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TGF-β1 increases invasiveness of SW1990 cells through Rac1/ROS/NF-κB/IL-6/MMP-2

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ABSTRACT

Human pancreatic cancer invasion and metastasis have been found to correlate with increased levels of active matrix metalloproteinase 2 (MMP-2). The multifunctional cytokine transforming growth factor beta 1 (TGF-β1) has been shown to increase both secretion of MMP-2 and invasion by several pancreatic cancer cell types. In the present study, we investigated the signaling pathway involved in TGF-β1-promoted MMP-2 secretion and invasion by human pancreatic cancer cells SW1990. Using specific inhibitors, we found that stimulation of these tumor cells with TGF-β1 induced secretion and activation of the collagenase MMP-2, which was required for TGF-β1-stimulated invasion. Our results also indicate that signaling events involved in TGF-β1-enhanced SW1990 invasiveness comprehend activation of Rac1 followed by generation of reactive oxygen species through nicotinamide adenine dinucleotide phosphate-oxidase, activation of nuclear factor-kappa beta, release of interleukin-6, and secretion and activation of MMP-2.

1. Introduction

Pancreatic cancer is a devastating malady with poor survival and ranks as the fourth most common cause of cancer mortality. This aggressive disease is often diagnosed at advanced stages due to its proclivity for early metastasis [1,2].

Cancer metastasis involves tumor cells crossing tissue barriers such as the basement membrane, comprised largely of type I collagen. Matrix metalloproteinase 2 (MMP-2) is a type-IV collagenase secreted by cells as a pro-enzyme and then activated in the extracellular milieu to execute its proteolytic activity [3]. High expression and activation levels for this proteinase have been found in human pancreatic cancer tissues [4,5]. Furthermore, these elevated levels of active MMP-2 showed to correlate with tumor invasion and metastasis of pancreatic carcinoma [5,6].

The multifunctional cytokine transforming growth factor beta 1 (TGF-β1) plays a dual role in the process of carcinogenesis acting as a suppressor of epithelial cell tumorigenesis at early stages, but promoting tumor progression by enhancing migration, invasion, and survival of the tumor cells during the later stages [7]. TGF-β1 has been shown to increase both secretion of MMP-2 and invasion by several pancreatic cancer cell types, including the well to moderately differentiated adenocarcinoma cells SW1990 [8,9].

In the present study, we investigated the signaling pathway involved in TGF-β1-promoted MMP-2 secretion and invasion by human pancreatic cancer cells SW1990.

2. Materials and methods

2.1. Antibodies and reagents

Mouse anti-interleukin-6 (anti-IL-6) monoclonal antibody was from R&D Systems (Minneapolis, MN). Dulbecco's Modified Eagle Media (DMEM) and fetal calf serum (FCS) were from GIBCO/Invitrogen (Carisbad, CA). Trans-well chambers (8 μm pore-size) and Matrigel were from BD Biosciences (San Jose, CA). Recombinant human TGF-β1 was from R&D Systems. NSC23766 was from Tocris (Bristol, UK). Specific inhibitors of nicotinamide adenine dinucleotide phosphate-NADPH (DPI), reactive oxygen species-ROS (NAC) and MMP (Ilomastat), were from Sigma Chemical Co (St. Louis,

Abbreviations: DPI, diphenyleneiodonium; IL-6, interleukin-6; ILO, Ilomastat; MMP, matrix metalloproteinase; NAC, *N*-acetylcysteine; NADPH, nicotinamide adenine dinucleotide phosphate-oxidase; NF-κB, nuclear factor-kappa beta; NSC, NSC23766; PDTC, pyrridoline dithiocarbamate; ROS, reactive oxygen species; TGF-β1, transforming growth factor-beta 1.

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MO). CM-H_2DCFDA was from Molecular Probes (Burlington, Ontario, Canada).

2.2. Cell culture

Human pancreatic cancer cells SW1990 (ATCC, Manassas, VA) were maintained in DMEM supplemented with 10% heat-inactivated fetal calf serum at 37 °C in a 5% CO_2 humidified atmosphere.

