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Type I collagen synergistically enhances PDGF-induced smooth muscle cell proliferation through pp60^{src}-dependent crosstalk between the α2β1 integrin and PDGFβ receptor

Scott T. Hollenbeck[a,*], Hiroyuki Itoh[a], Otway Louie[a], Peter L. Faries[a,b], Bo Liu[a], K. Craig Kent[a,b,*]

[a] Columbia Weill Cornell Division of Vascular Surgery, Weill Medical College of Cornell University, USA
[b] Columbia Weill Cornell Division of Vascular Surgery, Columbia College of Physicians and Surgeons, USA

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Abstract

Smooth muscle cells (SMCs) are exposed to both platelet-derived growth factor (PDGF) and type I collagen (CNI) at the time of arterial injury. In these studies we explore the individual and combined effects of these agonists on human saphenous vein SMC proliferation. PDGF-BB produced a 5.5-fold increase in SMC DNA synthesis whereas CNI stimulated DNA synthesis to a much lesser extent (1.6-fold increase). Alternatively, we observed an 8.3-fold increase in DNA synthesis when SMCs were co-incubated with CNI and PDGF-BB. Furthermore, stimulation of SMCs with PDGF-BB produced a significant increase in ERK-2 activity whereas CNI alone had no effect. Co-incubation of SMCs with PDGF-BB and CNI resulted in ERK-2 activity that was markedly greater than that produced by PDGF-BB alone. In a similar fashion, PDGF-BB induced phosphorylation of the PDGF receptor β (PDGFRβ) and CNI did not, whereas concurrent agonist stimulation produced a synergistic increase in receptor activity. Blocking antibodies to the α2 and β1 subunits eliminated this synergistic interaction, implicating the α2β1 integrin as the mediator of this effect. Immunoprecipitation of the α2β1 integrin in unstimulated SMCs followed by immunoblotting for the PDGFRβ as well as Src family members, pp60^{src}, Fyn, Lyn, and Yes demonstrated coassociation of α2β1 and the PDGFRβ as well as pp60^{src}. Incubation of cells with CNI and/or PDGF-BB did not change the degree of association. Finally, inhibition of Src activity with SU6656 eliminated the synergistic effect of CNI on PDGF-induced PDGFRβ phosphorylation suggesting an important role for pp60^{src} in the observed receptor crosstalk. Together, these data demonstrate that CNI synergistically enhances PDGF-induced SMC proliferation through Src-dependent crosstalk between the α2β1 integrin and the PDGFRβ.

Keywords: α2β1 integrin; PDGF receptor β; Synergy; Cell proliferation; Src tyrosine kinase

Following vascular interventions, smooth muscle cells (SMCs) within the arterial wall transition from a quiescent-contractile to a synthetic-proliferative phenotype [1]. This phenotypic modulation ultimately leads to the development of intimal hyperplasia. Growth factors, inflammatory cytokines, and extracellular matrix (ECM) proteins have all been implicated as factors that incite this hyperplastic response. Many of these agonists are capable of stimulating SMC proliferation by binding surface receptors and activating intracellular signaling pathways that induce cell cycle progression [2]. Understanding the complex interactions between these various mitogens will provide greater insight into the mechanism of intimal hyperplasia.

Platelet-derived growth factor (PDGF) is a well-known SMC agonist that acts through a tyrosine kinase

* Corresponding authors. Fax: +1 212 746 5812.
E-mail addresses: sth9005@med.cornell.edu (S.T. Hollenbeck), kckent@med.cornell.edu (K.C. Kent).

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receptor. PDGF-BB, the most mitogenic of the PDGF isotypes, binds the PDGF receptor β (PDGFRβ) inducing a conformational change that results in receptor auto-phosphorylation [3]. The phosphorylated receptor then binds to intracellular signaling proteins including the Src family of non-receptor tyrosine kinases, which in turn activate downstream pathways implicated in cellular proliferation including Ras and mitogen-activated protein kinase (MAPK) [3]. MAPK proteins are serine threonine kinases that have been associated with numerous cellular activities. The most widely studied isotypes of MAPK are the extracellular signal-regulated protein kinases (ERK), ERK-1 and ERK-2. Our laboratory and others have demonstrated that growth factors such as PDGF activate MAPK. Moreover, MAPK has been shown to be essential for PDGF-induced SMC migration as well as proliferation [4].

