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Claudin-1 expression is induced by tumor necrosis factor-α in human pancreatic cancer cells

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Abstract. Claudin-1 is a membrane protein with four transmembrane domains, that is exclusively localized at cellular tight junctions. Recent studies have reported that claudin-1 plays an important role in cancer invasion and metastasis. However, the significance of claudin-1 in pancreatic cancer is still unknown. In the present study, we investigated the role of claudin-1 expression in pancreatic cancer growth using the PANC-1 human pancreatic cancer cell line. Treatment with tumor necrosis factor-α (TNF-α), an inflammatory cytokine, resulted in increased detection of 89 kDa products of poly-(ADP-ribose) polymerase (PARP), a marker of apoptosis, and decreased PANC-1 cell proliferation by 23%. Expression of claudin-1 was up-regulated by TNF-α in a concentration-dependent manner in PANC-1 cells. PANC-1 cells treated with TNF-α and siRNA against claudin-1 showed a 15% increase in proliferation; i.e. the cells transfected with siRNA against claudin-1 showed resistance to TNF-α-induced apoptosis. These results suggest that claudin-1 expression is responsible for TNF-α-dependent growth signals and the proliferation of pancreatic cancer cells.

Introduction

Tight junctions of cells serve as a barrier that prevents solutes and water from passing through the paracellular pathway, and as a fence between the apical and basolateral plasma membranes in epithelial cells. Tight junctions consist of transmembrane proteins such as claudins and many peripheral membrane proteins (1,2). Claudins play crucial roles in the formation and maintenance of the tight junctions. Claudins were first discovered in 1998, and the claudins family consists of at least 24 newly discovered members, the expression of which depend on cell type and tissue (2,3). Claudins are connected with the actin cytoskeleton and participate in intracellular signaling (4,5). In this context, the downward or upward regulation of claudins may have a role in cancer development. Alterations of claudin expression have been noted in several tumor types such as colorectal, ovarian, and breast cancer, suggesting their involvement in carcinogenesis (6-11).

Pancreatic cancer is one of the most lethal cancers, with a 3-year survival rate of ≤10% (12-17). Factors responsible for this poor prognosis include: a) difficulty in early diagnosis due to anatomical location and lack of early symptoms; b) limitations of conventional cancer therapies including surgery, chemotherapy, radiation therapy, and immune therapy; c) rapid spreading of tumors to the surrounding organs, causing obstructive jaundice; and d) frequent incidence of metastasis even from a small primary tumor ≤2 cm in diameter. Pancreatic cancer ranks fifth as a cause of cancer-related mortality in the USA and Japan. Modulation of the aggressive cell proliferation of pancreatic cancer is one of the most significant issues in modern medicine (18,19).

Tumor necrosis factor-α (TNF-α) is a cytokine that induces a diverse range of biological responses, including the induction of apoptosis and anti-proliferative effects in various cells (20,21). Interleukin (IL)-1, IL-6, and TNF-α are produced after cancer treatments such as surgical intervention and non-specific immunotherapy (22). These cytokines produced either locally or systematically may directly affect the growth and the metastasis of tumor cells. The effects of TNF-α on the expression of tight junction proteins are still unknown.

In this study, we examined the expression of claudin-1 in PANC-1 human pancreatic cancer cells treated with TNF-α and investigated the functions of claudins in the apoptosis of cancer cells.

Materials and methods

Cell culture and treatment. PANC-1 human pancreatic cancer cells were obtained from the American Type Culture

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Abbreviations: TNF-α, tumor necrosis factor-α; PARP, poly (ADP-ribose) polymerase; IFN-γ, interferon-γ

Key words: claudin-1, tumor necrosis factor-α, PANC-1, poly (ADP-ribose) polymerase