2.3. Invasion assay

The upper compartment of trans-well chambers were coated with Matrigel and placed in 6-well plates. SW1990 cells were washed with phosphate buffer saline (PBS) and then detached with 5 mM EDTA in PBS. Detached cells were washed once more in PBS, resuspended in serum-free media and added to the upper compartment of the chamber (1×10^5 cells/well). Conditioned medium was placed in the lower compartment of the chambers. To determine the effect of specific inhibitors, cells were pretreated with the inhibitor for 1 h before they were added to the chamber, and then fresh inhibitor was added to the well. After 24 h of incubation at 37 °C, the cells on the upper surface were completely removed by wiping with a cotton swab, and then the filter was fixed with methanol and stained with crystal violet solution. Cells that had migrated from the upper to the lower side of the filter were counted with a light microscope on 50 fields/filter. Invasiveness levels were expressed as fold-increase over the levels in control conditions.

2.4. Rac1 activity assay

For measurement of Rac1 activation in cell lysates from SW1990 cells, equal amounts of protein per sample (determined by use of a Protein Assay Kit from Bio-Rad Laboratories; Hercules, CA) were analyzed using the specific Rac1 G-LISA™ Activation Assay Kit (Cytoskeleton; Denver, CO) according to the manufacturer's instructions. This luminescence G-LISA™ is an ELISA based assay that allows measuring the GTP-bound (active) form of small G-proteins. Active Rac1 levels were expressed as fold-increase over the active Rac1 levels in control conditions.

2.5. MMP-2 activity assay

For quantification of active and total (pro and active) MMP-2 in the supernatants of SW1990 cells, the high-sensitivity MMP-2 Activity Biotrak Assay System kit (Amersham Biosciences; Piscataway, NJ) was used according to the manufacturer's instructions. This activity assay is based on an antibody-capture technique that sequesters the metalloproteinase. Bound endogenously active metalloproteinase activates a detection enzyme, which in turn activates a detectable chromogenic substrate. Total metalloproteinase activity (endogenously active plus the activatable proenzyme) is measured by the addition of α-aminophenylmercuric acetate, which artificially activates the inactive form of the metalloproteinase. Total-MMP-2 levels were expressed as fold-increase over the total-MMP-2 levels in control conditions. Active-MMP-2 levels were expressed as fold-increase over the active-MMP-2 levels in control conditions.

2.6. NF-κB activity assay

For determination of the p65 and p50 subunits of nuclear factor-kappa beta (NF-κB) in nuclear extracts from SW1990 cells, the NF-κB (p65) and NF-κB (p50) Transcription Factor Assay Kits (Cayman Chemical Company; Ann Arbor, MI) were used according to the manufacturer's instructions. NF-κB activation levels were expressed as fold-increase over the levels in control conditions.

2.7. IL-6 assay

Release of interleukin-6 (IL-6) in the supernatant of SW1990 cells was measured using the IL-6 Kit (Assay Designs; Ann Arbor, MI) according to the manufacturer's instructions. IL-6 levels were expressed as fold-increase over the levels in control conditions.

2.8. ROS production

Free radical production was determined by incubating SW1990 cells in the presence of 10 μM CM-H_2DCFDA. Fluorescence was measured in a stirred cuvette at 37 °C in a Hitachi H-7000 (Hitachi Limited, Tokyo, Japan) spectrofluorometer with excitation at 488 nm and emission at 530 nm. ROS levels were expressed as fold increase over ROS production in control conditions.

2.9. Statistical analysis

Data correspond to at least three independent experiments, each of which was done in triplicate. Results are presented as mean ± standard error (SE). The data for each condition were subject to analysis of variance (ANOVA) followed by Dunnet post hoc test when comparing three or more conditions, or evaluated using Student's *t*-test when comparing only two conditions. Significant differences were considered with values of $p < 0.05$.