Highly structured extracellular matrix (ECM) proteins are found in the adventitia, media, and basement membrane of normal blood vessels. Following arterial injury, the architecture of the matrix is altered both by degradation as well as synthesis of a number of matrix components [5,6]. ECM proteins occurring as both soluble monomers and polymerized fibrils are found in the injured artery and have unique abilities to regulate cellular function [7]. Moreover, there is growing evidence that a dynamic interaction occurs between SMCs and the various matrix proteins during matrix remodeling [8,9]. Matrix proteins interact with the cell via integrins, a family of cell surface, heterodimeric receptors composed of combinations of various α and β subunits [10,11]. Matrix proteins bind to the extracellular domain of integrins whereas the intracellular or cytoplasmic domain mediates intracellular signaling. Ligand binding to integrin receptors can initiate multiple intracellular events, including activation of MAPK and tyrosine phosphorylation of cytoskeletal associated proteins.

At the time of arterial injury, SMCs are simultaneously exposed to both growth factors and matrix proteins, and it is postulated that interactions between these agonists may be complex. In fact, there are numerous well-characterized examples of crosstalk between integrin and growth factor receptors [12–16]. We have demonstrated, previously, that PDGF and ECM proteins can stimulate human vascular SMC migration in a synergistic manner [16]. There is considerable overlap between the signaling cascades of both receptor types providing many potential sites of interaction. Both growth factors and integrins activate the Ras–Raf–MAPK pathway, the Rho family of GTPases, PI3K, as well as ribosomal S6 kinase [17].

Given the common pathways shared by integrins and growth factor receptors, we postulated that PDGF and ECM proteins might stimulate cellular proliferation in a synergistic manner. In these studies, we examine the effects of ECM proteins on PDGF-induced SMC proliferation. Our results demonstrate that collagen type I (CNI) synergistically enhances PDGF-induced SMC proliferation, an effect that appears to be mediated through Src-dependent crosstalk between the α2β1 integrin and the PDGFRβ.

Materials and methods

General materials. Human recombinant PDGF-BB was purchased from Upstate Biotechnologies (Lake Placid, NY). Collagen type I from calf skin, collagen type IV (CNIV), laminin, smooth muscle specific actin immunostaining kit, ethylene glycol-bis(b-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), adenosine 5′-triphosphate (ATP, disodium salt), sodium dodecyl sulfate (SDS), Nonidet-P40, and Triton X-100 were from Sigma Chemical (St. Louis, MO). Polystyrene-coated tissue culture plates were purchased from BD Falcon (Franklin Lakes, NJ). Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS), fetal bovine serum (FBS), trypsin–EDTA, penicillin/streptomycin/amphotericin B solution, L-glutamine, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes), and myelin basic protein (MBP) were from Gibco-BRL (Gaithersberg, MD). Protein A–Sepharose (PAS) was from Pharmacia Biotechnology. (Uppsala, Sweden). [methyl-^{3}H]thymidine was purchased from Dupont-NEN (Boston, MA). The enhanced chemiluminescence (ECL) system was from Amersham Life Science (Little Chalfont, UK). Mouse IgG and SU6656 were from Calbiochem (San Diego, CA).

Antibodies. Rabbit polyclonal anti-human PDGFRβ, mouse monoclonal anti-phospho-MBP, rabbit polyclonal anti-Fyn, rabbit polyclonal anti-Lyn, rabbit polyclonal anti-Yes, and mouse monoclonal anti-human pp60^{src} (clone GD11) were purchased from Upstate Biotechnologies (Lake Placid, NY). Mouse monoclonal anti-phosphotyrosine antibody (PY20) was from Transduction Laboratories (Lexington, KY). Mouse monoclonal anti-human integrin α2 (clone P1E6), β1 (clone JB1A), and α2β1 (clone JBS2) antibodies were from Chemicon International (Temecula, CA). Rabbit polyclonal antibody to the extracellular-signal related protein kinase 2 (ERK2) isotype of MAPK and rabbit polyclonal c-src (SRC-2) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Bio-Rad (Hercules, CA).

