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Up-regulated type I collagen expression by the inhibition of Rac1 signaling pathway in human dermal fibroblasts

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ABSTRACT

Tissue remodeling is known to play important roles in wound healing. Although Rac1 is reported to be one of the key signaling molecules in cutaneous wound healing process, the exact mechanisms of Rac1-mediated tissue remodeling is still unknown. This study investigated the role of Rac1 in the regulation of extracellular matrix in cultured human dermal fibroblasts obtained by skin biopsy from three healthy donors. Protein levels of type I collagen in cultured human fibroblasts were increased by the treatment with Rac1 inhibitor NSC23766 in a dose-dependent manner. However, the mRNA levels of α2(I) collagen was not altered by the inhibitor. On the other hand, by the addition of inhibitor, half-lives of type I collagen protein were increased and MMP1 levels were reduced. These data suggest that blockade of Rac1 signaling results in accumulation of type I collagen due to decreased collagenase activity. This study also suggests that controlling Rac1 signaling is a new therapeutic approach to chronic/untreatable ulcer.

Introduction

The extracellular matrix (ECM) is composed of proteoglycans (e.g. decorin and lumican), fibrous proteins (collagen and elastin), and substrate adhesion molecules (fibronectin and vitronectin). ECM forms a complex, three-dimensional network to fill the extracellular space between cells and to give structural support to resident cells in an organ-specific manner. In addition, ECM macromolecules are bioactive and can modulate cellular events such as adhesion, migration, proliferation, differentiation, and survival by interacting with the cells and generating signals through feedback loops [1,2].

In the tissue remodeling process or wound healing process, ECM molecules need to be rapidly accumulated during the formation of early granulation tissue and during the final replacement by mature connective tissue. In general, the amount of ECM in the tissue might be controlled through balance among the ECM production, the ECM degradation by matrix metalloproteinases (MMPs), and the inhibition of MMPs by tissue inhibitor of metalloproteinases (TIMPs). MMPs cleave various ECM components including collagens and play important roles in physiologic processes of tissue remodeling or wound healing.

In the skin, ECM, MMPs and TIMPs are mainly produced by dermal fibroblasts. Numerous in vitro and in vivo studies have suggested that some cytokines regulate dermal fibroblast proliferation and ECM expression [3,4]. Transforming growth factor (TGF)-β has been shown to increase the expression of collagen types I, III VI, VII, X, fibronectin, proteoglycans, and TIMPs but decrease MMPs [5]. On the other hand, interleukin (IL)-1β inhibits collagen production and induces MMP expression [6,7], whereas it can enhance fibroblast proliferation [8–10]. In addition, the involvement of downstream signal molecules including Smads, protein kinase C, STAT6 or phosphaidylinositol 3-kinase in the regulation of ECM production have been also well investigated [5]. We assumed that Rac1 signaling pathway is also involved in the regulation of ECM.

Rho GTPases are a family of small G-proteins that belong to Ras superfamily of GTP hydrolases and have multiple roles in cell behaviors including the regulation of the morphology and cell cycle [11–14]. Rac1 and Cdc42 have been shown to regulate cortical actin organization and to be involved in the formation of lamellipodia and filopodia, respectively [15–17]. In contrast, RhoA acting through Rho-kinase (ROCK) promotes the formation of stress fibers and spindle-shaped cells [18,19]. Although it has been shown that Rac1 controls proliferative activity of normal human dermal fibroblasts, it remains unclear whether the Rac1 signaling pathway affects metabolism of ECM including collagens [20]. This study presents that Rac1 also takes part in the regulatory mechanisms of ECM expression in normal dermal fibroblasts.

Materials and methods

Reagents. Rac1 inhibitor NSC23766 and Rho-kinase inhibitor Y27632 were obtained from Calbiochem (Darmstadt, Germany).

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The antibodies for type I collagen (1301–01) and type III collagen (1330–01) were purchased from Southern Biotech (Birmingham, AL). Antibody for β-actin (sc-47778) was from Santa Cruz Biotechnology (Santa Cruz, CA). Cycloheximide was from Sigma (St. Louis, MO).

