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Induction of the myofibroblastic phenotype in human gingival fibroblasts by transforming growth factor-β1: role of RhoA-ROCK and c-Jun N-terminal kinase signaling pathways

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Background and Objectives: Myofibroblastic differentiation is an important event in gingival wound healing and chronic inflammation. Transforming frowth factor-β 1 (TGF-β1) is a potent growth factor that has been implicated in this process. Gingival myofibroblasts have an increased ability to remodel the extracellular matrix and this feature has been associated with changes in the distribution of F-actin and the expression of the myofibroblast marker α-smooth muscle actin. In the present study we have analyzed the role of TGF-β1 and the signaling routes activated by this factor in the cytoskeletal changes that characterize the myofibroblastic differentiation process in human gingival fibroblasts.

Materials and methods: The signalling pathways involved in myofibroblastic differentiation were studied in primary cultures of human gingival fibroblasts using several signal transduction inhibitors. RhoA activation was analyzed through a pull-down assay. Distribution of focal adhesions and actin cytoskeleton was assessed by means of immunofluorescence and western blot. A cell adhesion assay was performed in TGF-β1-stimulated cells. Smooth muscle actin expression was studied through western blot and imunofluorescence. c-Jun N-terminal kinase phosphorylation was assessed through western blot.

Results: Our observations show that TGF-β1 activated the GTPase RhoA, a key regulator of the actin cytoskeleton. As a consequence of this event, this growth factor stimulated the generation of actin stress fibers and the reinforcement of vinculin-enriched focal adhesions. These responses were blocked after inhibiting ROCK, the main target of RhoA activation. TGF-β1 also stimulated the adhesion of fibroblasts over fibronectin, an extracellular matrix molecule involved in myofibroblastic differentiation. Finally, induction of the myofibroblast marker

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α-smooth muscle actin by TGF-β1 was abolished by the c-Jun N-terminal protein kinase inhibitor SP600125, suggesting a role for this signaling pathway during the induction of this phenotype.

Conclusions: We propose that TGF-β1 may promote the differentiation of myofibroblasts through the stimulation of cell spreading and adhesion, the reinforcement of focal adhesions, the maturation of the actin cytoskeleton, and the induction of α-smooth muscle actin. Activity of RhoA-ROCK and c-Jun N-terminal protein kinase signaling pathways are probably involved in these cellular events.

Gingival fibroblasts play an active role in tissue remodeling events during wound healing and inflammation. Despite their somewhat xuniform appearance, fibroblasts exhibit a high degree of heterogeneity in tissues subjected to remodeling (1). A subpopulation of cells termed myofibroblasts express high levels of α-smooth muscle actin (α-sma) and are responsible for cell-mediated matrix contraction during wound healing and chronic inflammation (2,3). *In vivo*, wound myofibroblasts are thought to arise from quiescent α-sma negative fibroblasts (4). Transforming growth factor-β1 (TGF-β1) is a multifunctional growth factor expressed at high levels in gingival tissues subjected to inflammation and wound healing (5,6) and has been implicated in the induction of the myofibroblastic phenotype (7). Although the role of TGF-β1 on myofibroblast differentiation and α-sma expression has been studied previously (7,8), the cellular mechanisms activated by this growth factor during this event are still not completely understood. A striking feature of myofibroblasts is the development of a strong actin network reinforced by α-sma and the presence of enhanced cell adhesion mechanisms (3). Since TGF-β1 is able to regulate the actin cytoskeleton in several cell types (9,10), this growth factor has been proposed as a modulator of the changes observed during the differentiation of myofibroblasts (7).

TGF-β1 may modify cell behavior through activation of several signal transduction pathways including the Smads and the mitogen-activated protein kinases, extracellular signal-regulated kinase (ERK) and c-Jun N-terminal protein kinase (JNK) (11). RhoA, a small GTP-binding protein that regulates the dynamics of the actin cytoskeleton through the activation of the kinase ROCK (12), may be modulated by TGF-β1 in epithelial cells (13,14). In spite of the fact that TGF-β1 is highly expressed in gingival tissue (5), the role of this growth factor on the activation of the above-mentioned pathways has not been studied in gingival fibroblasts.

