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Type I Collagen Induces DEC Expression and Epithelial-Mesenchymal Transition (EMT) in Human Breast Cancer MCF-7 Cells

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ABSTRACT

DEC1 and DEC2 are basic helix-loop-helix (bHLH) transcription factors, and are involved in the regulation of apoptosis, cell proliferation, circadian rhythms and the response to hypoxia. Type I collagen is the most abundant collagen of the human extracellular matrix (ECM) and is known as an epithelial-mesenchymal transition (EMT) inducing factor. Here, we demonstrated that DEC1, DEC2 and mesenchymal markers (snail and α-smooth muscle actin) were up-regulated in MCF-7 human breast cancer cells cultured on the type I collagen-coated plates, while epithelial markers (E-cadherin and claudin-1) were down-regulated in mRNA levels. Furthermore, type I collagen increased cell proliferation and invasive potential and affected cell morphology. These results indicated that type I collagen up-regulated DEC1 and DEC2 concomitant with EMT processes in MCF-7 cells and proposed a possibility that DEC1 and DEC2 participated in type I collagen stimulated EMT.

Keywords: Type I collagen, Helix-loop-helix, MCF-7, Extracellular matrix

INTRODUCTION

Circadian rhythms are 24 h cycles of biological processes including sleeping, moving and eating. The rhythms are tightly regulated by molecular clock mechanisms based oscillations of clock genes including period (Per 1, Per 2, Per 3), cryptochromes (Cry 1, Cry 2, Cry 3), clock, aryl hydrocarbon receptor nuclear translocator-like (Arntl/Bmal1), casein kinase I (CKI/Tau) and differentiated embryo-chondrocyte I (Dec1/Bhlhb2/Sharp2/Stra13) and Dec2 (Bhlhb3/Sharp1) in the normal and cancer cells [1]. Disruption of circadian rhythms leads to various pathological conditions such as insomnia, cardiovascular disorders and cancer progression [2-4]. Recently, several reports have stated that the relationship between cancer metastasis including epithelial-mesenchymal transition (EMT) and circadian rhythms [5-7].

DEC1 and DEC2 are basic helix-loop-helix (bHLH) transcription factors that are involved in the regulation of circadian rhythms, apoptosis, cell proliferation and the response to hypoxia [8-17]. Previously, we reported that DEC1 mediated EMT which is the primary step leading to invasion and migration of various tumor cells [18]. DEC1 expression was increased during progression from normal to carcinoma in situ and invasive carcinoma [19] and mutant DEC1 prevented metastasis of cancer *in vivo* [14]. Based on the findings, DEC1 was thought to be a key factor of cancer invasion and metastasis. On the other hand, DEC2 was associated with the regulation of apoptosis and cell proliferation [20,21] and the expression of vascular endothelial growth factor (VEGF) gene [10]. However, roles of DEC1 and DEC2 in cancer progression are not well studied.

Extracellular matrix (ECM) is the extracellular part of animal tissue that usually contributes to dynamic cell behavior, pooling of growth factors, wound healing and tumor invasion. ECM is composed of an interlocking mesh of glycosaminoglycans and fibrous proteins such as collagens, elastins, fibronectins and laminins [22]. Type I collagen is one of the major proteins in ECM and promotes EMT in both physiological and pathological processes such

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as embryonic epithelium [23], breast cancer [24,25], lung cancer [26] and pancreatic cancer [12,27,28]. It was also reported EMT induced by type I collagen could be mediated by the ILK-dependent signaling pathway [29] and the transforming growth factor beta (TGF-β) signaling pathway [26]. But there are few reports about the downstream factors of EMT induced by type I collagen [30] and the relationship between EMT induced by type I collagen and DEC is still unknown.

In the present study, we investigated the effects of type I collagen on the expression of DEC1 and DEC2 in human breast cancer MCF-7 cells. Our results indicated that type I collagen up-regulated the expression of DEC1 and DEC2 and regulated EMT-associated genes expressions. These findings suggested that EMT induced by type I collagen might occur through DECs. An understanding of the interaction between type I collagen and clock gene DECs will help in comprehending the complex dynamics of tumor invasion and metastasis in cancer biology.