Cell culture. Human SMCs were harvested from explants of remnant portions of saphenous vein intended for coronary or peripheral arterial bypass grafting as previously described [18]. Cells were maintained at 37 °C and 5% CO_2 in DMEM supplemented with 10% FBS, 25 mM Hepes, 40 U/ml penicillin G, 40 μg/ml streptomycin, 100 ng/ml amphotericin B, and 4.8 mM L-glutamine. Cells in passages 2–5 were used for all experiments. SMC identity was verified by immunostaining with anti-human α-actin antibody.

Extracellular matrix preparation. For all experiments, ECM proteins were added to SMCs as soluble solutions. Type I collagen was solubilized in 0.1 N acetic acid at room temperature for 3 h. Type IV collagen was solubilized in 0.25% acetic acid at 8 °C for 3 h. Laminin was purchased in a Tris-buffered NaCl solution. Immediately prior to initiating experiments, soluble ECM proteins were diluted to desired concentrations in DMEM at 37 °C and then added to SMCs adherent to polystyrene coated plates. SMCs were then transferred to incubators at 37 °C for the remainder of the experiments.

Proliferation assay. Confluent SMCs in 100 mm plates were detached with 0.05% trypsin/EDTA, seeded onto polystyrene coated 24-well plates (10,000 cells/well) in 10% FBS–DMEM, and allowed to

attach overnight. SMCs were starved in serum-free DMEM for 72 h and then stimulated for 24 h with agonists as indicated. Following agonist stimulation, 2 μCi of [methyl-^{3}H]thymidine was added to each well for the remaining 24 h of the assay. Protein was precipitated with 10% trichloroacetic acid and radioactivity of incorporated [^{3}H]thymidine was determined by use of a liquid scintillation counter. In the assays with blocking integrin antibodies, quiescent SMCs were pre-incubated with 5 μg/ml of mouse monoclonal integrin antibodies or mouse IgG (as control) for 1 h, and then stimulated with designated agonists for 24 h. In the assays with SU6656, quiescent SMCs were pre-incubated with 1 μM SU6656 for 1 h and then stimulated with designated agonists for 24 h.

MAPK assay. The activity of MAPK was measured by the ability of an isotype of MAPK, ERK2, to catalyze the phosphorylation of MBP. Quiescent SMCs were stimulated with agonists for the designated period of time. Cells were rinsed twice with iced PBS and then incubated with RIPA buffer for 20 min on ice. Cell lysates were collected into microcentrifuge tubes, vortexed, and centrifuged at 4 °C for 20 min. Protein concentration was measured by a modification of the method of Lowry and equalized for all samples. Cell lysates were coincubated with an antibody against ERK2 overnight. The antibody–ERK-2 complex was conjugated to PAS for 2 h. The PAS–antibody–ERK2 conjugate was collected and washed 3 times in RIPA buffer, and twice in 150 mM NaCl/50 mM Tris–HCl. The immunoprecipitated pellet was resuspended in kinase buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$), and 8 μg MBP, and 10 μM ATP, and added to each sample. The phosphorylation of MBP by ERK2 was allowed to continue for 30 min at 30 °C. The reaction was terminated by the addition of 4× Laemmli sample buffer (250 mM Tris–HCl, pH 6.8, 8% SDS, 20% glycerol, and 10% 2-mercaptoethanol). Samples were then heated and denatured for 5 min, and then subjected to 12.5% SDS–PAGE. The phosphorylation of MBP was detected by immunoblotting with anti-phospho-MBP monoclonal antibody. The activity of MAPK was quantified by densitometry using NIH-Image software.

Immunoprecipitation. Quiescent SMCs were stimulated with designated agonists for set time periods and lysed in Nonidet P40 buffer (1% IGEPAL, 150 mM NaCl, 20 mM Tris–HCl, 2 mM PMSF, 10 μg/ml leupeptin, and 5 μg/ml aprotinin). Total protein concentration of the resulting supernatant was determined by a modification of the method of Lowry, and the protein amount of each sample was then equalized. The lysate was then pre-cleared with non-immune serum plus 50 μl PAS beads. Primary antibody was added and allowed to mix gently at 4 °C for 1 h. Precipitation of immune complexes was accomplished with the addition of 50 μl PAS beads. After 1 h of mixing followed by centrifugation, pellets were washed three times with NP-40 buffer and once with 50 mM Tris. For immunocomplex elution, the final pellet was re-suspended in 30 μl of sample buffer (1% SDS, 100 mM DTT, and 50 mM Tris) and heated to 95 °C for 3 min. Samples were then subjected to SDS–polyacrylamide gel electrophoresis (PAGE). The electrophoretically separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane, then, after blocking, incubated with primary antibodies as indicated followed by HRP-conjugated secondary antibodies. Labeled proteins were visualized using ECL. For studies designed to measure protein phosphorylation, membranes were stripped and re-blotted with an antibody against the protein. Western blot band intensity was determined using NIH image software and graphs were generated using Excel (Microsoft). PDGFRβ phosphorylation was calculated using the ratio of phosphorylated receptor to total receptor and fold increase was established by comparison to control.