In the present study we show that TGF-β1 may promote several cellular changes associated with the myofibroblastic phenotype including the formation of actin stress fibers and focal adhesions, responses that were regulated by the activity of the RhoA-ROCK signaling cascade. Moreover, induction of the myofibroblast marker α-sma by TGF-β1 was associated with the activation of the JNK signaling pathway. We propose that the activation of these signaling routes by TGF-β1 represents significant molecular events during gingival wound healing and chronic inflammation.

Materials and methods

Antibodies and reagents

Monoclonal antibody against RhoA was obtained from Cytosqueleton (Denver, CO, USA). Anti-vinculin, anti-α-sma and β-actin were supplied by Sigma (St Louis, MO, USA). Recombinant human TGF-β1 was purchased from US Biological (Swampscott, MA, USA). The Rho-kinase inhibitor Y27632 and PD98059 were obtained from Calbiochem (San Diego, CA, USA). SP600125 was purchased from Biolmol (Plymouth Meeting, PA, USA). Rabbit polyclonal anti-p-JNK was purchased from Upstate Biotechnology (Lake Placid, NY, USA) and mouse monoclonal anti-JNK-2 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Human plasma fibronectin was obtained from Calbiochem (San Diego, CA, USA).

Cell cultures

Primary cultures of human gingival fibroblasts were established by the explant method (15). Tissue explants were obtained from the retromolar tissue of one female patient undergoing extraction of a third molar at a private dental practice in Santiago, Chile. The tissue sample was harvested with the informed consent of the patient and the Ethical Commitee of the Faculty of Dentistry of the University of Chile approved the protocol for tissue obtention. No previous history of inflammation of the retromolar tissue was reported. No relevant pre-existing medical or drug histories were cited during the last 6 months. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (Gibco BRL), 100 μg/ml penicillin (Sigma), 100 μg/ml streptomycin (Sigma), and 50 μg/ml gentamycin at 37°C in a 5% CO_2 atmosphere. All experiments were performed using cells between the fourth and tenth passages.

Affinity precipitation of GTP-bound RhoA

Dr Keith Burridge (University of North Carolina, USA) kindly provided a construct that encodes the Rho

binding domain of Rhotekin (RBD) as a glutathione S-transferase fusion protein (GST-RBD). Cell cultures were stimulated with 10 ng/ml TGF-β1 (US Biological, Swampscott, MA, USA) for 7 min. Lysophosphatidic acid (Sigma) was used as a positive control of RhoA activation (16). Cells (10^7) were washed once with ice-cold phosphate-buffered saline (PBS) and lysed with 50 mM Tris–HCl, pH 7.6, 0.5 mM $MgCl_2$, 500 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.5% deoxycholate, 10 μg/ml each of aprotinin and leupeptin and 1 mM phenylmethylsulfonyl fluoride (PMSF). Lysates were clarified by centrifugation at 19,000 ***g*** for 10 min, and the supernatant was incubated on a roller for 1 h with 30 μg GST-RBD [the GST fusion protein containing the RhoA-binding domain (amino acids 7–89) of Rhotekin] bound to glutathione-agarose beads (Sigma). Samples were washed three times with lysis buffer and bound proteins were eluted by boiling in sample buffer and then were immunoblotted with RhoA monoclonal antibodies. Whole cell lysates were also immunoblotted for RhoA as loading controls.