METHODS

Cell culture and treatment

MCF-7 human breast cancer cells purchased from ATCC (American Type Culture Collection) were maintained in Dulbecco's Modified Eagle's Medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum and 1% penicillin and 1% streptomycin in 5% CO2 at 37°C. Cells were cultured on Biocoat Cell Environment Collagen I Cellware 6-well or 96-well plate (BD BioCoatTM, Belgium, UK) for type I collagen treatment.

Reverse transcription-polymerase chain reaction (RT-PCR)

MCF-7 cells were seeded on type I collagen-coated or non-coated 6-well plates for 24 h. Then total RNA was isolated from the cells using RNeasy RNA isolation kit (QIAGEN, Hilden, Germany). First-strand cDNA was synthesized from 1 μg of total RNA using ReverTra Ace (TOYOBO, Tokyo, Japan). Then, RT-PCR was performed using the aliquot of first-strand cDNA as a template under standard conditions using Taq DNA polymerase (QIAGEN). The PCR products were separated on 1.5% (w/v) agarose gels. The signal intensities were compensated by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal controls. The sequences and product sizes of the primer sets are shown in **Table 1**.

Table1. The sequences and product sizes of the primer sets used in RT-PCR.

Genes	Product size (bp)	Primer sequences
DEC1	534	F:5'-GTCTGTGAGTCACTCTTCATG-3' R:5'-GAGTCTAGTTCTGTTTGAAGG-3'
DEC2	502	F:5'-CACCTTTGACGTCTTTGGAG-3' R:5'-GAGAGTGGGAATAGATGCAC-3'
E-cadherin	200	F:5'-TGCCCAGAAAATGAAAAAGG-3' R:5'-GTGTATGTGGCAATGCGTTC-3'
snail	310	F:5'-GCGAGCTGCAGGACTCTAAT-3' R:5'-GCCTCCAAGGAAGAGACTGA-3'
slug	331	F:5'-GAGCATTTGCAGACAGGTCA-3' R:5'-TGAATTCCATGCTCTTGCAG-3'
Claudin-1	260	F:5'-CAGCTGTTGGGCTTCATTCTC-3' R:5'-ATCACTCCCAGGAGGATGCC-3'
α-SMA	307	F:5'-GGGAATGGGACAAAAAGACA-3' R:5'-GCGTCCAGAGGCATAGAGAG-3'
GAPDH	696	F:5'-CCACCCATGGCAAATTCCATGGCA-3' R:5'-AGACCACCTGGTGCTCAGTGTAGC-3'

F: forward primer; R: reverse primer

Cell morphology characterization

To observe the morphology changes of MCF-7 cells by type I collagen treatment, cell staining was performed using Cnt-ST-100 stain kit (CELLnTEC Advanced Cell Systems AG, Bern, Switzerland). MCF-7 cells were seeded on type I collagen-coated or non-coated 6-well plate and cultured for 24 h. Then, the medium of each well was aspirated and fixed with 4% paraformaldehyde for 20 min before being stained by Cnt-ST-II solution for 10 min. The cells were washed in PBS for twice. Then the cells were covered with cover glasses and photographed.

Cell invasion assay

Cell invasion assay was performed using BD BioCoat Matrigel invasion Chamber kit (Becton Dickinson, New Jersey, USA). MCF-7 cells were seeded on type I collagen-coated or non-coated 6-well plate. 24 h later, 1×10^5 cells/600 µl were added to the top chamber of a cell culture insert in a 24-well companion plate. After 48 h incubation, the chambers were collected and stained by Cnt-ST-II solution and invaded cells numbers on the membrane were counted. The number of cells that had migrated was quantified by counting them in ten random distinct fields using a light microscope.

Cell proliferation assay

The cell proliferation assay was performed using the MTS[3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]assay. MCF-7 cells were seeded on type I collagen-coated or non-coated 96-well plate (Becton Dickinson). These cells were cultured in 5% CO_2 at 37°C for indicated time (24, 48, 72 and 96 h). Then, the cells were added along with the Cell Titer 96 AQueous One Solution Reagent (Promega Corporation, Medison, WI, USA) to each well and were incubated at 37°C for additional 1 h. Absorbance at 490 nm was measured using a microplate reader.