Statistical analysis. All experiments were performed at least in triplicate. All values are provided as means ± standard deviation (SD). Comparison of means was made using unpaired Student's t test with Statview software (BrainPower, Calabasas, CA) on an Apple Macintosh system (Apple Computer, Cupertino, CA). For all comparisons, $p < 0.05$ was considered to indicate a significant difference.

Results

CNI synergistically enhances PDGF-BB-induced smooth muscle cell proliferation

We investigated the potential for laminin (1–50 μg/ml), CNI (1–50 μg/ml) or CNIV (1–50 μg/ml) to induce SMC proliferation. Of the ECM proteins studied, only CNI significantly enhanced tritiated thymidine incorporation. This effect was concentration dependent, first evident at 10 μg/ml (fold increase over control = 1.4 ± 0.1) and increased at 20 μg/ml (fold increase over control = 1.7 ± 0.4). The effect was most pronounced at 50 μg/ml (fold increase over control = 2.1 ± 0.5). We observed no increase in SMC proliferation when cells were stimulated with laminin or CNIV at any concentration. Although, the effect of CNI on SMC proliferation was reproducible, the overall response was small compared to our previous observations with PDGF-BB. We therefore evaluated whether CNI, in addition to its direct effect on SMC proliferation, might also synergistically enhance the response of SMCs to PDGF-BB. Thus, we stimulated SMCs with CNI (20 μg/ml) and/or PDGF-BB (5 ng/ml) for 24 h and measured tritiated thymidine incorporation. In comparison to non-stimulated SMCs, CNI alone produced a 1.6 ± 0.3-fold increase in DNA synthesis while the increase in DNA synthesis related to PDGF-BB was 5.5 ± 0.2-fold (Fig. 1). However, when SMCs were simultaneously exposed to CNI (20 μg/ml) and PDGF-BB (5 ng/ml), DNA synthesis was enhanced to a degree greater than would be anticipated by the combination of their additive effects (8.3 ± 0.9 fold actual increase versus 6-fold predicted additive effect). These data indicate

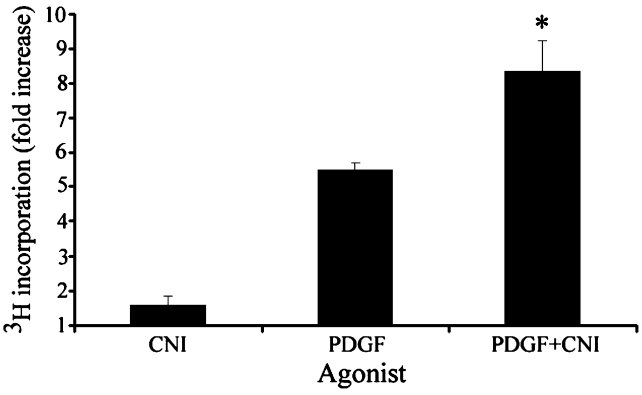

Fig. 1. CNI stimulates human vascular SMC proliferation alone and in combination with PDGF-BB. SMCs were stimulated with CNI (20 μg/ml) and/or PDGF-BB (5 ng/ml) for 24 h and DNA synthesis was assessed using tritiated thymidine incorporation during the final 4 h of treatment. Results are expressed as fold increase versus non-treated controls ± SD. Experiments were performed in triplicate and repeated in at least three cell lines from different donors (*$p < 0.05$, PDGF + CNI versus PDGF).

that CNI and PDGF-BB in combination produce a synergistic effect on SMC proliferation.