Immunofluorescence

Cells plated on coverslips were washed once with PBS and fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.25% Triton X-100 for 5 min and incubated with PBS containing 4% bovine serum albumin (Rockland, Gilbertsville, PA, USA) for 30 min at room temperature. Primary antibodies diluted in PBS containing 1% bovine serum albumin were used in a dilution of 1/100 for both anti-α-sma and anti-vinculin and were incubated for 30 min at room temperature. Afterwards, antigen–antibody complexes were washed and incubated with fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin G (Rockland). F-Actin was stained with Alexa fluor 594 Phalloidin-rhodamine (Molecular Probes, Eugene, OR, USA). Fluorescence images were collected on a Zeiss Axioplan microscope and photographed using a 63 × immersion objective and an Axiocam camera.

Cell spreading and adhesion assays

Gingival fibroblasts were stimulated, or not, with 10 ng/ml TGF-β1 for 12 h. Cells detached from the substratum by a brief exposure to trypsin/EDTA were plated onto fibronectin-coated plates (10 μg/ml) for 10 min. Cell spreading was stopped by pouring off the medium, washing adhered cells once with PBS, fixing them with methanol for 2 min, and then incubating with 0.2% crystal violet for 5 min (17). After several washes, adherent cells were photographed using a Sony DSC camera through a Zeiss Axiovert 25 inverted microscope. To quantify cell adhesion, crystal violet-stained cells were dissolved with 0.1% sodium citrate in 50% methanol. The absorbance at 570 nm was read on an ELX 800 Microplate reader as previously described (18).

Preparation of Triton-soluble and Triton-insoluble fractions

Cells in 60-mm culture dishes were lysed for 15 min with a buffer containing 50 mM HEPES pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$, 2 mM EGTA, 1% Triton X-100, 10% glycerol, 2 mM PMSF, 2 μg/ml pepstatin, 2 μg/ml leupeptin, and 1 mM sodium orthovanadate. The solubilized material (hereafter the Triton-soluble fraction) was obtained after centrifugation for 10 min at 19,000 ***g*** The material that remained adhered to the plastic dish after Triton solubilization was scraped with a rubber policeman in the presence of 1% NP-40, 0.5% sodium deoxycholate, 0.2% sodium dodecyl sulphate, 150 mM NaCl, 50 mM Tris HCl, pH 7.4 (RIPA). This suspension was boiled in sodium dodecyl sulfate–polyacrylamide gel electrophoresis sample buffer to dissociate proteins and then centrifuged at 19,000 ***g***. This supernatant corresponded to the Triton-insoluble fraction.

Western blot analysis

Assay of mitogen-activated protein kinase activation - JNK activation was determined by Western blotting using antibodies specific for phosphorylated forms of JNK. Total JNK levels were detected as loading controls. Fibroblasts were treated with 10 ng/ml TGF-β1 in DMEM without serum at various time-points and lysed in PBS containing 150 mM NaCl, 1% Triton X-100 pH 7.4 and 10 μg/ml leupeptin, 1 mM PMSF, 2 mM *N*-ethylmaleimide, 1 mM orthovanadate, and 1 mM NaF. Proteins were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride transfer membranes (PerkinElmer Life Sciences, Boston, MA, USA). Membranes were exposed to primary antibodies, secondary antibodies coupled to horseradish peroxidase, and finally developed with an enhanced chemiluminescence kit (Amersham Corp., Arlington Heights, FL, USA).

TGF-β1-stimulated α-sma expression — Serum-starved cells were stimulated with 10 ng/ml TGF-β1 for 3 days in the presence or absence of matogen-activated protein kinase kinase (MEK) (PD98059), JNK (SP600125), or ROCK (Y27632) inhibitors. α-sma and β-actin expression levels were detected using Western blots. Cells were lysed and processed for Western blot as described in the JNK activation assay.