RESULTS

Type I collagen significantly up-regulated the expression of both DEC1 and DEC2

Type I collagen affected the mRNA levels of DEC1, DEC2 as well as EMT-associated markers, while the basal expression of both DEC1 and DEC2 were low in mRNA levels **(Figure 1)**. Type I collagen also up-regulated the expression of mesenchymal markers such as snail and α-SMA, while type I collagen did not affect the expression of slug. The mRNA levels of E-cadherin and claudin-1 were decreased by type I collagen treatment. These results indicated that signaling pathways induced by type I collagen regulated the expression of DEC1 and DEC2, as well as that of EMT-associated genes in human breast cancer MCF-7 cells.

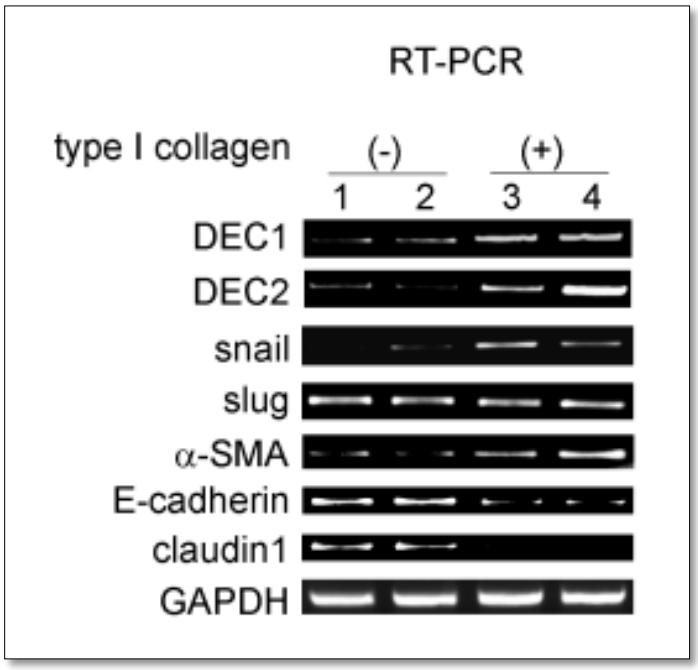

Figure 1. Type I collagen up-regulated expressions of DEC1, DEC2 and mesenchymal marker genes (snail, □-SMA), whereas down-regulated epithelial marker genes (E-cadherin, claudin-1). Human breast cancer MCF-7 cells were cultured for 24 h at 37°C on type I collagen-coated (lane 3 and 4) or non-coated (lane 1 and 2) 6-well plates. Then total RNA was isolated from each well and RT-PCR was performed using the primers as described in materials and methods. These experiments were performed duplicately.

Type I collagen altered the morphology of MCF-7 cells and promoted invasiveness of MCF-7 cells

Cancer cells with EMT are characterized by acquiring a fibroblast-like motile and invasive phenotype. We examined whether type I collagen induced EMT-like morphological changes in MCF-7 cells. MCF-7 cells cultured on the type I collagen-coated dish for 24 h showed a spindle-shaped morphology **(Figure 2A)**. Next we examined whether type I collagen affected invasive potentials of MCF-7 cells using cell invasion assay. Cell invasion of MCF-7 cells was significantly increased by type I collagen treatment compared to non-treated condition **(Figure 2B)**. These results indicated that type I collagen induced a mesenchymal phenotype in MCF-7 cells.