Cell cultures. Human fibroblasts were obtained by skin biopsy from three healthy donors as described previously [21,22]. Institutional review board approval and written informed consent were obtained according to the Declaration of Helsinki. Primary explant cultures were established in 25-cm^2 culture flasks in modified Eagle's medium (MEM) supplemented with 10% fetal calf serum (FCS), and 50 μg/ml amphotericin. Monolayer cultures were maintained at 37 °C in 5% CO_2 in air. Fibroblasts between the third and sixth subpassages were used for experiments.

Cell lysis and immunoblotting. Dermal fibroblasts were cultured until they were confluent, then the medium was collected. The remaining cells were washed with cold phosphate-buffered saline twice and lysed in Denaturing Cell Extraction Buffer (BIOSOURCE, Camarillo, CA), which contains SDS and Triton X-100 to denature both intracellular and surface-attached collagens. Aliquots of the conditioned media (normalized for cell numbers) or cell lysates (normalized for protein concentrations as measured by the Bio-Rad reagent) were subjected to electrophoresis on 10% sodium dodecyl sulfate–polyacrylamide gels and transferred onto PVDF filters. The PVDF filters were then incubated with antibody against type I collagen, type III collagen or β-actin. Then the filters were incubated with secondary antibody, and the immunoreactive bands were visualized using an ECL system (Amersham Biosciences) according to the manufacturer's recommendations. The densities of the bands were measured using Quantity One 1D analysis version 4.6.6 software on a ChemiDoc™ XRS System (Bio-Rad Laboratories, Hercules, CA).

RNA isolation and reverse transcription. Total RNA was extracted with ISOGEN (Nippon Gene, Tokyo, Japan), following the protocol provided by the manufacturer. And first-strand cDNA was synthesized by PrimeScript RT reagent Kit (Takara Bio Inc., Shiga, Japan) with both oligodT primer and random hexamers.

Quantitative real-time PCR. Quantitative real-time polymerase chain reaction (PCR) with a Takara Thermal Cycler Dice (TP800)® used primers and templates mixed with the SYBR Premix Ex Taq Kit (Takara Bio Inc). Primer sets for α2(I) collagen, GAPDH, and MMP1 were purchased from SABiosciences (Frederick, MD). Primer sets were prevalidated to generate single amplicons. One microliter of diluted first-strand cDNA product was used for amplification in triplicate in a 25 μl reaction solution containing 12.5 μl of SYBR Green/Fluorescein PCR master mix and 1 μl of each primer. RNA (not reverse transcribed) was used as negative control. DNA was amplified for 40 cycles of denaturation for 5 s at 95 °C, annealing for 30 s at 60 °C. Data generated from each PCR reaction were analyzed using Thermal Cycler Dice Real Time System ver2.10B (Takara Bio Inc). Specificity of reactions was determined by melting curve analysis. The relative fold changes of gene expression between each gene of interest and GAPDH were calculated by standard curve method. For each gene of interest, we analyzed at least 3 independent samples.

Statistical analysis. Student's *t*-test was used to evaluate statistical differences between groups. *P* values less than 0.05 were considered significant.

Results

The effect of Rac1 inhibitor on the expression of collagen proteins in normal fibroblasts

As an initial experiment, we examined the effect of NSC23766, a specific inhibitor of Rac1, on the levels of type I collagen protein in normal fibroblasts. The expression of both α1(I) procollagen and α2(I) procollagen was significantly increased about 2.5-fold by the treatment with NSC23766 as compared with untreated cells (Fig. 1A and B). In addition, the levels of type III collagen protein were also induced by the addition of NSC23766 (Fig. 1C). These results suggest that Rac1 signaling has inhibitory effects on the production of collagen proteins, and that the blockade of Rac1 by NSC23766 results in the increase of collagen expression. In con-

