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Claudin-6 enhances cell invasiveness through claudin-1 in AGS human adenocarcinoma gastric cancer cells

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ABSTRACT

Claudins participate in tissue barrier function. The loss of this barrier is associated to metalloproteases-related extracellular matrix and basal membranes degradation. Claudin-1 is a pro-MMP-2 activator and claudin-6 transfected AGS (AGS-Cld6) cells are highly invasive. Our aim was to determine if claudin-6 was direct or indirectly associated with MMP-2 activation and cell invasiveness. Cytofluorometry, cell fractioning, immunoprecipitation, gelatin-zymography, cell migration and invasiveness assays were performed, claudin-2, -6, -7 and -9 transfected AGS cells, anti-MMP-2, -9 and -14, anti-claudins specific antibodies and claudin-1 small interfering RNA were used. The results showed a significant ($p < 0.001$) overexpression of claudin-1 in AGS-Cld6 cell membranes. A strong MMP-2 activity was identified in culture supernatants of AGS-Cld6. Claudin-1 co-localized with MMP-2 and MMP-14; interestingly a significant increase in cell membrane and cytosol MMP-14 expression was detected in AGS-Cld6 cells ($p < 0.05$). Silencing of claudin-1 in AGS-Cld6 cells showed a 60% MMP-2 activity decrease in culture supernatants and a significant decrease ($p < 0.05$) in cell migration and invasiveness. Our results suggest that claudin-6 induces MMP-2 activation through claudin-1 membrane expression, which in turn promotes cell migration and invasiveness.

1. Introduction

Epithelial barrier function requires normal expression of tight junction (TJ) proteins; when the TJ structure looses cohesion transformed cells can be invasive [1]. Claudins are a family of TJ proteins which participate in tissue barrier development [2]. Gastric adenocarcinoma, the second most common cause of death by cancer [3], exhibits changes in claudin expression during its progression to metastatic adenocarcinoma; altered expression of claudin-4, -6, -7 and -9 in human gastric cancer cells has been associated with higher cancer invasiveness [4,5] and poor survival rates [6]. Likewise, it has been clearly established that cancer cell invasion is subordinated to extracellular matrix and basal membrane degradation by matrix metalloproteinase (MMP) 2 and 9 [7,8]. Therefore the regulation of MMP activity is crucial to understand the cancer cells invasive mechanisms. There is ample evidence suggesting that claudins regulate MMPs activity [9,10]. Previous results in claudin-6 transfected AGS cells revealed that endogenous induction of claudin-1 expression [5] enhances activation of pro-MMP2 in human embryonic kidney cells [11].

The aim of this work was to determine if MMP2 activation detected in the culture supernatants of claudin-6 transfected AGS cells, was induced by claudin-1 expression or if the latter serves as a mediator for an alternative molecule activator. Our results showed that the expression of claudin-1 correlates with a) the activation of the sheddase MMP14 in the cell membrane, b) the activation of MMP2, on AGS transfected cells, and c) an enhanced cellular invasiveness.

2. Material and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine, sodium pyruvate, insulin, Dulbecco´s Phosphate-Buffered Saline (PBS), streptomycin-penicillin, bovine albumin, mouse monoclonal anti-claudin-1 (1:100–1:1000) (#cat. 374900) and anti-MMP-2 (1:1000, #cat. 35-1300Z), polyclonal rabbit anti-claudin-2 (1:200, #cat. 516100), anti claudin-3 (1:100, #cat. GTX15102), anti claudin-4 (1:800, #cat. GTX15104), anti claudin-5 (1:400, #cat. GTX15106), anti claudin-7 (1:100, #cat. 349100), anti claudin-10 (1:100, #cat. 388400), anti MMP-14 (1:100–1:500 #cat. GTX73117),