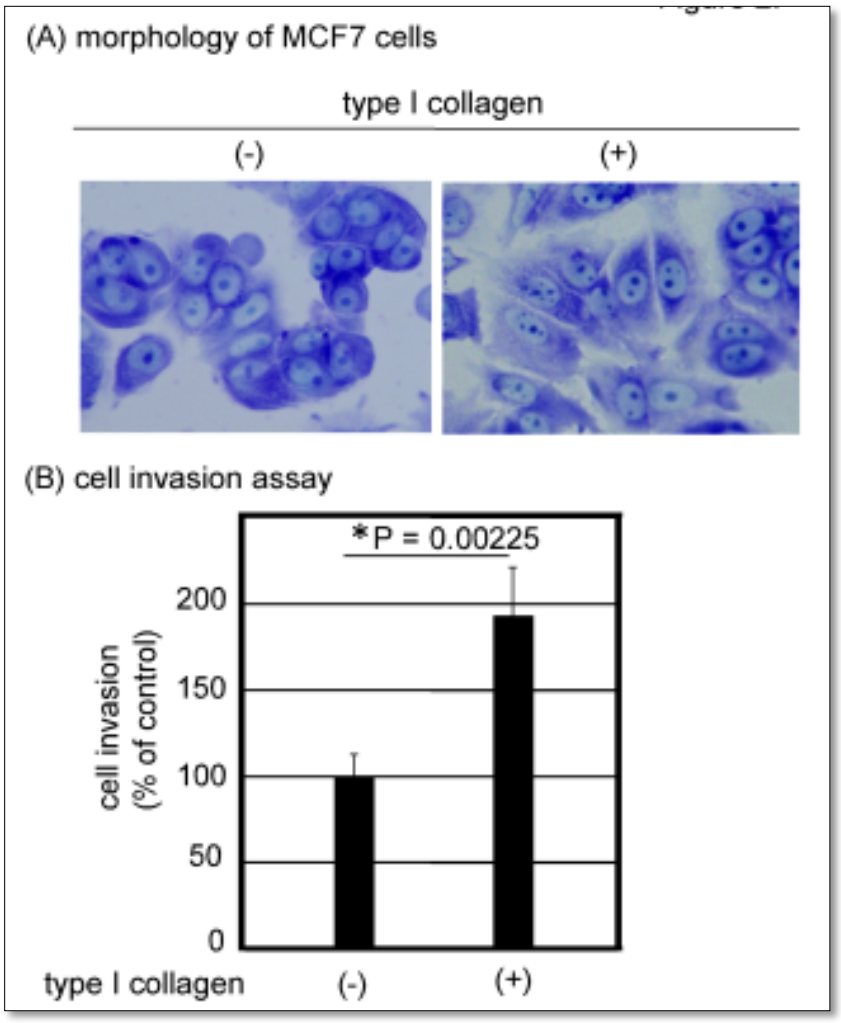

Figure 2. (A) Type I collagen changed the morphology of the human breast cancer MCF-7 cells to fibroblast-like shapes. MCF-7 cells were cultured on type I collagen-coated or non-coated plates for 24 h. Then, the cells were stained and photographed. (B) Type I collagen enhanced MCF-7 cells invasion. MCF-7 cells were cultured on type I collagen-coated or non-coated plate for 24 h. Next, the cells were transferred to the invasion chambers and cultured for additional 48 hours. Then, the chambers were stained and the numbers of invaded cells were counted. All of the data are means ± SEM (standard error of the mean) from four independent experiments, the number of invaded cells cultured on non-treated plates was used as control. Two-tailed Student t-test was used to determine the P value. $P<0.05$ was considered statistically significant. Graph was marked with an asterisk (*) if $P<0.05$.

Type I collagen promoted cell proliferation of MCF-7 cells

To investigate whether type I collagen affects cell proliferation of MCF-7 cells, we performed the MTS assay after culture for 24, 48, 72 and 96 h. The MTS assay showed that the proliferation of MCF-7 cells was induced by type I collagen treatment in any culture periods. Especially, cultured for 24, 48, 96 h showed significantly increased in the proliferation of MCF-7 cells by type I collagen treatment **(Figure 3)**. These results indicated that type I collagen promoted MCF-7 cells proliferation.