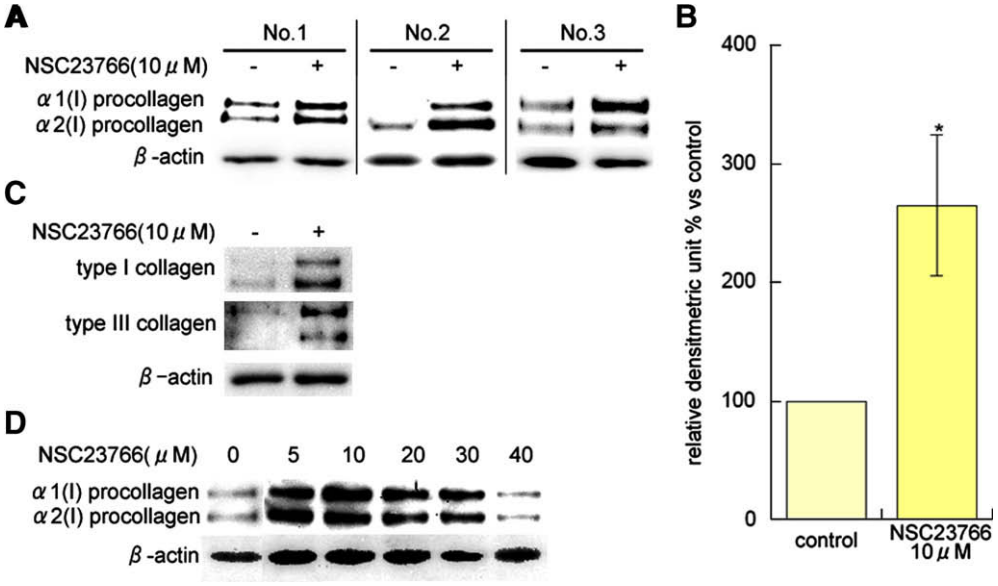

Fig. 1. The effect of Rac1 inhibitor on the expression of collagen proteins in normal fibroblasts. (A) Three human dermal fibroblasts were cultured independently until they were confluent, and then serum-starved for an additional 24 h. Cells were incubated in the presence or absence of 10 μM NSC23766 for 24 h, and immunoblotting was performed using anti-type I collagen antibody or anti-β-actin antibody. (B) The levels of type I collagen quantitated by scanning densitometry are shown relative to the levels in untreated cells. Data are expressed as the mean ± SD of three independent experiments. * $p < 0.05$ as compared with the value in untreated cells. (C) The effect of NSC23766 on the number or morphology of human dermal fibroblasts. Confluent quiescent dermal fibroblasts were treated as the above. The levels of type III collagen as well as type I collagen were analyzed by immunoblotting. (D) Dose-dependent effects of NSC23766 on the levels of type I collagen protein. Human dermal fibroblasts were serum-starved for 24 h and incubated in the presence or absence of indicated doses of NSC23766. Immunoblotting was performed as described above.

trast, phase-contrast microscopy revealed no prominent difference in the morphology between NSC23766-treated fibroblasts and untreated cells, whereas the cell number was slightly decreased by the inhibitor, consistent with previous reports (data not shown) [20].

Next, we investigated the dose-dependency of the effect of NSC23766 on the type I collagen protein expression. Human dermal fibroblasts were cultured until they were confluent, and then serum-starved for an additional 24 h. Cells were subsequently incubated for 24 h in the presence or absence of the indicated doses of NSC23766. The levels of type I collagen protein expression were induced maximally by treatment with 10 μM NSC23766, but the response decreased when 40 μM NSC23766 was added (Fig. 1D). Based on the result, in the following experiments we exposed cells to 10 μM NSC23766 to obtain maximal effect.

On the other hand, we also investigated the effect of Y27632, a specific inhibitor of Rho kinase. Although both Rho and Rac1 are known to be the same subgroup of the Ras superfamily of GTP hydrolases as described above, the levels of type I collagen protein were not affected by the treatment with Y27632 (data not shown). Taken together, the regulation of the collagen expression by Rac1 is thought to be kinase-specific.

The effect of Rac1 inhibitor on α2(I) collagen mRNA levels

To determine whether the effect of NSC23766 on type I collagen protein was paralleled with the corresponding mRNA levels, we investigated the effect of NC23766 on mRNA expression levels of α2(I) collagen (COL1A2) by quantitative real-time PCR. As shown in Fig. 2, NSC23766 did not induce the levels of COL1A2 mRNA. This indicates that NSC23766 up-regulates collagen protein expression without changing its mRNA level. The steady-state level of protein can be affected by the mRNA level and/or half-lives of the protein. Therefore, NSC23766 may induce type I collagen expression via the alteration of protein degradation activity. To confirm the possibility, de novo protein synthesis was blocked with cyclohexamide in the presence or absence of NSC23766. As shown in Fig. 3, the protein half-lives of existing type I collagen was significantly increased by adding NSC23766. Thus, Rac1 signaling may have reducible effect on collagen expression by decreasing the protein half-lives.

