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Dual inhibition of Src family kinases and Aurora kinases by SU6656 modulates CTGF (connective tissue growth factor) expression in an ERK-dependent manner

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ABSTRACT

Src kinases are regulators of the expression of connective tissue growth factor (CTGF/CCN2), which plays a role in fibrotic injuries. The aim of the present study was to evaluate the potential of SU6656, a dual inhibitor of Src family and Aurora kinases, to interfere with the synthesis of this pro-fibrotic factor.

SU6656 impaired TGF-β-mediated upregulation of CTGF mRNA and protein in proximal epithelial HKC-8 cells, and also reduced CTGF expression in cells exposed to autocrine growth factors. In association with the inhibition of Src family kinases and diminished focal adhesion kinase activity, adherence of the cells was reduced. Furthermore, SU6656 interfered with Aurora kinase activity resulting in inhibition of cell division and formation multilobular nuclei after 24 h. Comparable alterations were observed in primary tubular cells. When cell division was inhibited by SU6656 or ZM447439, a specific inhibitor of Aurora kinases, CTGF levels were back to control or even increased after 48 h. The activity of RhoA-Rho kinase and ERK signaling was analyzed to delineate the signaling pathways responsible for the biphasic regulation of CTGF. While Rho kinase was not significantly altered by SU6656, ERK activity was inhibited in the early phase and increased after 24–48 h. ERK activity correlated with secreted CTGF. As ZM447439 increased ERK activity only after 48 h, cellular reorganization is likely responsible for triggering the ERK-dependent upregulation of CTGF.

Taken together, in non-transformed epithelial cells, SU6656 modulates the expression of the pro-fibrotic factor CTGF in a time-dependent manner by inhibition of Src kinases and Aurora kinases.

1. Introduction

Non-receptor tyrosine kinases of the Src family are involved in basic cellular processes including cell survival, proliferation and migration (Frame, 2004). Upon translocation to plasma membranes, Src family kinases (SFK) interact with receptor tyrosine kinases and adhesion receptors such as integrins or E-cadherin (Guarino, 2010). Multiple downstream signaling pathways are being activated, primarily Ras/ERK and phosphatidyl inositol 3 kinase/AKT (PI3K/AKT) signaling related to survival and proliferation, as well as Rho/Rac signaling leading to alterations of the cytoskeleton. Hyperactivation of Src kinases leads to uncontrolled growth and has been associated with many types of human cancers (Guarino, 2010). Therefore, several inhibitors have been developed to suppress Src kinase activity in tumors. The most effective ones inhibit not only Src kinases, but also other growth-promoting kinases, with dual inhibition being more effective clinically (Musumeci et al., 2012).

To study the molecular effects of SFK in vitro, inhibitors as well as genetic approaches are being used. While genetic knockdown of SFK is highly specific, it also implies downregulation of SFK protein which will disrupt protein–protein interactions. Thus, in addition to inhibiting SFK activity, biological actions which are mediated by SFK as part of protein complexes may also be affected. Pharmacological inhibition on the other hand bears the potential of targeting other kinases but SFK (Bain et al., 2003). This does not only refer to tyrosine kinases which resemble SFK, such as Kit or Bcr-Abl (Tatton et al., 2003), but also serine threonine kinases such as TGF-β receptors. The often used inhibitors PP1 (Maeda et al., 2006) and PP2 (Maeda et al., 2006; Ungefroren et al., 2011) have been shown to inhibit TGF-β receptors I and II directly. This challenges some of the data which include SFK activation in TGF-β-mediated effects

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based on inhibitor studies. By contrast, inhibition of TGF-β receptors was not observed with SU6656, which seemed to be more specific for SFK (Blake et al., 2000). However, while not inhibiting TGF-β receptors, SU6656 has recently been reported to inhibit Aurora kinases which are critical for cell division by regulating spindle check point activation during mitosis (Arai et al., 2012; Riffell et al., 2011; Sanchez-Bailon et al., 2012; Tamm et al., 2012).

Connective tissue growth factor (CTGF, CCN2) is a profibrotic protein the expression of which is regulated via multiple signaling pathways in a cell type dependent manner (Cicha and Goppelt-Struebe, 2009; Samarakoon et al., 2010). Using PP2 and siRNA against c-Src Zhang et al. provided evidence for Src being a major signaling component for CTGF induction by TGF-β in osteoblasts (Zhang et al., 2010). In our own studies, we showed that regulation of CTGF by Src/focal adhesion kinase/PI3K signaling in fibroblasts was dependent on the cell culture conditions. Only in fibroblasts embedded in 3D collagen gels was CTGF regulated by this pathway, as compared to cells cultured on plastic, which were insensitive to SFK inhibition in terms of CTGF regulation (Graness et al., 2006).

In renal tubular epithelial cells, CTGF has been implicated in TGF-β-mediated mesenchymal alteration of these cells during tubular injury (Boor and Floege, 2011; Phanish et al., 2010). Short-term activation of CTGF expression by TGF-β was sensitive to inhibition by PP2 in proximal tubular cells HK-2 (Kroening et al., 2009b). In those studies the inhibition of TGF-β receptors by PP2 was not yet taken into consideration, as this effect of PP2 was reported only recently. Therefore, in order to further delineate the role of SFK in CTGF expression in epithelial cells we inhibited SFK with SU6656, which does not directly interfere with TGF-β signaling. Furthermore, we analyzed implications of SU6656-mediated inhibition of Aurora kinases in the regulation of long-term expression of CTGF.