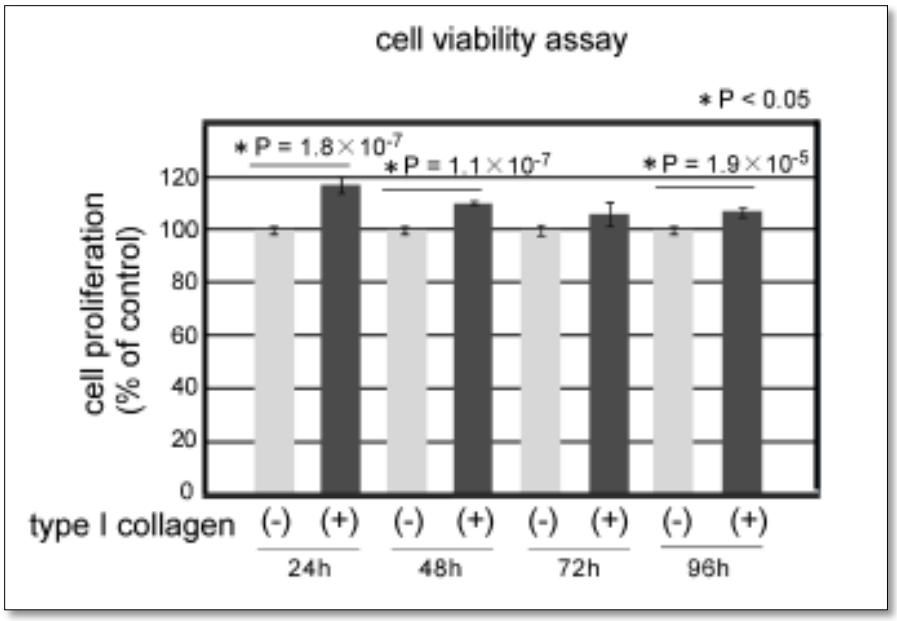

Figure 3. Type I collagen induced cell proliferation of the human breast cancer MCF7 cells. MCF7 cells were cultured on Type I collagen-coated or non-coated 96-well plate for 24-96 h. Then, MTS regents were added to MCF7 cells, and each absorbance (OD_{490} nm) was measured as described in materials and methods. All of the data are means ± SEM from six independent experiments and expressed percent of the control: absorbance of non-collagen-treated cells was used as control. Two-tailed Student t-test was used to determine the P value. $P<0.05$ was considered statistically significant. Graph was marked with an asterisk (*) if $P<0.05$.

DISCUSSION AND CONCLUSION

In the present study, we demonstrated type I collagen induced EMT in human breast cancer MCF-7 cells as follows:

i. Decreased epithelial markers and increased mesenchymal markers of MCF-7 cells on the type I collagen-coated plates,

ii. MCF-7 cells morphology were changed to fibroblast-like shapes on the type I collagen-coated plates, and

iii. Invasive potential of MCF-7 cells were increased by type I collagen treatment. This is the first report to describe the up-regulated expression of DEC1 and DEC2, concomitant with EMT-associate factors.

EMT is a process by which epithelial cells lose their cell polarity and cell-to-cell adhesion, and gain invasive and migratory properties to become mesenchymal cells. EMT is essential for numerous developmental processes including mesoderm formation and neural tube formation. EMT has also been shown to occur in wound healing in organ fibrosis and in the initiation of metastasis for cancer progression [31]. In the molecular levels, EMT is associated with up-regulated transcription factors such as snail and slug, decreased adhesion molecules (e.g. E-cadherin, β-catenin, claudin) and increased mesenchymal markers (e.g. N-cadherin, vimentin, α-smooth muscle actin). In our present study, type I collagen induced EMT and the mRNA levels of slug showed no significant changes, although slug has been known as one of the EMT-associated genes [32]. We have not found any reports that type I collagen up-regulates slug gene expression and we speculated that the mRNA level of slug is not directly affected by type I collagen.

We demonstrated that type I collagen also up-regulated DEC1 and DEC2 expressions, concomitant with inducing EMT-associated factors. The up-regulated DEC1 and DEC2 are thought to participate in EMT processes in MCF-7 cells treated with type I collagen. Previously, we reported that DEC1 knockdown inhibited EMT processes [18] and DEC1 was the downstream factor of PI3K-Akt signaling pathway [33]. Type I collagen has shown to promote EMT through ILK-Akt signaling pathway [29]. Therefore, DEC1 is thought to be up-regulated by crosstalk between PI3K-Akt and ILK-Akt signaling pathways, and to promote EMT by type I collagen treatment. In the near future, we will try to clarify the molecular mechanisms of DEC1, as well as the significance of DEC2 in EMT process induced by type I collagen. Elucidation of relationship between DECs and EMT may provide new insights into breast cancer therapeutic strategies.

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