3. Results

3.1. Rac1 mediates TGF-β1-induced SW1990 invasion through MMP-2 secretion and activation

Acting as a molecular switcher cycling between a GDP-bound inactive state and a GTP-bound active state, the member of the Rho family of small GTPases Rac1 plays an essential role in a variety of cellular events [10]. Importantly, Rac1 was shown to mediate invasion through a collagen barrier by activating MMP-2 in different cell types [11–13]. We therefore examined the activation of Rac1 in SW1990 cells challenged with TGF-β1.

Stimulation of these cells with TGF-β1 induced a 5.6 ± 0.4-fold-increase in Rac1 activity (Fig. 1A). As control, the Rac1 inhibitor NSC23766 (NSC) reduced Rac1 activity in both TGF-β1-stimulated and unstimulated cells (fold-increase of 1.2 ± 0.2 and 0.7 ± 0.1, respectively; Fig. 1A).

We next analyzed the influence of Rac1 on TGF-β1-mediated MMP-2 secretion and activation by SW1990 cells. Stimulation of these cells with TGF-β1 increased both secretion and activation of MMP-2, as reflected by the 1.8 ± 0.2 and 2.6 ± 0.3-fold-increase over control conditions in total- and active-MMP-2, respectively (Fig. 1B and C). However, pretreatment with NSC not only reduced MMP-2 secretion in both TGF-β1-stimulated and unstimulated cells (1.1 ± 0.1 and 0.7 ± 0.1-fold-increase in total-MMP-2, respectively; Fig. 1B), but also blocked MMP-2 activation (Fig. 1C). As expected, the MMP inhibitor Ilomastat (ILO) blocked MMP-2 activation in both TGF-β1-stimulated and unstimulated cells (Fig. 1C), without affecting MMP-2 secretion, as shown respectively by 1.1 ± 0.2 and 2.3 ± 0.3-fold-increase in total-MMP-2 (Fig. 1B).

We then evaluated the ability of SW1990 cells to cross a Matrigel basement membrane as a measure of cell invasiveness. The basal invasive capacity of these cells experienced a 1.9 ± 0.3-fold-increase by stimulation with TGF-β1 (Fig. 1D). Remarkably, NSC decreased both basal and stimulated invasive capacity (0.7 ± 0.1 and 0.9 ± 0.2-fold-increase, respectively; Fig. 1D). Accordingly,