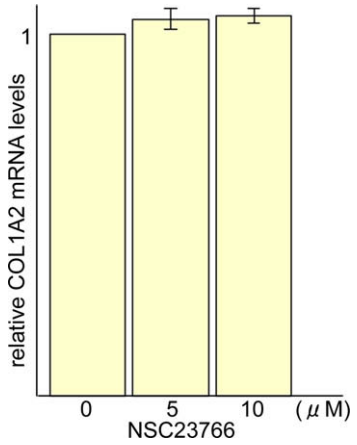

Fig. 2. The effect of NSC23766 on the α2(I) collagen mRNA expression in human normal skin fibroblast. Cultured fibroblasts were incubated in the presence or absence of 10 μM NSC23766 under the same conditions for 24 h and the quantitative real-time PCR of α2(I) collagen (COL1A2) and GAPDH mRNA expression was performed. Mean relative transcript levels of COL1A2 normalized to GAPDH in the same samples are shown relative to the level in untreated cells (1.0). Data are expressed as the mean ± SD of three independent experiments.

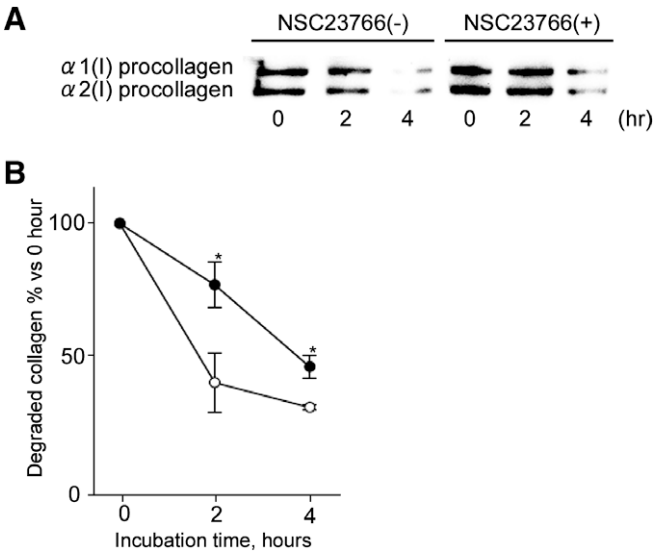

Fig. 3. Half-lives of type I collagen protein in the presence or absence of NSC23766. (A) Confluent quiescent cells were incubated in the presence or absence of 10 μM NSC23766 for 24 h before the addition of cyclohexamide (10 μg/ml). Cells were harvested at the indicated time points after cyclohexamide was administrated. The levels of type I procollagen were determined by immunoblotting. One experiment representative of three independent experiments is shown. (B) The type I collagen protein levels quantitated by scanning densitometry was expressed as a percentage of the value at time 0 and plotted on a scale. The bold line indicates levels in NSC23766-treated cells, and the solid line indicates control (untreated) levels. Statistical analysis was performed comparing the value of each time point with the Student's *t*-test. $^{*}p < 0.05$ as compared with the value in untreated cells.

The molecular mechanisms of increased half-lives of type I collagen in the presence of Rac1 inhibitor

As described above, the amount of type I collagen might be controlled fully or partly through balance among the production of type I collagen protein from fibroblasts, the degradation of the protein by MMPs, and the inhibition of MMPs by TIMPs. When MMP expression is up-regulated, protein half-lives of type I collagen expression should be decreased; on the other hand, collagen expression will be increased when TIMP expression is up-regulated. Among MMPs, MMP1 (collagenase-1), the interstitial collagenase, is expressed by a large variety of cells and is thought to be one of the collagenases mainly involved in the degradation of type I collagen [23,24]. To determine the possibility that MMP1 may participate in the alteration of type I collagen half-lives by NSC23766, we determined the effect of Rac1 inhibitor on MMP1 expression. As shown in Fig. 4, the mRNA expression levels of MMP1 were suppressed by NSC23766 significantly. On the other hand, the expression of TIMP was not affected by the addition of inhibitor (data not shown). This indicates that NSC23766 can modify the balance between MMP and TIMP expression. Taken together, Rac1 signaling may be essential for MMP1 expression, and blockade of Rac1 may down-regulate MMP1 levels, resulting in collagen up-regulation.

