3$ (Figure 2d, f and h, respectively). Exposure to each compound for 15 min at the tested concentration did not inhibit the growth of MDCK cells (data not shown).

Immunofluorescent staining of Cx 43

We examined the effect of ATRA on the distribution of Cx 43 protein in MDCK cells by immunofluorescent staining. Linear fluorescent plaques were localised at the plasma

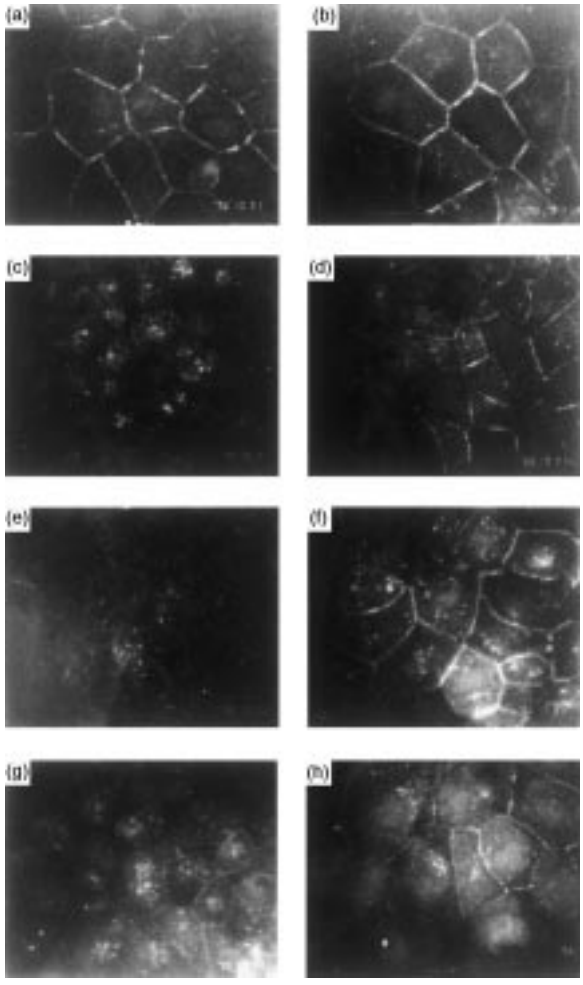

Figure 3. Localisation of Cx 43 in MDCK cells altered by TPA, DMN or $KBrO_3$ exposure with or without ATRA. Cells grown in wells of Lab-Tec chamber slides were fixed and stained with monoclonal antibody against Cx 43 by an indirect immunofluorescent method. (a) MDCK cells treated with 0.1% DMSO for 3 days; (b) MDCK cells treated with 10^{-6} M ATRA for 3 days; (c) MDCK cells exposed to 1.6 μM TPA for 15 min; (d) ATRA+TPA; (e) MDCK cells exposed to 1% (W/V) DMN for 15 min; (f) ATRA+DMN; (g) MDCK cells exposed to 5 mM $KBrO_3$ for 15 min; (h) ATRA+$KBrO_3$.

membrane of 0.1% DMSO-treated MDCK cells (Figure 3a). Pretreatment with 10^{-6} M ATRA for 3 days did not remarkably alter the distribution of Cx 43 protein (Figure 3b). Cx 43 membranous plaques were less evident in MDCK cells exposed to TPA (Figure 3c), DMN (Figure 3e) or $KBrO_3$ (Figure 3g) and some plaques were detected in the perinuclear cytoplasmic compartment. However, Cx 43 protein was distributed at the plasma membrane and the perinuclear cytoplasmic area when MDCK cells were pretreated with ATRA for 3 days before the carcinogens were added (Figure 3d, f and h).

Western blots of Cx 43

We investigated the effect of ATRA on the expression and phosphorylation of Cx 43 protein by Western blotting. MDCK cells exhibited three clearly distinct immunoreactive bands (43, 45 and 47 kDa; defined as P0, P1 and P2) (Figure 4, lane 1), when electrophoresed. ATRA upregulated Cx 43 protein expression in MDCK cells (lane 2). To determine whether this heterogeneity is due to protein phosphorylation, the lysate of MDCK cells treated with TPA was subjected first to phosphatase digestion and then to electrophoresis. As seen in Figure 4, lane 9, phosphatase digestion resulted in a single strong band at 43 kDa. TPA increased the phosphorylation of Cx 43 protein (lane 3), whereas DMN inhibited this process (lane 5). $KBrO_3$ did not affect the phosphorylation state of Cx 43 (lane 7). ATRA upregulated Cx 43 protein expression without affecting Cx 43 phosphorylation in MDCK cells (lane 2, 4, 6 and 8, respectively).