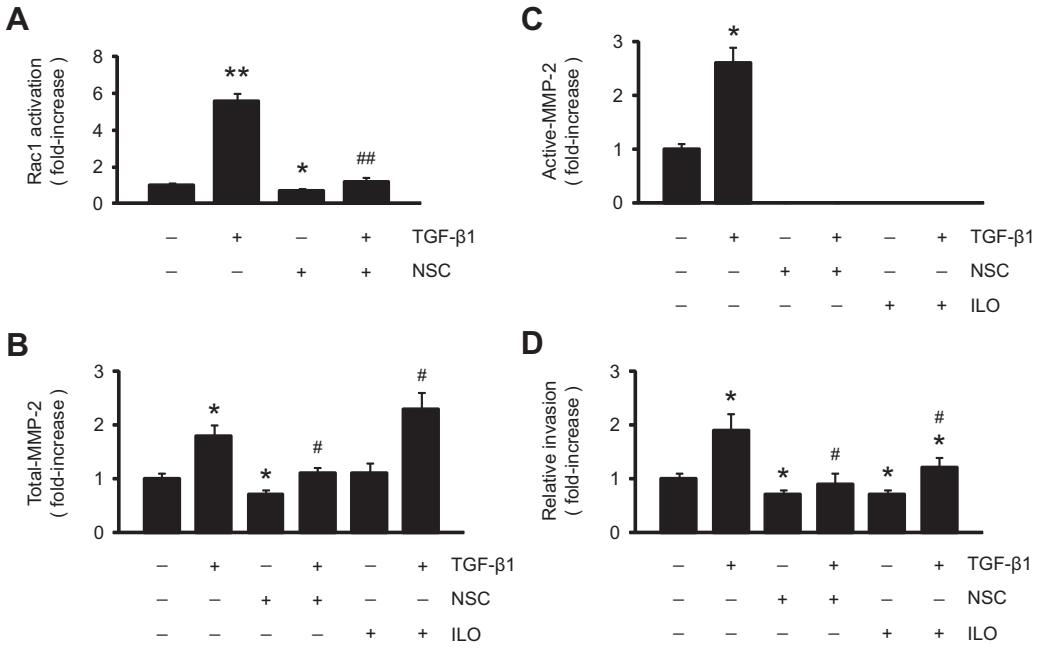

Fig. 1. Rac1 mediates TGF-β1-induced SW1990 invasion through MMP-2 secretion and activation. (A) SW1990 cells were stimulated for 15 min with TGF-β1 (20 ng/ml) or vehicle as control. To determine the effect of Rac1 inhibition, cells were pretreated with NSC (50 μM) for 1 h before and along the experiment. Rac1 activation levels were assessed in cell lysates. (B–D) SW1990 cells were stimulated with TGF-β1 (20 ng/ml) or vehicle as control, and cultured for 24 h. To determine the effect Rac1 and MMP inhibition, cells were pretreated with NSC (50 μM) or ILO (20 μM), respectively, for 1 h before and along the experiment. Total (B) and active (C) MMP-2 levels were measured in cell supernatants. (D) Cellular invasiveness through Matrigel by SW1990 cells. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to unstimulated control cells in the absence of inhibitors. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared to TGF-β1-stimulated cells in the absence of inhibitors.

ILO also diminished both basal and stimulated invasive capacity (0.7 ± 0.1 and 1.2 ± 0.2-fold-increase, respectively; Fig. 1D).

3.2. NADPH-generated ROS act downstream of Rac1 in TGF-β1-challenged SW1990 cells

Rac GTPase is the upstream signal protein for nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-dependent ROS generation, which has been shown to induce MMP-2 activation in various cell types [13–15]. In Fig. 2A, we presented ROS production in SW1990 cells. Basal ROS levels for control cells were substantially increased by stimulation with TGF-β1 (2.1 ± 0.3-fold-increase). Pretreatment with NSC not only decreased ROS levels in unstimulated cells, but also in TGF-β1-stimulated cells (0.6 ± 0.1 and 0.8 ± 0.1-fold-increase, respectively). As expected, the NADPH oxidase inhibitor DPI as well as the ROS inhibitor NAC also diminished ROS levels in unstimulated cells (0.5 ± 0.1 and 0.5 ± 0.1-fold-increase, respectively), and in TGF-β1-stimulated cells (0.7 ± 0.2 and 0.6 ± 0.1-fold-increase, respectively).

We then proceeded to evaluate the effects of NADPH and ROS inhibition on MMP-2 activity and cell invasiveness. As shown in Fig. 1B and C, TGF-β1 augmented total- and active-MMP-2 (1.9 ± 0.2 and 2.7 ± 0.3-fold-increase, respectively; Fig. 2B and C). Pretreatment of SW1990 cells with either DPI or NAC produced a decrease in total-MMP-2 secretion in both unstimulated and TGF-β1-stimulated cells (0.8 ± 0.1 and 1.0 ± 0.2-fold-increase for DPI; 0.8 ± 0.1 and 0.9 ± 0.1-fold-increase for NAC, respectively; Fig. 2B). Remarkably, absence of MMP-2 activation was also found in cells pretreated with either DPI or NAC in both unstimulated and TGF-β1-stimulated cells (Fig. 2C). In invasion assays, while TGF-β1 increased SW1990 invasiveness (2.1 ± 0.3-fold-increase; Fig. 2D), pretreatment with either DPI or NAC of both unstimulated and TGF-β1-stimulated cells reduced invasive capacity (0.8 ± 0.1 and 1.1 ± 0.2-fold-increase for DPI; 0.7 ± 0.1 and 1.0 ± 0.2-fold-increase for NAC, respectively; Fig. 2D).