Discussion

In this study, we tested the effect of Rac1 inhibitor on the levels of collagen produced by dermal fibroblasts in vitro. We found that treatment with the inhibitor of Rac1 increased the collagen expression and reduced MMP1 expression. These results lead us to believe that Rac1 signaling maintains MMP1 expression and participates in the degradation of the collagen protein.

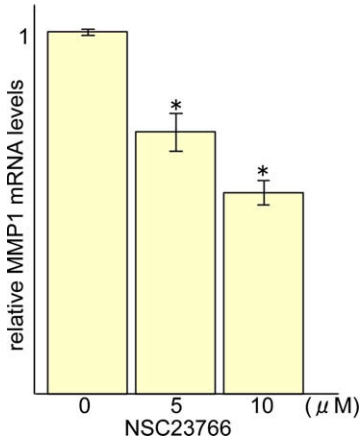

Fig. 4. The effect of NSC23766 on MMP1 expression levels in human normal skin fibroblast. Total RNA was extracted from cultured fibroblast treated with or without NSC23766 for 24 h. The mRNA expression levels of MMP1 were determined by quantitative real-time PCR as described in 'Materials and methods'. Mean relative transcript levels of MMP1 normalized to GAPDH in the same samples are shown relative to the level in untreated cells (1.0). Data are expressed as the mean ± SD of three independent experiments. $^{*}p < 0.05$ as compared with the value in untreated cells.

MMP1 is an interstitial collagenase secreted by fibroblasts and other cells that catalyses degradation of collagen. Among several prenylated proteins, the Rho family of GTPases has been implicated in the regulation of MMP1 expression. For example, it has been reported that Rac1 activity is required for the induction of MMP1 mediated by inactivation of the α5β1 integrin in rabbit synovial fibroblasts [25]. Moreover, down-regulation of Cdc42 by siRNA led to up-regulation of MMP1 in human skin fibroblasts via Rac1 signaling [26]. Human smooth muscle cells transfected with siRNA against Rac1 showed less secretion of MMP1 [27]. Consistent with these reports, our results suggest that the cultured normal human dermal fibroblasts treated with Rac1 inhibitor expresses less MMP1 than cells without the inhibitor.

Wound healing is a complex process, involving inflammation, fibroplasia, neovascularization, collagen deposition, epithelialization and wound contraction. Collagen synthesis in healing wounds has been documented by increased concentrations of fibroblasts, type I collagen mRNA, increased production of growth factors, and decreased activity of MMPs [28,29]. During wound healing, types I and III collagens which are produced and deposited by fibroblasts increase the tensile strength of the wound [30]. On the other hand, elevated expression of MMPs, decreased proliferation rate of fibroblasts and decreased expression of growth factors have been found in nonhealing chronic wounds [31].

There have been many studies about a potential function for Rac1 in tissue remodeling or wound healing. In vitro studies showed Rac1 is also essential for dermal fibroblasts proliferation in normal human dermal fibroblasts [20]. Rac1 is necessary for migration of primary rat embryo fibroblasts, lung fibroblasts and Madin–Darby canine kidney epithelial cell [32–34]. In vivo studies suggests Rac1-knockout mice showed reduced rates of wound closure [35]. Previous studies demonstrated the involvement of estrogen or Akt in the pathogenesis of keloid [36–38]. Because estrogen and Akt are known to decrease or inactivate Rac1 [39,40], respectively, they may promote the pathological scar formation by inhibiting Rac1 pathway and enhancing the deposition of collagens. On the other hand, our study suggests the Rac1 signaling can induce cell proliferation as well as MMP1 expression in normal human dermal fibroblasts. To note, as described above, IL-1β is reported to have similar effects on dermal fibroblasts; induction of MMP1 expression, reduction of collagen expression, and proliferation of fibroblasts. There have been several reports indicating the relation between IL-1β and Rac1 signaling [41,42]. Our results may indicate that such IL-1β effects may be mediated by Rac1 signaling.

Taken together, we showed the possibility that regulation of Rac1 signaling may led to new treatment of the wound healing and scar formation though controlling the activity of MMPs or the amount of ECM. Further studies are needed to elucidate the detailed mechanism of collagen regulation by Rac1.

In conclusion, this study sheds light on the regulation of type I collagen by the Rho family of GTPases. Controlling Rac1 signaling may be a new therapeutic approach to chronic/untreatable ulcer.

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