Collection (ATCC, Manassas, VA, USA). Cells were cultured in RPMI 1640 medium (Gibco, Breda, The Netherlands) and supplemented with 10% fetal bovine serum. They were cultured at 37˚C in a humidified atmosphere of 95% air and 5% CO_2. Cells were incubated with recombinant human TNF-α (R&D systems, Minneapolis, MN, USA) and interferon-γ (IFN-γ) (R&D systems) at various concentrations and periods.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated using an RNeasy RNA isolation kit (Qiagen, Hilden, Germany). First-strand cDNA was synthesized from 1 μg of total RNA using Rever Tra Ace (Toyobo, Osaka, Japan). PCR was performed using an aliquot of first-strand cDNA as a template under standard conditions with Taq DNA polymerase (Takara, Shiga, Japan). The cDNAs for human claudin-1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were amplified for up to 25 cycles. The primers used were as follows: Claudin-1-F: 5'-CAGCTGT TGGGCTTCATTCTC-3', claudin-1-R: 5'-ATCACTCCC AGGAGGATGCC-3', GAPDH-F: 5'-CCACCCATGG CAAATTCCATGGCA-3' and GAPDH-R: 5'-AGACCA CCTGGTGCTCAGTGTAGC-3'. The predicted sizes of the amplified products for claudin-1 and GAPDH were 277 bp and 696 bp, respectively. The PCR products were separated on 1.5% (w/v) agarose gels.

Short interference RNA (siRNA). Short interference RNA (siRNA) against claudin-1 was used for the knockdown of claudin-1 gene expression. The sequences for the siRNA against claudin-1 were as follows: sense 5'-r (GCAUGGUA UGGCAAUAGAA) d (TT)-3' and antisense 5'-r (UUCUAU UGCCAUACCAUGC) d (TG)-3'. The sequences for the negative control siRNA were as follows: sense 5'-r (UUCU CCGAACGUGUCACGU) d (TT)-3' and antisense 5'-r (ACGUGACACGUUCGGAGAA) d (TT)-3'. The siRNA was transfected into cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). Transfection, cells were then incubated for 48 h.

Western blot analysis. Cells were seeded at $5x10^4$ cells per 35-mm well and siRNA was transfected into cells using Lipofectamine 2000 reagent. The cells were incubated for 48 h and treated with TNF-α, IFN-γ, or TNF-α/IFN-γ combination (each 50 ng/ml). Cells were then lysed using M-PER lysis buffer (Pierce, Rockford, IL, USA), and proteins were prepared for Western blot analysis. Protein concentrations were determined by BCA (bicinchoninic acid) assay. The lysates (20 μg protein) were subjected to SDS-PAGE, and proteins were transferred to PVDF membranes. The membranes were incubated with antibodies specific for claudin-1 (1:1,000) (Zymed, South San Francisco, CA, USA), claudin-4 (1:15,000) (Zymed), poly (ADP-ribose) polymerase (PARP, 1:1,000) (Cell Signaling Tech, Danvers, MA, USA) or actin (Sigma, St. Louis, MO, USA) followed by a horseradish peroxidase-conjugated secondary antibody. An ECL Plus Western Blotting Detection System (Amersham, Uppsala, Sweden) was used for detection.

MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay. Cells were seeded at $2.5x10^4$ in 96-well plates. Cells were transfected with siRNA against claudin-1. After 2 days, cells were treated with TNF-α (50 ng/ml) and incubated for 24 h. The cells were added (Cell Titer 96 AQuenous One Solution Reagent) (Promega Corporation, Madison, WI, USA) to each well, and were incubated at 37˚C for an additional 1 h. The absorbance (OD_{490} nm) was measured using a 96-well plate reader.

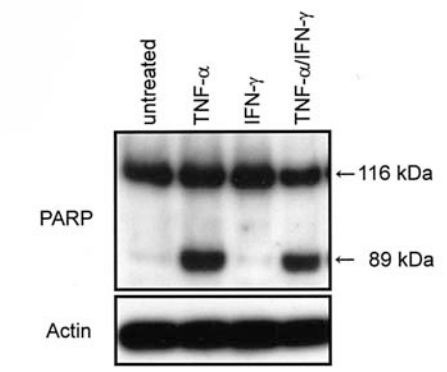

Figure 1. Detection of cleaved PARP in PANC-1 cells by Western blotting. Cells were treated with TNF-α, IFN-γ, or TNF-α/IFN-γ combination (each 50 ng/ml) for 24 h. Untreated cells detected full-length PARP (116 kDa). TNF-α or TNF-α/IFN-γ combination showed increased PARP cleavage (89 kDa).