Results

RhoA activation, actin cytoskeleton re-organization and focal adhesion formation by TGF-β1

Since myofibroblasts display a well-developed actin cytoskeleton network, we investigated whether RhoA, a well-known regulator of cytoskeletal changes (12), is activated by TGF-β1 in human gingival fibroblasts. To test this, semiconfluent cultures of gingival fibroblasts were stimulated with 10 ng/ml TGF-β1 for 7 min and the activation of RhoA was assayed as described in the Materials and methods. As a positive control, cells were stimulated with 10 μM lysophosphatidic acid. As shown in Fig. 1(A), TGF-β1 stimulation induced a potent activation of RhoA as demonstrated by a pull-down assay, which allows the identification of the GTP-bound form of this protein.

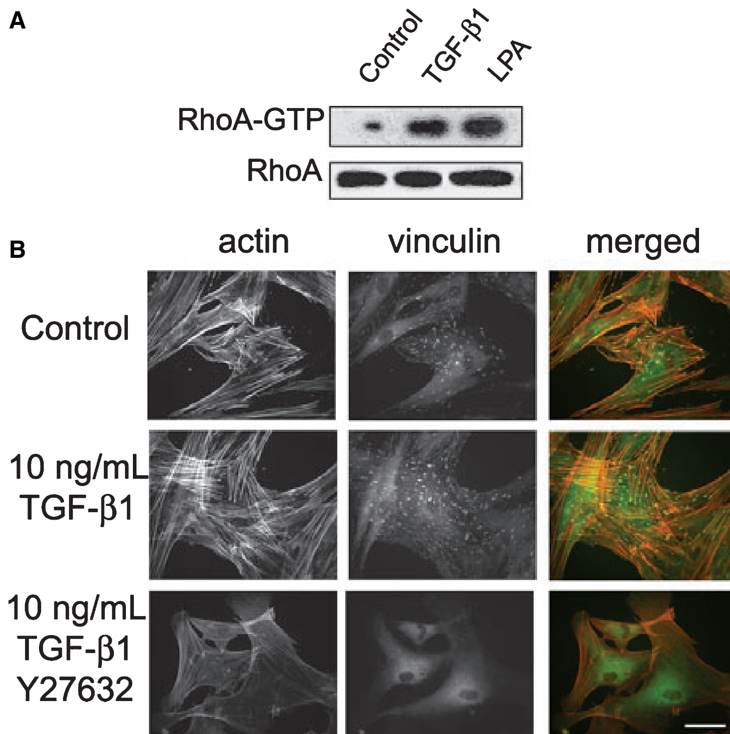

Fig. 1. RhoA activation, actin cytoskeleton re-organization, and focal adhesion formation by TGF-β1 in human gingival fibroblasts. (A) Cultures of gingival fibroblasts were stimulated with 10 ng/ml TGF-β1 for 7 min. As a positive control, cells were stimulated with 10 ng/ml LPA. RhoA activation was assessed by a pull-down assay, which allows the identification of the GTP-bound form of this protein. As a loading control, total levels of RhoA were detected. (B) Gingival fibroblasts were stimulated with 10 ng/ml TGF-β1 for 12 h and processed for immunofluorescence. Y27632 was added 30 min before TGF-β1 stimulation. The actin cytoskeleton was identified after staining with Alexa fluor Phalloidin-rhodamine (red) and distribution of vinculin was detected through immunofluorescence (green). Results are representative of at least two independent experiments. Bar = 10 μm.

To analyze the morphological consequences of TGF-β1-induced RhoA activation, serum-starved gingival fibroblasts were stimulated with 10 ng/ml TGF-β1 for 12 h and processed for immunofluorescence. As observed in Fig. 1(B), TGF-β1-stimulated cells displayed strong actin stress fibers and high levels of focal adhesions, as revealed by staining of the actin cytoskeleton or immunostaining for vinculin. When the ROCK inhibitor Y27632 was added before TGF-β1 stimulation, the cytoskeletal changes promoted by the factor were abrogated, suggesting that RhoA and its kinase ROCK are implicated in these cellular responses. Y27632-treated cells had almost no focal adhesions and a poorly developed actin network, suggesting that this inhibitor also abolished the basal ROCK activity in these cells.