Northern blots of Cx 43

To determine whether the effect of ATRA upon Cx 43 protein was due to increased levels of Cx 43 mRNA, Northern blot analysis was performed in MDCK cells pre-incubated for 3 days with or without ATRA and exposed to TPA, DMN or $KBrO_3$. Total RNA was hybridised with 1.4 kb fragments of human Cx 43 cDNA. A 3.1 kb band appeared under conditions of high stringency (Figure 5), a size that corresponds to that of the Cx 43 transcript. There was no remarkable difference in the expression of Cx 43 mRNA after exposure to the carcinogens (lanes 3, 5, 7). However, ATRA upregulated Cx 43 mRNA expression in MDCK cells regardless of the presence or absence of these compounds. These data, in combination with the results shown in Figure 4, indicate that ATRA enhances junctional communication by increasing the transcription of Cx 43 mRNA followed by a concomitant increase in protein expression.

DISCUSSION

We report here that ATRA, to some extent, can prevent the disruption of GJIC function in MDCK cells exposed to TPA or the renal carcinogens tested. TPA, which is a well-known tumour promoter for skin and liver, disrupted GJIC in MDCK cells inducing Cx 43 phosphorylation. DMN induced Cx 43 hypophosphorylation and $KBrO_3$ did not affect Cx 43 phosphorylation, but both disrupted GJIC in MDCK cells, indicating that the function of Cx 43 protein was inhibited by different pathways. ATRA upregulated the expression of Cx 43 protein without affecting the phosphorylation state in MDCK cells exposed to each compound. These compounds decreased cell-membrane-associated Cx 43 levels and increased the cytoplasmic Cx 43 concentration. ATRA enhanced the expression of Cx 43 protein at the plasma membrane. These results indicate that one mechanism of the antitumour effect of ATRA in renal epithelial cells

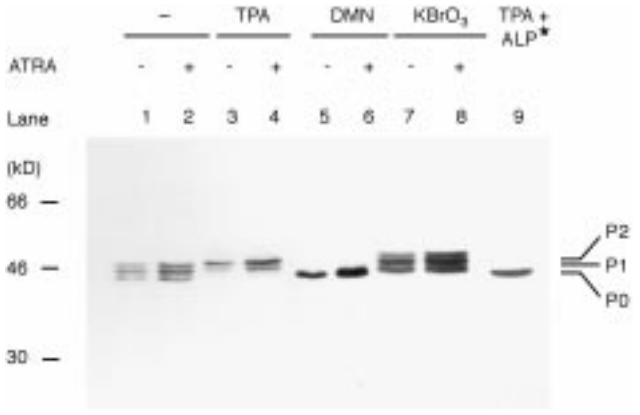

Figure 4. Changes of Cx 43 protein expression in MDCK cells was altered by TPA, DMN or $KBrO_3$-exposure with or without ATRA. Each protein of total cell lysate was electrophoresed, transferred on to a PVDF and the membrane was immunoblotted with the anti-Cx 43 polyclonal antibody as described in Materials and Methods. Lane 1, MDCK cells treated with 0.1% DMSO for 3 days; lane 2, MDCK cells treated with 10^{-6} M ATRA for 3 days; lane 3, MDCK cells exposed to 1.6 μM TPA for 15 min; lane 4, ATRA+TPA; lane 5, MDCK cells exposed to 1% (W/V) DMN for 15 min; lane 6, ATRA+DMN; lane 7, MDCK cells exposed to 5 mM $KBrO_3$ for 15 min; lane 8, ATRA+$KBrO_3$; lane 9, total cell lysate exposed to TPA treated with alkaline phosphates (10 U/ml) for 2 h. ALP*: alkaline phosphatase.