2. Materials and methods

2.1. Materials

DMEM/Ham's F12 medium was purchased from Biochrom AG (Berlin, Germany), DMEM medium and Hank' BSS from PAA Laboratories (Coelbe, Germany), insulin-transferrin-selenium supplement from Gibco (Karlsruhe, Germany), fetal calf serum (FCS) from PAN Biotech (Aidenbach, Germany), triiodothyronine from Fluka (Buchs, Switzerland), hydrocortisone from Sigma (Munich, Germany), epidermal growth factor from PeproTech (Hamburg, Germany), TGF-β1 from tebu-bio (Offenbach, Germany), H1152 and U0126 were obtained from Calbiochem (Munich, Germany), SU6656 and ZM447439 from Enzo Life Sciences.

2.2. Cell culture

HKC-8 cells were cultured as described previously (Kroening et al., 2009a). Human primary tubular epithelial cells (hPTECs) were isolated from renal cortical tissues collected from healthy

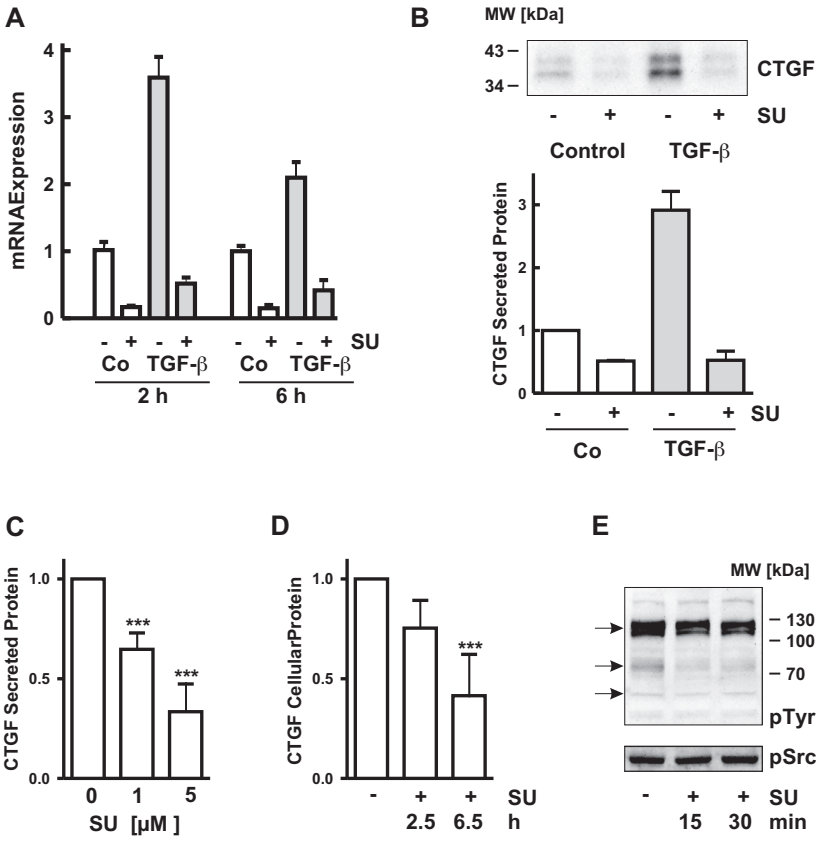

Fig. 1. Src kinase inhibition by short-term SU6656 treatment prevents TGF-β-induced and basal expression of CTGF. (A) Epithelial HKC-8 cells were preincubated with SU6656 (5 μM) for 30 min and then stimulated wit TGF-β (2 ng/ml) for 2 and 6 h. CTGF mRNA was analyzed by quantitative real time RT-PCR. Expression of CTGF in control cells was set to 1 at each time point. Data are means of two experiments analyzed in duplicate. Stimulation by TGF-β and inhibition by SU6656 were significant with at least $p < 0.01$, ANOVA with Tukey's multiple comparison test. (B) CTGF was precipitated in the cell culture supernatants at the 6 h time point shown in Fig. 1A. Expression of CTGF was detected by Western blot analysis. Data summarized in the graph are means ± half range of two experiments. (C) CTGF protein was detected in the cell culture supernatants of unstimulated cells incubated with 1 μM ($n = 3$) and 5 μM ($n = 9$) SU6656 for 6.5 h. $^{***}p < 0.001$, ANOVA with Dunnett's multiple comparison test. (D) Unstimulated HKC-8 cells were incubated with 5 μM SU6656 for 2.5 h ($n = 3$) and 6.5 h ($n = 5$). CTGF was detected in the cellular homogenates. $^{***}p < 0.001$ compared to controls, ANOVA with Dunnett's multiple comparison test. (E) Unstimulated HKC-8 cells were incubated with 5 μM SU6656 for 15 and 30 min. Tyrosine-phosphorylated proteins were detected in cellular homogenates by Western blotting. The blot was reprobed with an antibody directed against phospho-Src family (pTyr-416).