3.3. TGF-β1-stimulated ROS activate NF-κB in SW1990 cells

The transcription factor nuclear factor-kappa beta (NF-κB) is one of the main signal-transduction molecules activated in response to oxidant stress [16]. Thus, we investigated the activation of NF-κB in SW1990 cells. Results presented in Fig. 3A and B revealed that both basal NF-κB (p65) and NF-κB (p50) activity in control cells were increased by stimulation with TGF-β1 (2.1 ± 0.3 and 1.9 ± 0.2-fold-increase, respectively). Pretreatment with NAC decreased both NF-κB (p65) and NF-κB (p50) activity in TGF-β1-stimulated cells (0.9 ± 0.1 and 0.8 ± 0.1-fold-increase, respectively). As expected, the NF-κB inhibitor PDTC diminished both basal and TGF-β1-stimulated NF-κB (p65) and NF-κB (p50) activity in SW1990 cells (0.7 ± 0.1 and 0.8 ± 0.1-fold-increase for p65, and 0.6 ± 0.1 and 0.7 ± 0.1-fold-increase for p50, respectively).

Next, we evaluated the effects of NF-κB inhibition on MMP-2 activity and cell invasiveness. As shown in the previous experiments, TGF-β1 augmented total- and active-MMP-2 (1.9 ± 0.2 and 2.6 ± 0.3-fold-increase, respectively; Fig. 3C and D). Pretreatment of SW1990 cells with PDTC produced a decrease in total-MMP-2 secretion in both unstimulated and TGF-β1-stimulated cells (0.8 ± 0.1 and 1.1 ± 0.1-fold-increase, respectively; Fig. 3C). Once again, lack of MMP-2 activation was also found in cells pretreated with PDTC in both unstimulated and TGF-β1-stimulated cells (Fig. 3D). In invasion assays, the raise observed for TGF-β1-stimulated cell invasiveness was prevented by PDTC pretreatment (1.8 ± 0.3 and 1.0 ± 0.2-fold-increase, respectively; Fig. 3E).

3.4. NFκB-induced IL-6 release is required for secretion and activation of MMP-2

NF-κB is known to induce the gene expression of the pleiotropic cytokine interleukin-6 (IL-6) [17,18], which has been shown to increase invasiveness of several cell types through MMP-2 [19–21]. We then examined the secretion of IL-6 by SW1990 cells. While