Statistics. Data were presented as a mean ± SD. Statistical significance was determined by Student's t test. Comparisons with $p \leq 0.05$ were considered statistically significant.

Results

Effects of TNF-α and IFN-γ in PANC-1 cells. PANC-1 cells were seeded at $1x10^5$ cells per 35-mm well and treated with TNF-α, IFN-γ, or TNF-α/IFN-γ combination (each 50 ng/ml). Cells were incubated for 24 h and cleaved PARP was examined using Western blot analysis (Fig. 1). PARP, an important indicator of apoptosis, is cleaved into 89 kDa products by caspase-3. Untreated cells detected full-length PARP (116 kDa), but did not demonstrate cleaved PARP (89 kDa). PANC-1 cells treated with TNF-α or TNF-α/IFN-γ combination demonstrated increased PARP cleavage (89 kDa).

PANC-1 cells were seeded in 96-well plates, treated with TNF-α, IFN-γ, or TNF-α/IFN-γ combination (50 ng/ml), and incubated for 24 h. MTS-assay demonstrated that the cells treated with TNF-α and TNF-α/IFN-γ combination showed 23% ($p=0.000012$) and 37% ($p=0.0000034$) decrease in proliferation ($p \leq 0.05$), respectively compared to untreated control cells (Fig. 2). In contrast, PANC-1 cells treated with IFN-γ showed an 11% decrease in proliferation. Therefore, TNF-α induced apoptosis in PANC-1 cells.

Up-regulation of claudin-1 expression by TNF-α in PANC-1 cells. PANC-1 cells were treated with various concentrations of TNF-α for 24 h, or 10 ng/ml of TNF-α treatment for 2, 8, and 24 h. Gene expression of claudin-1 was evaluated by RT-PCR analyses. The mRNA expression of claudin-1 was up-regulated by TNF-α in a concentration-dependent manner (Fig. 3A) and claudin-1 mRNA expression was further

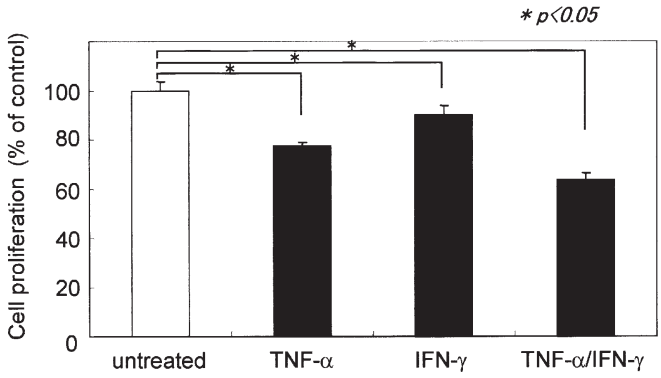

Figure 2. Proliferation of PANC-1 cells by MTS-assay. Cells were treated with TNF-α, IFN-γ, or TNF-α/IFN-γ combination (each 50 ng/ml) for 24 h. MTS-assay demonstrated that the cells treated with TNF-α IFN-γ or TNF-α/IFN-γ combination showed 23, 11 or 37% decrease in proliferation ($p \leq 0.05$), respectively compared with untreated control cells.

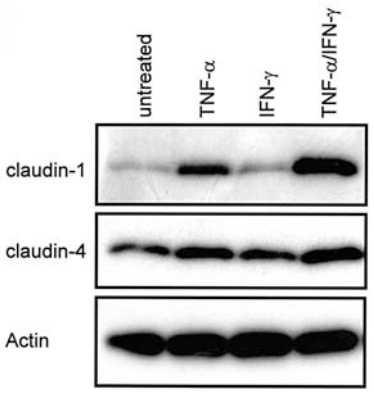

Figure 4. Expression of claudin-1 and claudin-4 in PANC-1 cells by Western blotting. The cells were treated with TNF-α, IFN-γ, or TNF-α/IFN-γ combination (each 50 ng/ml) and lysed. Claudin-1 protein was up-regulated by TNF-α or TNF-α/IFN-γ combination, while there were no significant changes in claudin-4 protein levels.