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horseradish peroxidase labeled, donkey anti-mouse IgG (1:500–1:1000, #cat. Sc2314) and goat anti-rabbit IgG (H+L) HRP (1:500–1:1000, #cat. A10547), Cy5 labeled goat anti-rabbit polyclonal (1:800, #cat. A10523), Alexa fluor 488 labeled goat anti-mouse (1:400, #cat. A11029), as well as Super Script One-Step kit were from Invitrogen (Life Technologies Corp, Carlsbad, CA, USA). Geneticyn (G418) was from GIBCO. Goat, rabbit or mouse IgG HRP- conjugated antibodies were from Zymed-Life Technologies (Grand Island, NY, USA). Mouse monoclonal anti Na/K-ATPase α-1 (F-2)(1:300, #cat. SC-514614), anti PKC (1:200, #cat. 624302), anti β-tubulin (D-10)(1:200, #cat. SC-5274) and claudin-1 siRNA made of a pool of three specific sequences (19–25 nt)(claudin-1 siRNA (h) #cat. sc-43040) and a control fluorescence-conjugated siRNA were from Santa Cruz Biotech (CA, USA). Polyclonal rabbit anti-MMP-14 was from GeneTex Inc. (Irvine, CA, USA). APC goat anti-mouse IgG1 (1:400, #cat. 406610) was from Cell Signaling Technology Inc. (Danvers, MA, USA). Anti-actin antibody was a kind gift of Dr. José Manuel Hernández, CINVESTAV-México. Tween-20, Triton X-100, phosphatase inhibitor cocktail 1 and 2, trypsin inhibitor, leupeptin, and aprotinin were from Sigma-Aldrich (St. Louis, MO, USA). Protein assay kit was from Bio-Rad Laboratories (Hercules, CA, USA). Santa Super Signal West Dura Chemiluminescent Substrate was from Thermo Fisher VScientific Inc. (Waltham, MA, USA). Point final RT-PCR (Super Script One-Step) was from Invitrogen. Petri dishes and 6 and 12 well culture plates were from Corning (NY, USA).

2.2. Cell culture

1×10^6 human gastric adenocarcinoma cell line AGS cells (CRL-1739, ATCC, USA) were cultured in sterile P-100 Petri dishes with DMEM supplemented with 5% FBS, 0.1 U/ml insulin, 1% streptomycin-penicillin solution, 2 mmol/L L-glutamine, and 2 mmol sodium pyruvate solution at 37 °C in a humid environment containing 5%CO_2, until reaching >90% confluence. Afterwards 8×10^5 cells/well were seeded in 6 well Tissue Culture Plates (Biocompare, San Francisco, CA, USA) for 48 h until they reached 95% confluence, that represent 2×10^6 cells/well. All the experiments were immediately performed with this confluent AGS cell cultures in their third passage. Claudin-2, -6 (AGS-Cld6), -7 and -9 as well as GFP transfected AGS cells [5] were cultured with DMEM supplemented as mentioned plus 400 μg/ml of G-418. AGS cells expressing the GFP-empty vector (AGS-GFP) were used as control. Western blot analysis was performed using confluent AGS cells. All the experiments were performed with AGS cells grown from 2nd passage frozen vials maintained in liquid nitrogen. Results are expressed as the mean of three different experiments.

2.3. Cell fractioning and immunoblot analysis

Cell fractioning was performed as already described [6]. Briefly, cells were scrapped from the plates with cold lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, pH 7.6) and suspended in 1 ml of a buffer A (20 mM Tris, 0.25 M Dextrose, 10 mM EGTA and 2 mM EDTA) supplemented with 0.5% of phosphatase inhibitor cocktail 1 and 2, 1 mg/ml trypsin inhibitor, 2 μg/ml leupeptin and 2 μg/ml aprotinin (Sigma-Aldrich, USA). Cells were sonicated for 1 min and ultra centrifuged for 30 min at 156,553*g* at 4 °C before collecting the supernatant. The precipitate was suspended in 150 μl of buffer A supplemented with protease and phosphatase inhibitors plus 1% Triton X-100, incubated for 1 h at 4 °C with gentle agitation, and ultra centrifuged for 30 min at 156,553*g*. The supernatant (membrane fraction) was kept at 4 °. The precipitate was suspended in 150 μl of lysis buffer (150 mM NaCl, 50 mM Tris, 1 mM EGTA, 1 mM EDTA, 1% IGEPAL, 0.1% Sodium deoxycholate, 0.1% SDS) supplemented with protease and phosphatase inhibitors, incubated for 1 h at 4 °C with gentle agitation, and ultra centrifuged for 30 min at 156,553*g*. The supernatant (cytoskeleton fraction) was kept at 4 °C. Protein concentration was determined for each fraction using Bio-Rad´s kit. To corroborate that the isolated cell fraction was clean specific fraction markers (PKC for cytosol, Na/ATPase for membrane, and β-tubulin for cytoskeleton) were used.