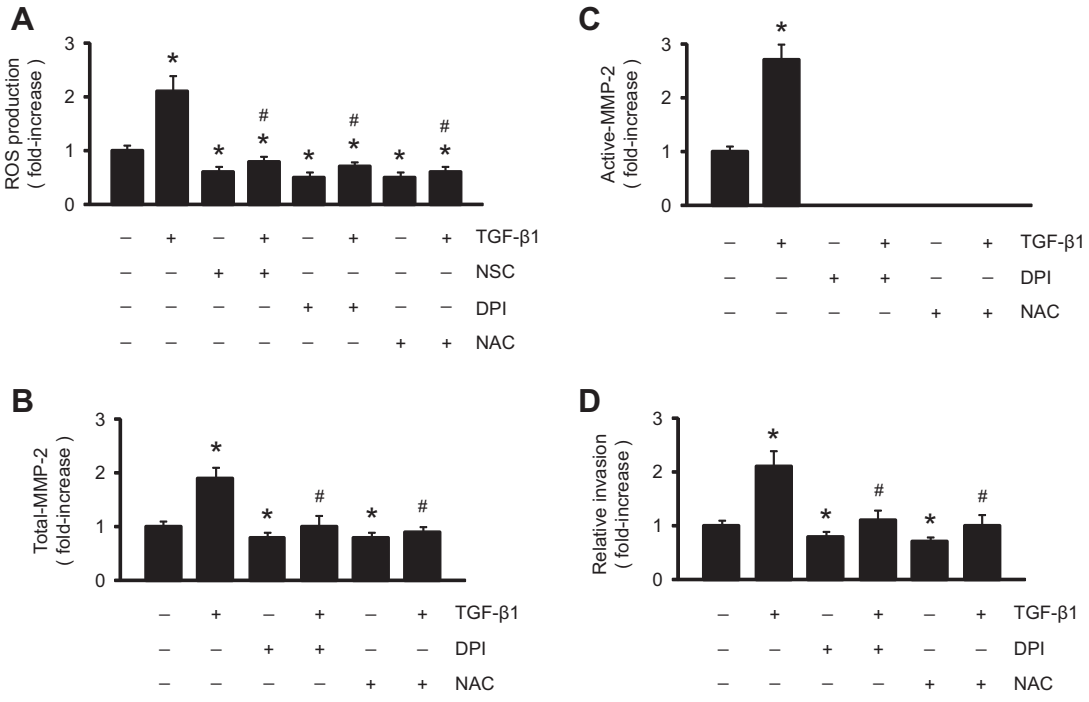

Fig. 2. NADPH-generated ROS act downstream of Rac1 in TGF-β1-challenged SW1990 cells (A) SW1990 cells were stimulated for 30 min with TGF-β1 (20 ng/ml) or vehicle as control. To determine the effect of Rac1, NADPH and ROS inhibition, cells were pretreated with NSC (50 μM) or DPI (15 μM) or NAC (30 mM), respectively, for 1 h before and along the experiment. (B–D) SW1990 cells were stimulated with TGF-β1 (20 ng/ml) or vehicle as control, and cultured for 24 h. To determine the effect of NADPH and ROS inhibition, cells were pretreated with DPI (15 μM) or NAC (30 mM), respectively, for 1 h before and along the experiment. Total (B) and active (C) MMP-2 levels were measured in cell supernatants. (D) Cellular invasiveness through Matrigel by SW1990 cells. $^{*}p < 0.05$ compared to unstimulated control cells in the absence of inhibitors. $^{\#}p < 0.05$ and compared to TGF-β1-stimulated cells in the absence of inhibitors.