From these results we may conclude that TGF-β1 is able to stimulate the generation of focal adhesions and stress fiber formation in human gingival fibroblasts through activation of the RhoA–ROCK signaling pathway.

Modulation of gingival fibroblast adhesion and spreading by TGF-β1

To confirm the stimulatory effect of TGF-β1 on the assembly of vinculin-enriched focal adhesions suggested in Fig. 1(B), serum-starved cells were stimulated with 10 ng/ml TGF-β1 for 12 h. Cells were lysed and processed as described in the Materials and methods section. As shown in Fig. 2(A), the presence of vinculin in the Triton-insoluble fraction was increased in TGF-β1-stimulated cells, suggesting that the growth factor may stimulate the establishment of strong focal adhesions enriched in vinculin. This difference in the distribution of surface-associated vinculin was not a result of an enhanced expression of the protein as shown by immunoblot analysis (Fig. 2A).

To determine whether the TGF-β1-stimulated phenotypical changes were able to modulate the adhesive or spreading behavior of these cells, serum-starved human gingival fibroblasts were stimulated with 10 ng/ml of the growth factor for 12 h. Cells were washed twice with PBS and detached by a brief exposure to a trypsin/EDTA solution. Trypsin was inactivated by adding DMEM supplemented with 10% fetal bovine serum and cells were allowed to recover for 15 min in an Eppendorf tube for 10 min. TGF-β1-stimulated and control cells were seeded onto fibronectin-coated plates and allowed to attach for 10 min. As observed in Fig. 2(B), TGF-β1 strongly stimulated the adhesion of human gingival fibroblasts when compared to vehicle-treated cells. As Fig. 2(C) shows, TGF-β1-stimulated cells displayed obvious signs of cell spreading. In contrast, vehicle-stimulated cells remained with a rounded morphology, lacking detectable cellular extensions.

Signaling pathways involved in α-sma induction in human gingival fibroblasts

Previous studies have demonstrated that TGF-β1 may stimulate α-sma expression in human gingival fibroblasts (19). In the present study we investigated the signaling pathways presumptively involved in TGF-β1-stimulated α-sma production. For this purpose, serum-starved gingival fibroblasts were exposed to the MEK1/2 inhibitor PD98059 (40 μM), the JNK inhibitor SP600125 (10 μM), or the ROCK inhibitor Y27632 (10 μM) for 30 min before the stimulation with 10 ng/ml TGF-β1 for 3 days. This stimulation time was derived from previous studies that have demonstrated

