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Full paper

Effect of enhanced expression of connexin 43 on sunitinib-induced cytotoxicity in mesothelioma cells

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ABSTRACT

Connexin (Cx) makes up a type of intercellular channel called gap junction (GJ). GJ plays a regulatory role in cellular physiology. The Cx expression level is often decreased in cancer cells compared to that in healthy ones, and the restoration of its expression has been shown to exert antiproliferative effects. This work aims to evaluate the effect of the restoration of connexin 43 (Cx43) (the most ubiquitous Cx subtype) expression on sunitinib (SU)-induced cytotoxicity in malignant mesothelioma (MM) cells. Increased Cx43 expression in an MM cell line (H28) improved the ability of SU to inhibit receptor tyrosine kinase (RTK) signaling. Moreover, higher Cx43 expression promoted SU-induced apoptosis. The cell viability test revealed that Cx43 enhanced the cytotoxic effect of SU in a GJ-independent manner. The effect of Cx43 on a proapoptotic factor, Bax, was then investigated. The interaction between Cx43 and Bax was confirmed by immunoprecipitation. Furthermore, higher Cx43 expression increased the production of a cleaved (active) form of Bax during SU-induced apoptosis with no alteration in total Bax expression. These findings indicate that Cx43 most likely increases sensitivity to SU in H28 through direct interaction with Bax. In conclusion, we found that Cx43 overcame the chemoresistance of MM cells.

1. Introduction

Malignant mesothelioma (MM) is an aggressive tumor arising from mesothelial cells. The main risk factor is asbestos. MM displays a long latency period that can take up to 40 years to develop following the first exposure to asbestos (1). Although it is a rare tumor at present, it is becoming more common worldwide (2). For example, the incidence of MM is now 2000–3000 per year in the United States, and approximately 70,000 new MM cases are expected over the next 20 years (3). In Europe, the peak year of MM incidence is expected to fall between 2015 and 2020, with an estimated 250,000 new cases over the next 40 years (3). Surgery is recommended for patients in clinical stage I; however, majority of patients are diagnosed in an advanced stage, and surgery is not suitable for them (4). Thus, systemic therapy is the only treatment option for these patients. Unfortunately, MM is usually a chemo and radioresistant tumor, which contributes to its poor prognosis (4). The combination of pemetrexed and cisplatin is the only FDA-approved regimen for MM, and its impact on the survival of patients is modest (5). No agents have shown an improvement in survival as a second line therapy; therefore, new therapeutic strategies for the treatment of MM need to be established.

With a view to overcoming the chemoresistance of MM, we turned our attention to connexin (Cx). Cx is a four-transmembrane protein, and forms an intercellular channel called gap junction (GJ). Cellular homeostasis, cell growth, and cell differentiation are mediated via GJ intercellular communication (GJIC) (6). GJ allows the direct transfer of several second messengers and small molecules between apposed cells, including Ca^{2+}, cyclic AMP (cAMP), cGMP, inositol 1,4,5-triphosphate (IP3), and glutathione (7). It has been frequently observed that GJIC is aberrant, and Cx expression is decreased in many cancer cells (8). Many *Cx* gene transfection experiments have suggested that Cx-mediated GJIC works as a tumor suppressor in cells that originate in tissues in which it is normally

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expressed (9). Recently, it has also been shown that connexin 43 (Cx43) could reduce cell growth in the absence of GJIC (10). Therefore, Cx exerts a tumor suppressive effect through both, GJ-dependent and GJ-independent mechanisms. We have previously shown that Cx43, the major Cx subtype in mesothelial cells, enhances cisplatin-induced cytotoxicity in MM cells in a GJ-independent manner (11).

Although the platinum-based chemotherapy has been mainly used for the MM patients at present, the identification of key growth factors, glycoproteins, and genetic mutations in MM tumorigenesis have generated interest in the development of new agents to target these oncogenic abnormalities (1). Vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) have been reported to play an important role in the growth of MM cells via autocrine loops (12,13). Moreover, it has also been shown that VEGF expression was higher in MM tissues than in healthy ones, with a correlation between higher VEGF expression level and worse prognosis (14). Based on these observations, it is rational to target the signaling pathways stimulated by these growth factors.

Sunitinib (SU) is a multi-targeted tyrosine kinase inhibitor (TKI), which suppresses the activation of VEGF receptors (VEGFRs 1–3), PDGF receptors (PDGFRs α and β), and other growth factor receptors (15). It is approved for the treatment of advanced renal cell carcinoma, imatinib-resistant or intolerant gastrointestinal stromal tumors, and advanced pancreatic neuroendocrine tumors (15,16). Recently, its antitumor activity was investigated in patients with other solid malignancies such as breast cancer (17), glioblastoma (18), and MM (19,20). However, SU displayed a limited activity in these patients. Therefore, there is a need for investigation into methods for making SU more appropriate as a therapeutic agent for these tumors, including MM.

Based on these facts, this report describes the effect of increased Cx43 expression on SU-induced cytotoxicity in MM cells.