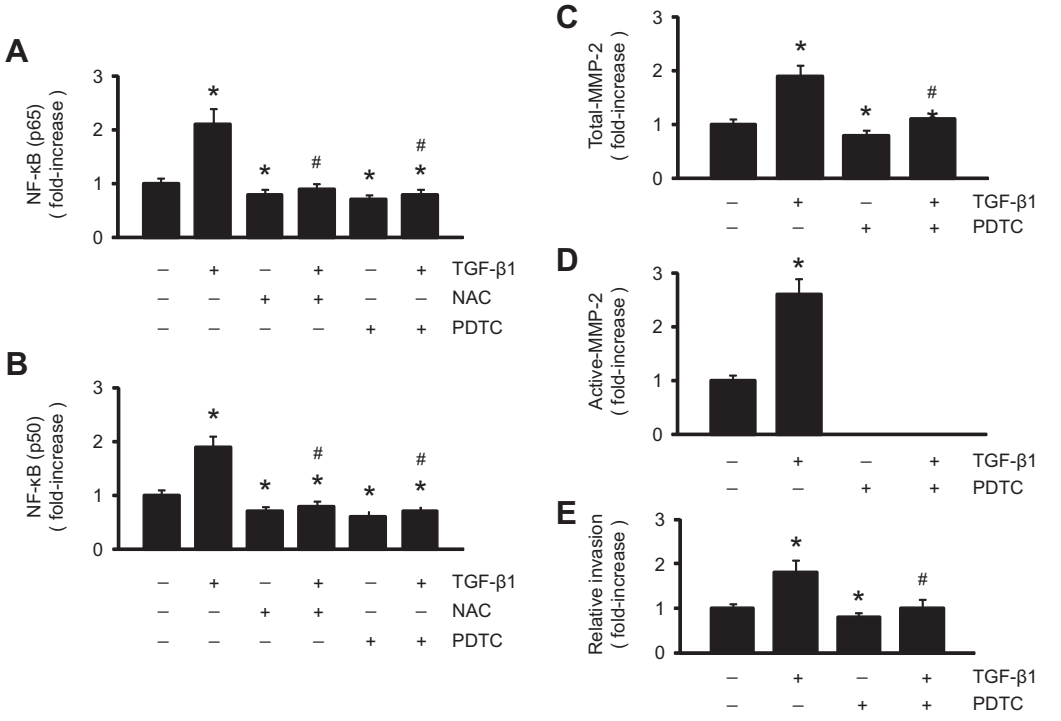

Fig. 3. TGF-β1-stimulated ROS activate NF-κB in SW1990 cells (A and B) SW1990 cells were stimulated for 1 h with TGF-β1 (20 ng/ml) or vehicle as control. To determine the effect of ROS and NF-κB inhibition, cells were pretreated with NAC (30 mM) or PDTC (50 μM), respectively, for 1 h before and along the experiment. NF-κB activation levels were assessed in nuclear extracts. (C–E) SW1990 cells were stimulated with TGF-β1 (20 ng/ml) or vehicle as control, and cultured for 24 h. To determine the effect of NF-κB inhibition, cells were pretreated with PDTC (50 μM) for 1 h before and along the experiment. Total (B) and active (C) MMP-2 levels were measured in cell supernatants. (D) Cellular invasiveness through Matrigel by SW1990 cells. $^{*}p < 0.05$ compared to unstimulated control cells in the absence of inhibitors. $^{\#}p < 0.05$ and compared to TGF-β1-stimulated cells in the absence of inhibitors.

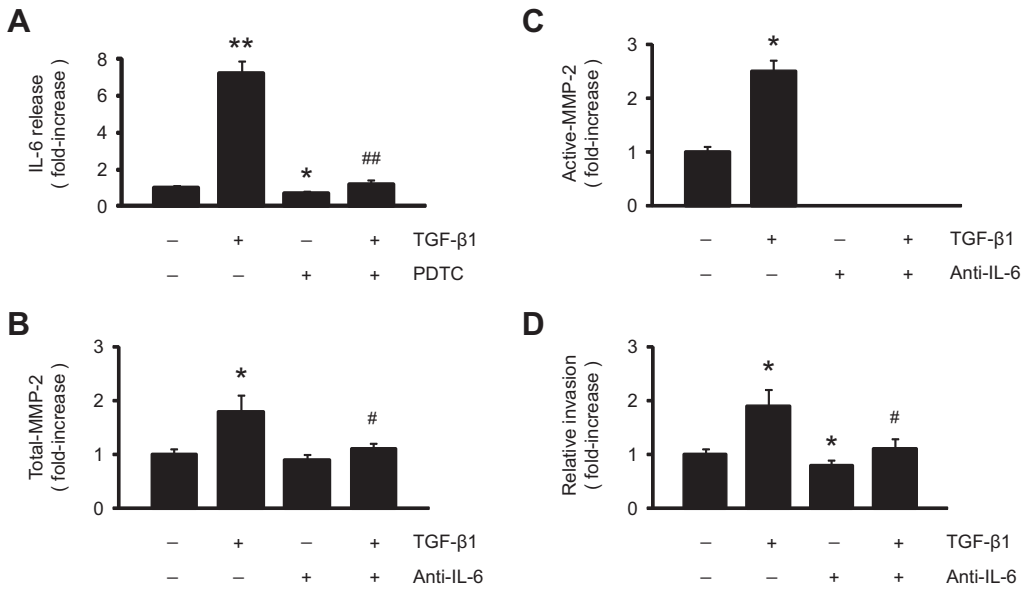

Fig. 4. NFκB-induced IL-6 release is required for secretion and activation of MMP-2 (A) SW1990 cells were stimulated with TGF-β1 (20 ng/ml) or vehicle as control, and cultured for 24 h. To determine the effect of NF-κB inhibition, cells were pretreated with PDTC (50 μM) for 1 h before and along the experiment. IL-6 levels were measured in cell supernatants. (B–D) SW1990 cells were stimulated with TGF-β1 (20 ng/ml) or vehicle as control, and cultured for 24 h in the presence of anti-IL-6 neutralizing antibody (10 μg/ml) or control IgG. Total (B) and active (C) MMP-2 levels were measured in cell supernatants. (D) Cellular invasiveness through Matrigel by SW1990 cells. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to unstimulated control cells in the absence of inhibitors. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared to TGF-β1-stimulated cells in the absence of inhibitors.