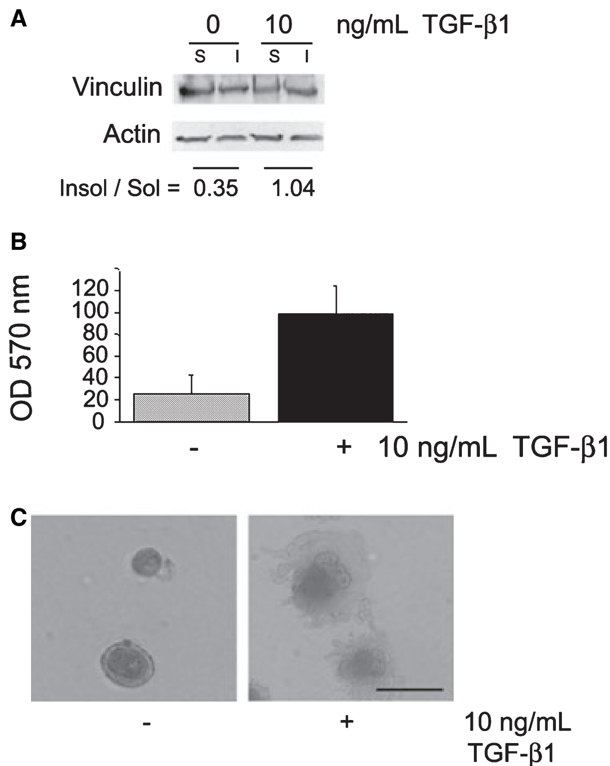

Fig. 2. Modulation of gingival fibroblast adhesion and spreading by TGF-β1. (A) Human gingival fibroblasts seeded over fibronectin were stimulated with 10 ng/ml TGF-β1 for 12 h and lysed as described in the Material and methods. Presence of vinculin in the Triton-soluble and insoluble fractions was detected through Western blot. β-actin levels were used as a loading control. The Triton-soluble/insoluble ratio was determined after the digital analysis of vinculin expression normalized against β-actin expression levels. (B) Control and TGF-β1-stimulated cells were seeded over a fibronectin matrix for 10 min. Cell adhesion was quantified after staining cells with crystal violet. Cells were lysed and absorbance was read at 570 nm (C) Under the same experimental conditions as described in B, cell spreading was evaluated through direct microscopy of crystal violet-stained cells. Bar = 10 μm.

that α-sma is induced after 72 h of TGF-β1 treatment in gingival fibroblasts (19). As revealed by Western blotting of the cell lysates, TGF-β1 stimulated α-sma expression in these cells and only the JNK inhibitor was able to attenuate this response (Fig. 3A). Neither the MEK1/2 (PD98059) nor the ROCK (Y-27632) inhibitors were able to modify the induction of α-sma in these cells. This result was confirmed by an immunofluorescence experiment in which we observed that the JNK inhibitor completely abrogated the expression of α-sma in TGF-β1-stimulated cells (Fig. 3B). To test whether the JNK signaling route was activated by TGF-β1, we analyzed the time–course phosphorylation of JNK through Western blotting in human gingival fibroblasts. As shown in Fig. 3(C), TGF-β1 activated JNK with a peak of phosphorylation detected between 15 and 30 min of stimulation.

Discussion

During gingival wound healing and chronic inflammation, a specific subpopulation of cells known as myofibroblasts is differentiated (1). Myofibroblasts probably originate from local resting fibroblasts, which are stimulated by soluble growth factors and/or molecular components of the extracellular matrix (7,19,20). Among the growth factors expressed in the gingiva, TGF-β1 is abundantly produced when this tissue is subjected to inflammation or wound healing (5,6). This growth factor has also been involved in the induction of the myofibroblastic phenotype (7). In the present study, we have analyzed the cellular mechanisms induced by TGF-β1 during differentiation of gingival myofibroblasts.

A striking feature of the myofibroblastic phenotype is the formation of increased cell adhesions onto insoluble substrates present in the extracellular matrix and the generation of strong stress fibers along the axis of the cells (21). A morphological continuity called fibronexus, a specialized form of focal adhesion enriched with vinculin, has been described between actin stress fibers that contain α-sma and the extracellular matrix, which contains fibronectin (21,22). The Rho family of GTPases has been involved in the regulation of cytoskeletal organization and therefore in cell morphology (12). Rho family members such as Cdc42, Rac, and RhoA are part of the Ras superfamily of proteins that cycle between an active, GTP-bound state and an inactive, GDP-bound state (12). Rac and Cdc42 control membrane ruffling, lamellipodia formation, and filopodia extensions (23–25). Considering that RhoA, and its main target Rho kinase (ROCK) have been shown to be essential for the formation of focal complexes and actin bundling (26–28), we investigated whether the signaling pathway initiated by RhoA activation was controlled by TGF-β1 in gingival fibroblasts. The present study shows that stimulation of human gingival fibroblasts by TGF-β1 activates RhoA and stimulates the formation of strong actin stress fibers and the distribution of vinculin into focal adhesion contacts, changes that were sensitive to the ROCK inhibitor Y-27632. Moreover, Y-27632 also abolished the basal assembly of focal adhesions and the organization of the actin cytoskeleton, suggesting that ROCK is also involved in the maintenance of these phenotypical features under non-stimulated conditions. These data strongly suggest that the RhoA–ROCK signaling pathway, activated by TGF-β1, is the main component of the Rho GTPase family involved in the cytoskeletal changes observed during myofibroblastic differentiation.