PDGF and CNI synergistically activate MAPK

To better understand the mechanism that facilitates the synergistic interaction between CNI and PDGF-BB, we evaluated the effect of these two agonists on the signaling protein, MAPK. Our laboratory and others have found that PDGF-induced human SMC proliferation is highly dependent on MAPK activation [4]. Thus, we hypothesized that the synergistic effect of CNI and PDGF-BB on SMC proliferation might occur through enhancement of MAPK activity. To test this hypothesis we evaluated the activity of an isotype of MAPK (ERK-2 kinase) following stimulation of SMCs with CNI (20 μg/ml) and/or PDGF-BB (5 ng/ml) at time points ranging from 1 to 180 min. While CNI alone had no effect on ERK-2, PDGF-BB alone produced a significant increase in ERK-2 activity at all time points tested (Fig. 2). Co-stimulation of SMCs with CNI and PDGF-BB, however, produced levels of ERK-2 activity that were significantly greater than that produced by PDGF-BB alone. This enhanced effect was most pronounced at 30 and 60 min (increase above PDGF alone $= 1.3 \pm 0.7$- and 1.2 ± 0.6-fold, respectively). These findings suggest that synergy between CNI and PDGF-BB occurs at the level of MAPK activation or more proximally.

PDGF and CNI synergistically enhance phosphorylation of PDGFRβ

We next evaluated the effect of these two agonists on PDGFRβ phosphorylation, a proximal event in the PDGF-BB signaling cascade. To assess PDGF-BB-induced receptor phosphorylation, we immunoprecipitated SMC lysates for the PDGFRβ followed by immunoblotting with an antiphosphotyrosine antibody. Stimulation with CNI (20 μg/ml) alone had no effect on receptor phosphorylation whereas PDGF-BB (5 ng/ml) produced a marked increase in phosphorylated PDGFRβ (Fig. 3). When SMCs were stimulated simultaneously with CNI and PDGF-BB, PDGFRβ tyrosine phosphorylation was significantly enhanced compared with that induced by PDGF-BB alone. This effect was evident at time points ranging from 1 to 120 min ($p < 0.05$) and most pronounced at 3, 5, and 15 min post agonist stimulation (fold increase above PDGF-BB alone $= 5.2 \pm 2$, 4.1 ± 1.9, and 5.0 ± 2.9, respectively; $p < 0.05$). Thus, the synergistic effect of CNI on PDGF-BB-induced human vascular SMC proliferation appears to occur as proximal as the PDGFRβ receptor.

Synergy between CNI and PDGF-BB requires activation of the α2β1 integrin

The ECM protein CNI is known to bind to a variety of integrins including α1β1, α2β1, α3β1, and α11β1 [19,20]. Of these four integrins, we found α2β1 to be prominently expressed in cultured human saphenous vein SMCs [21]. Therefore, we evaluated the role of α2β1 in CNI's potentiation of the PDGF-BB mitogenic signal. To accomplish this, we measured PDGFRβ tyrosine phosphorylation following stimulation with CNI and/or PDGF-BB in the presence or absence of blocking antibodies to the α2 or β1 integrin subunits. Pre-incubation of SMCs for 1 h with non-immune mouse IgG, anti-α2 or anti-β1 antibodies (5 μg/ml) did not alter PDGFRβ phosphorylation in response to PDGF-BB