basal IL-6 release in control cells was increased by stimulation with TGF-β1 to values of 7.2 ± 0.7-fold-increase, pretreatment with PDTC decreased both unstimulated and TGF-β1-stimulated IL-6 release (0.7 ± 0.1 and 1.2 ± 0.2-fold-increase, respectively; Fig. 4A).

We next proceeded to analyze the effects of IL-6 inhibition on MMP-2 activity and cell invasiveness. As shown in the previous experiments, TGF-β1 augmented total- and active-MMP-2 (1.8 ± 0.3 and 2.5 ± 0.2-fold-increase, respectively; Fig. 4B and C). Incubation of SW1990 cells with an anti-IL-6 antibody produced a decrease in total-MMP-2 secretion in TGF-β1-stimulated cells (1.1 ± 0.1-fold-increase; Fig. 4B). We also found that MMP-2 activation was abolished in both unstimulated and TGF-β1-stimulated cells incubated with the anti-IL-6 antibody (Fig. 4C). In invasion assays, the increase observed for TGF-β1-stimulated cell invasiveness was diminished by incubation with the anti-IL-6 antibody (1.9 ± 0.3 and 1.1 ± 0.2-fold-increase, respectively; Fig. 4D).

4. Discussion

In the present study, we showed that TGF-β1stimulation of pancreatic cancer cells SW1990 induced secretion and activation of MMP-2, which were required for TGF-β1-stimulated cell invasion. Our results also indicate that signaling events involved in TGF-β1-enhanced SW1990 invasiveness comprehend activation of Rac1 followed by generation of ROS through NADPH-oxidase, activation of NF-κB, release of IL-6, and secretion and activation of MMP-2.

MMPs are main characters in cancer progression [3]. The crucial role of MMP-2 can particularly be appreciated in the corresponding knockout mouse, which presents highly reduced tumor angiogenesis and tumor growth [22]. Different stimuli have been demonstrated to promote secretion and activation of MMP-2 in diverse pancreatic cancer cell types [13,8,23]. Amongst these stimuli, TGF-β1 was shown to increase not only MMP-2, but also invasiveness of responsive pancreatic cancer cell lines PANC-1 and IMIM-PC1 [8]. However, TGF-β1 failed to augment both MMP-2 and invasiveness in non-responsive cell lines CAPAN-1, MiaPaca2 and IMIM-PC2 [8].

The small GTPase Rac1 amplifies the invasive capacity of several cancer tumor cells [11,13,23–26]. Here, we established that TGF-β1 is able to activate Rac1 with the consequent NADPH oxidase-mediated production of ROS in the responsive cells SW1990. Interestingly, it was recently demonstrated that activation of NF-κB by Rac1/ROS mediates TGF-β1-induced expression of MMP-9 and cell invasion by transformed keratinocytes [27].

The transcription factor NF-κB up-regulates the expression of various tumor promoting cytokines, including IL-6 [17,18]. Importantly, exogenous IL-6 showed to increase both MMP-2 production and invasiveness by the TGF-β1 responsive cells CAPAN-2 [21].

In summary, our findings provide a mechanism supporting TGF-β1 as a key factor for the proclivity of pancreatic cancer to early metastasis, endorsing the relevance of TGF-β1 in pancreatic cancer progression. Additionally, the critical role that we found for Rac1 mediating TGF-β1-enhanced SW1990 invasiveness, makes Rac1 also attractive as a potential pharmacological target for antitumoral therapy in pancreatic cancer.

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