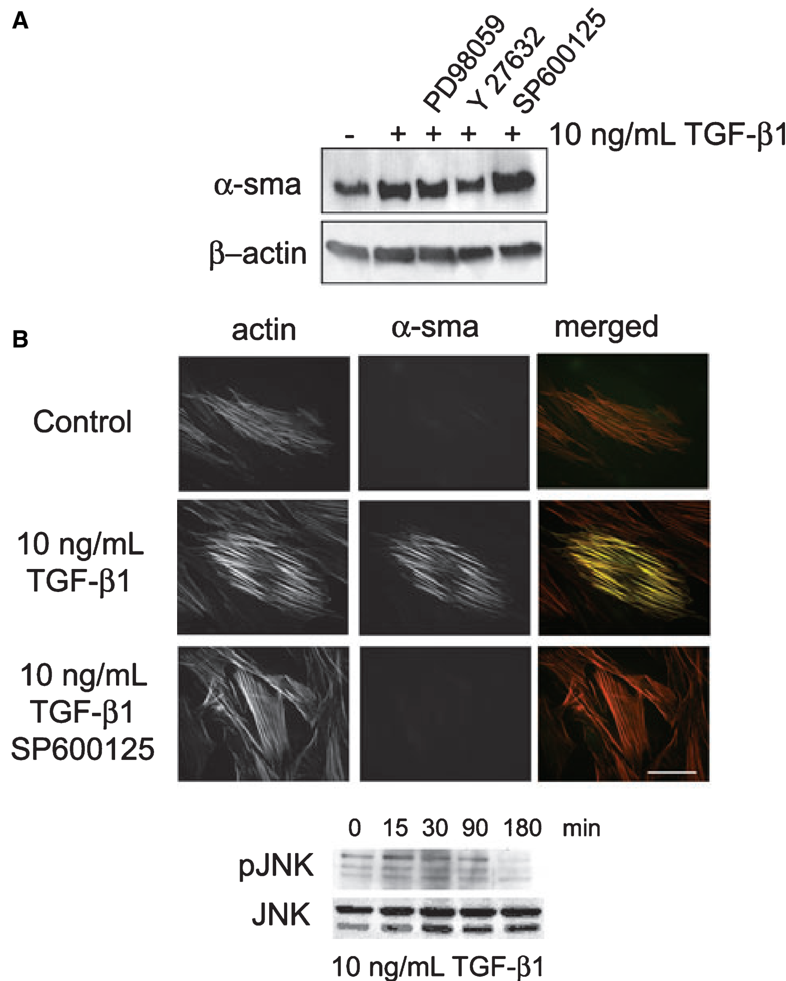

Fig. 3. Signaling pathways involved in α-sma induction in human gingival fibroblasts. Gingival fibroblasts were stimulated with 10 ng/ml TGF-β1 for 3 days. Cell cultures were exposed to the MEK1/2 inhibitor PD98059 (40 μM), the JNK inhibitor SP600125 (10 μM) or the ROCK inhibitor Y27632 (10 μM) for 30 min before TGF-β1 stimulation. (A) α-sma expression was detected through Western blotting of the cell lysates and β-actin was used as a loading control. (B) α-sma and total actin were detected through immunofluorescence (green) and Alexa fluor Phalloidin-rhodamine staining (red), respectively. Bar = 10 μm. (C) JNK activation was evaluated through Western blot of TGF-β1-stimulated cells using specific antibodies against the phosphorylated forms of JNK. Total JNK levels were detected as a loading control. These results are representative of three independent experiments.