For Western blot analysis 20–50 μg of protein was resolved on 10% or 15% SDS/PAGE and transferred to nitrocellulose membranes in a Bio-Rad semi-dry blotting system for 1 h at 120 mA. Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline (TBS) for 1 h, washed twice with TBS and incubated with the relevant primary antibody diluted in Tween 20/TBS (TTBS) overnight at 4 °C (anti claudin-1 (1:500), anti-MMP-2 (1:1000), anti-MMP-9 (1:500), anti-MMP-14 (1:500), anti α-actin (1:500)). Membranes were washed with TTBS, and incubated with horseradish peroxidase labeled secondary antibodies (goat anti-mouse IgG (1:2000), or goat anti-rabbit IgG (1:1000)) diluted in TBS for 1 h at room temperature. After these processes membranes were washed thrice with TTBS and once with TBS before antibody binding was detected by chemiluminiscence using the Pierce kit (Supersignal West Dura Extended Duration Substrate) as substrate. Equal protein loading was confirmed in all the experiments by determining β-actin as loading control. All experiments were run in triplicate. The quantitative analysis of the Western blot bands was performed with images obtained from the chemiluminiscence sheets using the MiniBis Pro (DNR Bio-Imaging System, Jersulamen, Israel) and analyzed using DNR GelQuant software that calculates the intensity of the gel band measuring the amount of pixels/sq.in. The values were determined for each band. The results are expressed as dots per point (DPP) and intensity.

2.4. Immunoprecipitation

1×10^6 AGS, Claudin-6 transfected, or AGS-GFP were seeded in culture plates with DMEM supplemented with 5% FBS, 1% streptomycin-penicillin solution, 1 mmol/L L-glutamine, insulin and sodium pyruvate solution at 37 °C in a humid environment containing 5%CO_2 until 90% confluence was reached. Cells were collected, washed three times in cold PBS, suspended in IP lysis buffer (50 mM Tris, 150 mM NaCl, 0.5% Triton X-100, 1 mg/ml trypsin, 5 mg/ml leupeptin and 10 μl/ml of phosphatase inhibitors cocktail) and centrifuged for 10 min at 13,000 rpm at 4 °C. Protein concentration was determined and adjusted to a 1 mg/ml final concentration. One mg of protein was incubated with 10 μl of pre-immune serum and 10 μl of Sepharose G at 4 °C for 1 h with mild agitation and centrifuged for 3 min at 9000 rpm. The supernatant was collected and incubated with 1 μg of rabbit anti-claudin 1 antibody with mild agitation; overnight at 4 °C. Afterwards 25 μl of Sepharose G were added and after 2 h of incubation at 4 °C the supernatant was centrifuged for 2 min at 9000 rpm. The precipitate was washed twice with 600 mM NaCl and once with 150 mM NaCl solution (both of them supplemented with 0.5% of phosphatase inhibitor cocktail 1 and 2, 1 mg/ml trypsin inhibitor, 2 μg/ml leupeptin and 2 μg/ml aprotinin). Finally 25 μl of 4X Laemli buffer were added and the protein content was resolved on 15% SDS/PAGE and transferred to nitrocellulose membranes in a Bio-Rad semi-dry blotting system for 1 h at 120 mA. Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline (TBS) for 1 h, washed twice with TBS and incubated with the primary anti-claudin-1, anti-MMP2 or anti-MMP-14 antibody diluted in Tween 20/TBS (TTBS) overnight at 4 °C. Membranes were washed with TTBS, and incubated with horseradish peroxidase labeled secondary antibodies diluted 1:500 in TBS for 2 h at room temperature. After these processes membranes were washed thrice with TTBS and once with TBS before antibody binding was detected by chemiluminiscence using the Pierce kit (Supersignal West Dura Extended Duration Substrate) as substrate.

2.5. Flow cytometry

Once the AGS cells reached 90% confluence, they were washed