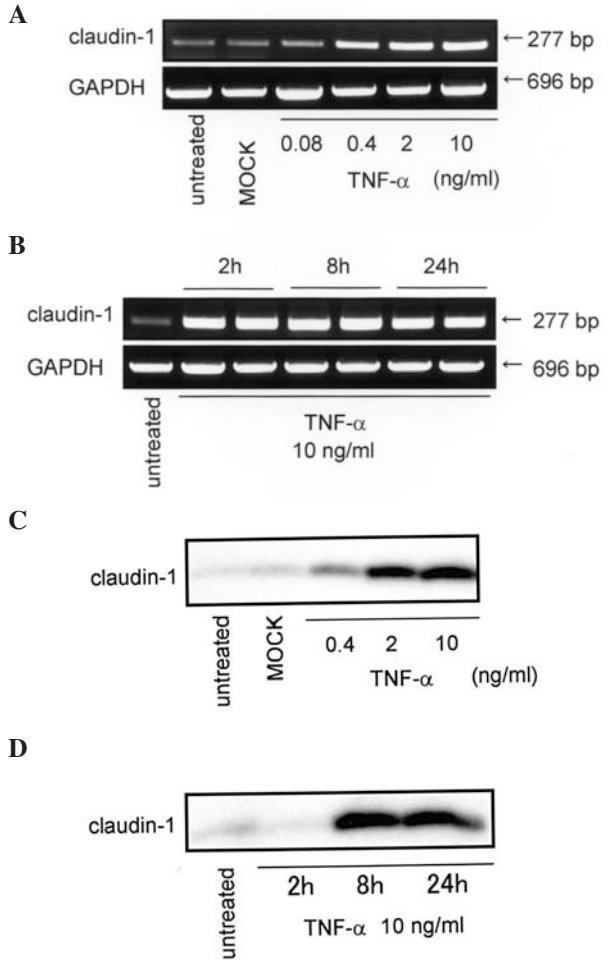

Figure 3. Expression of claudin-1 in PANC-1 cells. (A, RT-PCR) PANC-1 cells were treated with MOCK (buffer only) or 0.08, 0.4, 2, and 10 ng/ml TNF-α for 24 h. The mRNA expression of claudin-1 was up-regulated by TNF-α in concentration-dependent manner. (B, RT-PCR) PANC-1 cells were treated with TNF-α (10 ng/ml) for 2, 8, and 24 h. Claudin-1 mRNA expression was increased by TNF-α treatment for 2, 8, and 24 h. (C, Western blotting) PANC-1 cells were treated with MOCK or 0.4, 2, and 10 ng/ml TNF-α for 24 h. Claudin-1 protein expression was up-regulated by TNF-α in a concentration-dependent manner. (D, Western blotting) PANC-1 cells were treated with TNF-α (10 ng/ml) for 2, 8, and 24 h. Claudin-1 protein expression was up-regulated by TNF-α for 8 and 24 h.

increased by TNF-α treatment for 2, 8, and 24 h (Fig. 3B). We also examined protein expression of claudin-1 by TNF-α. Claudin-1 was up-regulated by TNF-α in a concentration-dependent manner (Fig. 3C) and claudin-1 protein expression was increased by TNF-α for 8 and 24 h, but not 2 h (Fig. 3D).

Effects of TNF-α, IFN-γ, or the TNF-α/IFN-γ combination on the expression of claudin-1 protein in PANC-1 cells. Cells were treated with 50 ng/ml TNF-α, IFN-γ, or TNF-α/IFN-γ combination for 24 h and proteins were lysed. Claudin-1 protein was up-regulated by TNF-α or TNF-α/IFN-γ combination treatment. However, there were no significant changes of claudin-4 protein levels by the TNF-α or IFN-γ treatment (Fig. 4).