Since cell adhesion is involved in events such as wound healing and myofibroblast differentiation, we also studied whether TGF-β1 was able to modulate the adhesive phenotype of human gingival fibroblasts. Our observations show that cell adhesion and spreading over fibronectin are strongly promoted after stimulation with this growth factor. This effect may be explained by the re-distribution of vinculin towards the focal adhesions, which allows the generation of strong adhesive contacts with the extracellular matrix. These results are in agreement with previous studies, which have shown that TGF-β1 may increase the expression levels of several integrin subunits, vinculin, and F-actin in human peritoneal fibroblasts (9) and may reinforce the formation of vinculin-containing fibronexus adhesion complexes in palmar aponeurosis myofibroblasts (10).

It is known that α-sma is one of the best markers of the myofibroblastic phenotype (29–31). We have analyzed the intracellular signal transduction pathways that regulate the induction of α-sma during the stimulation of human gingival fibroblasts by TGF-β1. Induction of α-sma in gingival fibroblasts depends on the interaction of the cell with the ECM and the level of tension of the actin cytoskeleton (28). Moreover, expression of α-sma depends on the adhesive state of the cell and signaling via focal adhesion kinase (32). These data suggest that the molecular events regulating the dynamic state of the actin cytoskeleton are probably involved in the induction of α-sma. However, contradictory studies have shown that induction of α-sma in pancreatic stellate cells can be attenuated after treatment with the ROCK inhibitor Y27632 (33). On the other hand, in an epithelial–mesenchymal transition model of kidney cells, TGF-β1-stimulated α-sma induction was dependent on the activity of RhoA but was not interrupted after inhibiting ROCK (14). Our results show that TGF-β1-induced α-sma expression is not affected by the ROCK inhibitor Y27632, suggesting that this kinase might not be directly involved in this event. The variability observed in the above-presented studies may be explained by the fact that regulation of the α-sma promoter is a complex process (34) and that it shows substantial tissue specificity (35). Further studies should be performed to determine whether RhoA is indeed involved in α-sma induction in human gingival fibroblasts.

TGF-β1 is able to activate several signaling pathways which may lead to expression or repression of genes involved in wound healing and inflammation (36). This growth factor exerts its effects through heterodimeric receptor complexes composed of type I and type II serine/threonine kinase receptors (11). Besides the role played by the Smads pathway (the prototypic signaling route activated by TGF-β1), activation of the mitogen-activated protein kinase signaling pathways ERK, p38, and JNK by TGF-β1 may also mediate several responses to this growth factor in distinct cell types (11). In human lung fibroblasts, TGF-β1-induced α-sma expression is sensitive to the JNK inhibitor CEP-1347, but is

not affected after blocking the ERK pathway (37). Similarly, our results show that inhibition of JNK by SP600125, but not the inhibition of ERK, is able to attenuate the induction of α-sma in human gingival fibroblasts after TGF-β1 treatment. These findings suggest that α-sma expression by TGF-β1 may be regulated by the same signaling pathway in cells derived from distinct tissue origins such as lung or gingiva.

It is interesting to note that the JNK signaling pathway has been involved in the production of fibronectin after TGF-β1 stimulation (38). Expression of a spliced form of fibronectin (ED-A fibronectin) is an early and crucial event detected during myofibroblastic differentiation that involves α-sma expression (20). It remains to be determined whether activation of JNK by TGF-β1 may modulate α-sma induction through expression of ED-A fibronectin or by other unknown molecular mechanisms.

Finally, we may conclude that TGF-β1 promotes the induction of the myofibroblastic phenotype through the activation of the RhoA–ROCK and JNK signaling pathways in human gingival fibroblasts. These phenotypical changes include the ability of cells to adhere and spread over fibronectin, the reinforcement of focal adhesion complexes, the organization of actin stress fibers, and the further induction of the myofibroblast marker α-sma. These cellular and molecular events may represent significant steps involved in the acquisition of an ECM-remodeling phenotype during physiological and pathological tissue remodeling in gingival fibroblasts.

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