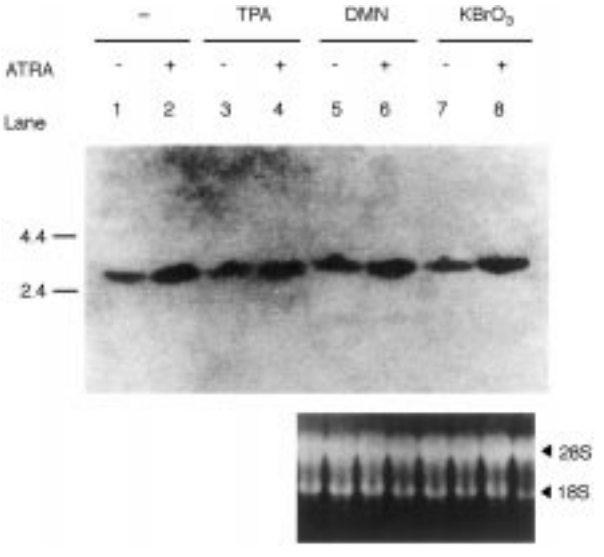

Figure 5. The steady-state level of Cx 43 mRNA was markedly elevated by ATRA pretreatment irrespective of TPA, DMN or $KBrO_3$ exposure. Total RNA from each cultured cell was electrophoresed and transferred on to a membrane. The membrane was hybridised with human Cx 43 cDNA probes as described in Materials and Methods. Lane 1, MDCK cells treated with 0.1% DMSO for 3 days; lane 2, MDCK cells treated with 10^{-6} M ATRA for 3 days; lane 3, MDCK cells exposed to 1.6 μM TPA for 15 min; lane 4, MDCK cells treated with 10^{-6} M ATRA for 3 days before TPA exposure; lane 5, MDCK cells exposed to 1% (W/V) DMN for 15 min; lane 6, MDCK cells treated with 10^{-6} M ATRA for 3 days before DMN exposure; lane 7, MDCK cells exposed to 5 mM $KBrO_3$ for 15 min; lane 8, MDCK cells treated with 10^{-6} M ATRA for 3 days before $KBrO_3$ exposure.

is the recovery of GJIC function by the enhanced expression and the normalised distribution of Cx 43 protein.

The connexin family, which is the principal membrane component of gap junctions, is composed of at least 13 proteins. Cx 43 is a major gap junctional protein in the human kidney, and it is expressed in glomerular epithelial, renal tubular and surrounding cells [3]. Cx 43 expression in human renal cancer cell line is remarkably decreased [18]. The loss of GJIC function may be associated with renal tubular cell carcinogenesis like other types of cancers [19].

DMN and $KBrO_3$ are renal carcinogens. Oxidative damage by $KBrO_3$ and DNA damage by DMN are thought to be responsible for their carcinogenic effects [20, 21]. We previously demonstrated that Cx 43 mRNA and protein expression was unaffected in MDCK cells chronically exposed at lower concentrations (0.1% DMN and 0.5 mM $KBrO_3$) [17]. In this study, acute exposure to each compound at higher concentrations induced inappropriate localisation of Cx 43 protein.

Recently, de Feijter described that the altered localisation of Cx 43 was mediated in part by changes in its phosphorylation [24]. Although changes in phosphorylation of Cx 43 protein were not observed in cells exposed to $KBrO_3$, Cx 43 protein expression was altered from the plasma membrane to the cytoplasm. This finding suggests that the inappropriate localisation of gap junction protein in the presence of $KBrO_3$ is not mediated through the phosphorylation of Cx 43 protein. ATRA increased the Cx 43 protein level at the plasma membrane, and some Cx 43 protein remained at the perinuclear cytoplasmic area. This finding suggests that ATRA upregulates Cx 43 protein in MDCK cells and that some of the Cx 43 localised at the plasma membrane-maintained GJIC. Although the TPA/DMN/$KBrO_3$ caused a variety of modifications of Cx 43 protein, ATRA maintained GJIC under any conditions tested, indicating that ATRA has a broad spectrum of activities that prevent the transformation of renal tubular epithelial cells. Thus, pretreatment with ATRA may just overwhelm the effects of the other compounds.

Several reports have shown that the regulation of Cx expression by retinoids depends on cell type [15, 16, 23, 24]. Retinoids enhance gap junctional communication and increase the expression of Cx 43 mRNA and protein in mouse fibroblast and rat liver cells. In contrast, retinoids decrease the expression of Cx 43 mRNA and protein, and functional gap junctions during the neuronal differentiation of p19 embryonal carcinoma cells and human pluriopotential teratocarcinoma cells [23, 24]. The reciprocal regulation of Cx 43 expression under the influence of retinoic acid may be due to the presence of other unknown transcriptional regulators in each cell type.

Evidence is accumulating that some of the connexin channels can be regarded as ''tumour suppressors''. Several studies have shown that the transfection of connexin genes into GJIC-deficient cancer cells decreases tumorigenicity in association with increased GJIC capacity [5]. Mesnil and associates suggested the possibility of using Cxs as both tumour suppressor genes and as diffusers of gancyclovir toxicity in therapeutic approach. Some Cxs not only suppress tumour growth but can also mediate the bystander effect that would be useful in gene therapy [25]. Park and associates noted that retinoids could augment the efficiency of cell killing by gene therapy through enhancing bystander effects [26]. In our study, ATRA enhanced GJIC and prevented the disruption of GJIC associated with renal carcinogenesis in renal epithelial cells. The present data suggest that ATRA can provide an effective means of prevention of renal cell carcinoma *in vitro*.

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