Effects of knockdown of claudin-1 on proliferation and apoptosis in PANC-1 cells. To examine the role of claudin-1 in cell proliferation and apoptosis, we performed the siRNA-mediated knockdown of claudin-1, and detected cleavage of PARP and cell proliferation with the Western blotting and the MTS-assay respectively. The expression of claudin-1 was down-regulated by siRNA against claudin-1 in PANC-1 cells (Fig. 5A).

Western blotting demonstrated no significant changes in cleaved PARP levels in TNF-α-untreated cells regardless of siRNA (Fig. 5B). In contrast, siRNA against claudin-1 decreased cleaved PARP in TNF-α-treated cells when compared with control siRNA.

MTS-assay revealed that siRNA against claudin-1 did not affect proliferation of TNF-α-untreated cell (Fig. 6A). However, cells treated with claudin-1 siRNA and TNF-α showed a 15% increase in proliferation compared to cells treated with a control siRNA ($p=0.00064$) (Fig. 6B).

Discussion

The claudins are tight junction proteins that participate in paracellular barrier and cellular connection functions (1,2). Expression and function of the claudin proteins in pancreatic cancer, have not yet been extensively clarified. In this study,

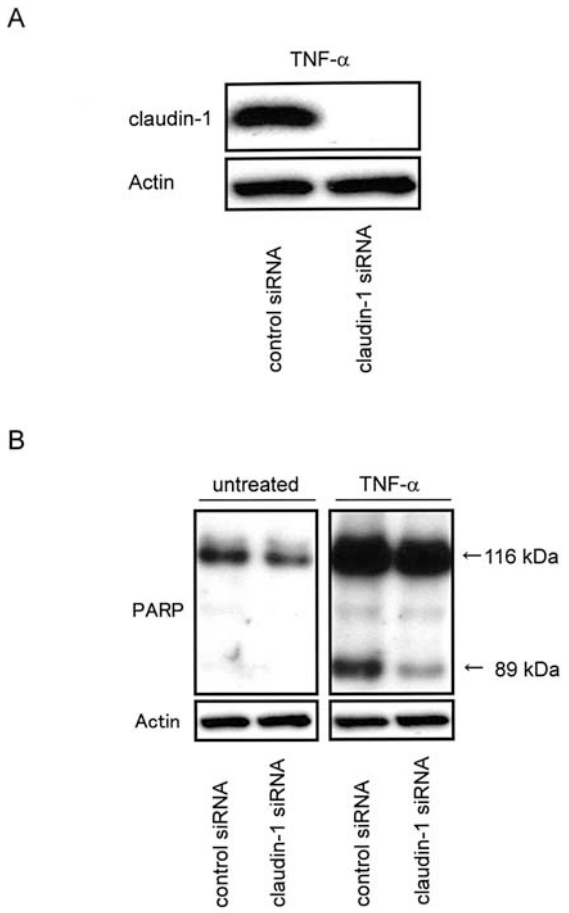

Figure 5. (A) Expression of claudin-1 in PANC-1 cells transfected siRNA against claudin-1 by Western blotting. The cells were transfected, siRNA against claudin-1 and incubated for 48 h. Claudin-1 expression was down-regulated by siRNA against claudin-1. (B) Detection of cleaved PARP in PANC-1 cells treated with or untreated with TNF-α. The cells were transfected siRNA against claudin-1 and incubated for 48 h. Cells were treated with TNF-α (50 ng/ml) or untreated with TNF-α, and incubated for 24 h. There were no significant changes in cleaved PARP levels in TNF-α-untreated cells. SiRNA against claudin-1 decreased cleaved PARP in TNF-α-treated cells when compared with control siRNA.