2. Materials and methods

2.1. Reagents

All cultures and reagents were purchased from Sigma (St. Louis, MO, USA) unless otherwise indicated. Sunitinib malate (SU) was dissolved in dimethyl sulfoxide (DMSO; Wako Pure Chemicals, Osaka) at a concentration of 10 mM and stored at −20 °C. A GJ inhibitor, 18β-glycyrrhetinic acid (GA), was dissolved in DMSO at a concentration of 40 mM and stored at −20 °C. 6-Carboxyfluorescein (6-CF) was dissolved in distilled water at a concentration of 10 mg/mL and stored at 4 °C. Dextran tetramethylrhodamine (DTMR; Invitrogen, Carlsbad, CA, USA) was dissolved in distilled water at a concentration of 50 mg/mL and stored at −20 °C. 3-(4,5-Dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Dojindo Laboratories, Kumamoto) was dissolved in distilled water at a concentration of 5 mg/mL and stored at −20 °C.

2.2. Cell culture, construct creation, and transfection

H28, a representative human MM cell line, was obtained from ATCC (Manassas, VA, USA) and grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin/100 μg/mL streptomycin, 0.01 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer solution, 1 mM sodium pyruvate, and 2.5 g/L D-glucose at 37 °C in 5% CO_2. All supplements were purchased from Gibco (Palo Alto, CA, USA) except for FBS (Biowest, Kansas City, MO, USA). Cx43-transfected H28 cells (H28-T) were produced as previously described (11). At that time, control vector-transfected cells (H28-W) were also produced, and we have confirmed that H28-W and H28 cells have the same level of Cx43 mRNA and protein expression and GJ function. Therefore, H28 and H28-T cells were used in this study.

2.3. Real-time polymerase chain reaction (PCR) analysis

Total RNA was isolated using a ChargeSwitch® Total RNA Cell Kits (Invitrogen), and cDNA was synthesized using a Super Script™ RNase H^- Reverse Transcriptase (Invitrogen). Real-time PCR was performed using an ABI StepOne™ Real-time PCR System (Applied Biosystems Japan Ltd., Tokyo) and SYBR® Premix Ex Taq™ (Takara Bio Inc., Shiga). The following primers were used: RPL32 [accession number (NM_000994)] sense (nucleotides-CATCTCCTTCTCGGCATCA) and anti-sense (nucleotides-AACCCTGTTGTCAATGCCTC); Cx43 [accession number (NM_000165)] sense (nucleotides-ACTCAACTGCTGGAGGGAAG) and anti-sense (nucleotides-GCACATGAGAGATTGGGAAA). The PCR reaction was performed at 95 °C for 10 s followed by 40 cycles at 95 °C for 5 s and 60 °C for 31 s. All data were normalized to the internal standard RPL32.

2.4. Immunoprecipitation and western blot analysis

A total of 1.5×10^5 H28 cells and 3.0×10^5 H28-T cells were seeded in 60 mm dishes. After 6 h incubation, the cells were cultured in 0.1% FBS medium for 24 h followed by treatment with 10 μM SU for each indicated period. Control cells were treated with 0.1% DMSO. Cells were collected by scraping and dissolved in ice-cold lysis buffer [50 mM Tris (pH 7.4) (Invitrogen), 150 mM sodium chloride (NaCl) (Wako Pure Chemicals), 1% Triton X (Wako Pure Chemicals), 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM ethylenediaminetetraacetic acid (Dojindo Laboratories), 1 mM phenylmethane sulfonyl fluoride, and 1% protease inhibitor cocktail]. Protein concentrations were determined by using a DC protein assay kit (Bio-Rad, Tokyo). For immunoprecipitation, total cell lysates (100 μg) pre-cleared with protein sepharose G (GE Healthcare, Buckinghamshire, UK) for 1 h at 4 °C, were immunoprecipitated with 3.2 μg anti-Cx43 antibody, 1 μg anti-Bax antibody or anti-rabbit antibody (negative control) for 1 h at 4 °C followed by incubation with protein sepharose G for 1 h at 4 °C. Immunoprecipitates were washed 5 times with lysis buffer followed by resuspension in sample buffer [250 mM Tris (pH 6.8), 40% sucrose (Wako Pure Chemicals), 20% 2-mercaptoethanol (Wako Pure Chemicals), 8% sodium dodecyl sulfate (SDS) (Wako Pure Chemicals), and 0.002% bromophenol blue (ICN Biomedicals, Eschwege, Germany)] and boiling for 5 min at 100 °C. Then, immunoprecipitates were purified by centrifugation at 2300 *g*. For western blotting, total cell lysates (10 μg or 25 μg) or 5 μL of immunoprecipitates were separated using a 7.5%, 10% or 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel. 30% Acrylamide solution was purchased from Bio-Rad. The separated proteins were then transferred to a polyvinylidene difluoride membrane (ATTO, Tokyo). After that, the membranes were blocked with 5% skim milk (Megmilk Snow Brand, Co., Ltd., Tokyo) in tris-buffered saline and tween 20 (TBS-T) [13.7 mM NaCl, 25 mM Tris, and 0.05% Tween 20 (Wako Pure Chemicals)] or 5% bovine serum albumin (BSA) in TBS-T for 1 h at room temperature or overnight at 4 °C. The membranes were then incubated with primary antibodies for 1 h at room temperature or overnight at 4 °C followed by incubation with secondary horse radish peroxidase (HRP)-conjugated antibodies for 1 h at room temperature. The primary antibodies are shown in Table 1. The detection was accomplished using an Immobilon™ Western Chemiluminescent HRP Substrate (Merck Millipore, Darmstadt, Germany) and an