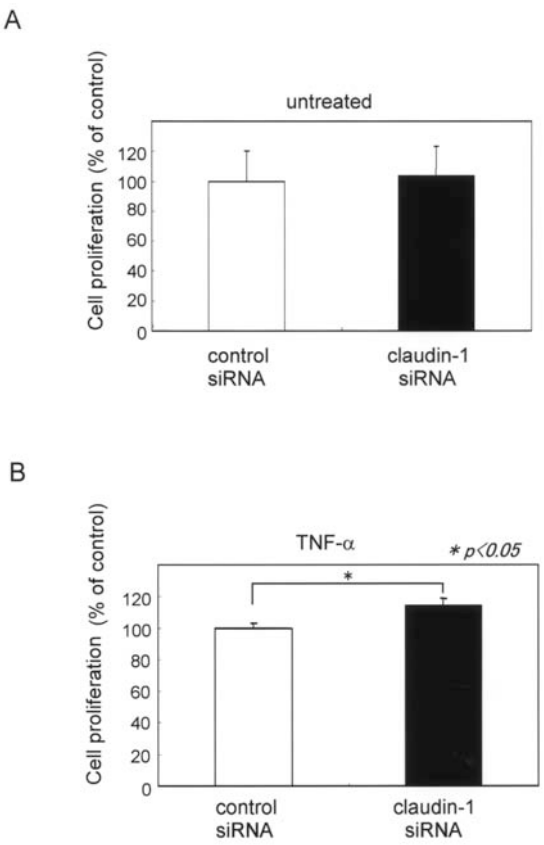

Figure 6. Proliferation of PANC-1 cells treated or untreated with TNF-α by MTS-assay. (A) The cells were transfected, siRNA against claudin-1 and incubated for 48 h. SiRNA against claudin-1 did not affect proliferation of TNF-α-untreated cells. (B) The cells were transfected, siRNA against claudin-1 and incubated for 48 h. After 2 days, cells were treated with TNF-α (50 ng/ml) and incubated for 24 h. Cells treated with claudin-1 siRNA and TNF-α showed a 15% increase in proliferation compared to cells treated with control siRNA.

we demonstrated the up-regulation of claudin-1 expression by TNF-α in a concentration-dependent manner in PANC-1 pancreatic cancer cells. In contrast, siRNA against claudin-1 increased the proliferation of PANC-1 cells treated with TNF-α. This is the first study to determine a relationship between TNF-α and claudin-1 expression in the human pancreatic cancer cells.

TNF-α is known to bind a receptor (tumor necrosis factor receptor, TNFR) on cells to transmit signals) (20,21). TNFR activation results in: a) the induction of apoptosis via activation of caspases after stimulation death domain proteins such as Fas-associated death domain (FADD) and TNFR1-associated death domain (TRADD); b) activation of nuclear factor-κB (NF-κB); and c) activation of p38 mitogen-activated protein kinase (MAPK) (23-26). In the present study, we found that TNF-α induced apoptosis and inhibited cell proliferation of human pancreatic PANC-1 cancer cells. Other cytokines such as IFN-γ did not significantly increase claudin-1 expression. Furthermore, TNF-α up-regulated protein levels of claudin-1, but not other claudins such as claudin-4. These results suggest that claudin-1 expression is responsible for TNF-α-dependent cell growth signals that lead to apoptosis and the inhibition of cell proliferation in pancreatic cancer cells.

In the present study, we also demonstrated that siRNA against claudin-1 affected the apoptosis and cell proliferation of PANC-1 cells. The siRNA-mediated knock-down of claudin-1 expression decreased apoptosis and inhibited stimulation of cell proliferation by TNF-α. Several nuclear oncogenes including *c-myc*, *c-fos* and *c-jun* participate in the regulation of cell proliferation (27,28). Alterations of claudin-1 expression might affect these factors. Based on the results of the present study, we speculated that claudin-1 may play an important role in the relationship between cell adhesion and TNF-α-dependent growth signals in pancreatic cancer cells. However, this hypothesis has not yet been tested. In the near future, we plan to clarify the mechanisms and significance of claudin-1 in apoptosis and cell proliferation of human cancer cells.

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