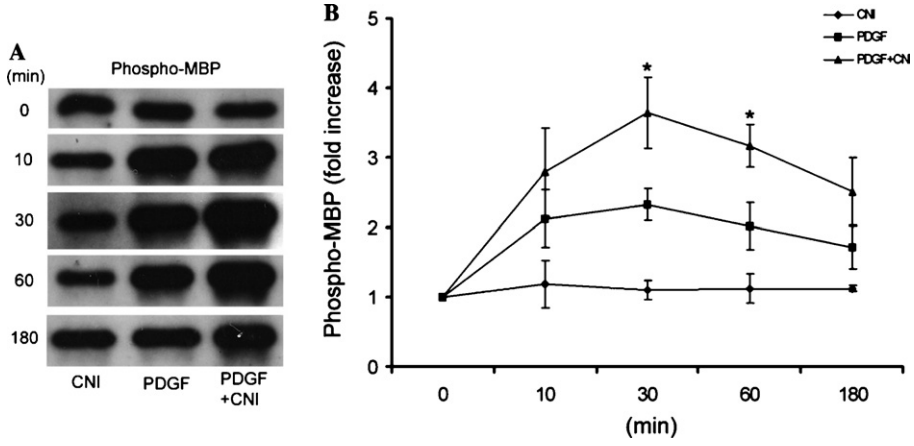

Fig. 2. Synergistic effect of CNI and PDGF-BB on ERK-2 kinase activity. (A) Human vascular SMCs were stimulated for 10, 30, 60, and 180 min with CNI (20 μg/ml) and/or PDGF-BB (5 ng/ml). ERK-2 was immunoprecipitated from cell lysates and co-incubated with its substrate, myelin basic protein (MBP), in kinase buffer. Phosphorylated MBP (Phospho-MBP) was assessed using western blotting with an anti-phosphorylated MBP antibody. The figure is a representative Western blot from these experiments. Membranes were stripped and probed with an anti-MBP antibody and no changes in protein levels were observed. (B) The graph represents a densometric analysis of the mean fold increase ± SD in MBP phosphorylation versus control ($N = 3$; using cells from different donors) (*$p < 0.05$, PDGF + CNI versus PDGF).

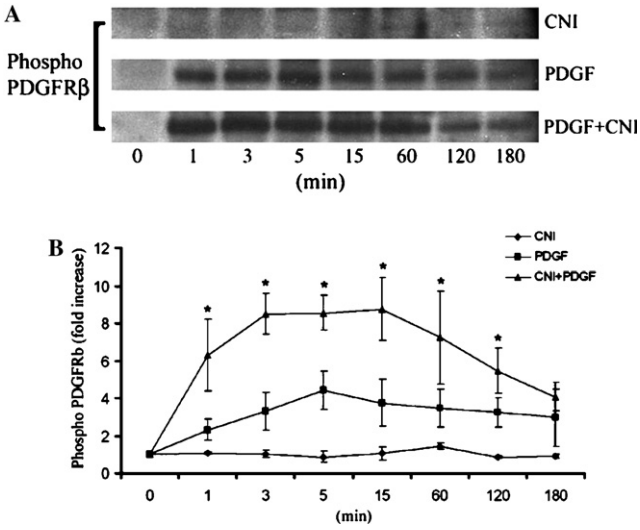

Fig. 3. Synergistic effect of CNI and PDGF-BB on autophosphorylation of PDGFRβ. (A) Human vascular SMCs were stimulated with CNI (20 μg/ml) and/or PDGF-BB (5 ng/ml) for 1, 3, 5, 15, 60, 120, and 180 min. PDGFRβ was immunoprecipitated from cell lysates and tyrosine phosphorylated PDGFRβ (phospho-PDGFRβ) was assessed by Western blotting with an anti-phosphotyrosine antibody. This figure is a representative Western blot. Membranes were stripped and probed with an anti-PDGFRβ antibody and no changes in protein levels were observed. (B) The graph represents a densometric analysis of the mean fold increase ± SD in PDGFRβ tyrosine phosphorylation versus control ($N = 3$; using cells from different donors) (*$p < 0.05$, PDGF + CNI versus PDGF).

(5 ng/ml) (Fig. 4). However, pre-incubation of SMCs for 1 h with blocking antibodies to either α2 or β1 completely eliminated the synergistic effect of CNI (20 μg/ml) on PDGF-BB-induced PDGFRβ phosphorylation. In parallel studies, pre-incubation of SMCs with blocking antibodies to α2 or β1 eliminated the synergistic effect of CNI on PDGF-BB-induced SMC DNA synthesis (data not shown). These findings indicate that the synergistic effect of CNI on PDGF-BB-induced phosphorylation of PDGFRβ is dependent on both the α2 and the β1 integrin subunits, thus implicating the collagen receptor α2β1 as the mediator of the interaction between CNI and PDGF-BB.

There is a coassociation between α2β1 and PDGFRβ

We next wished to identify the mechanism that enabled crosstalk between α2β1 and PDGFRβ. We first examined whether there might be a physical association between these two receptors. Unstimulated SMCs were lysed and immunoprecipitated with antibodies against either the α2β1 or PDGFRβ receptors followed by Western blotting for the PDGFRβ or the α2β1 integrin, respectively. We found a prominent association between the PDGFRβ and the α2β1 integrin under basal conditions (Figs. 5A and B). Exposure of SMCs to CNI (20 μg/ml) or PDGF-BB (5 ng/ml) individually or in

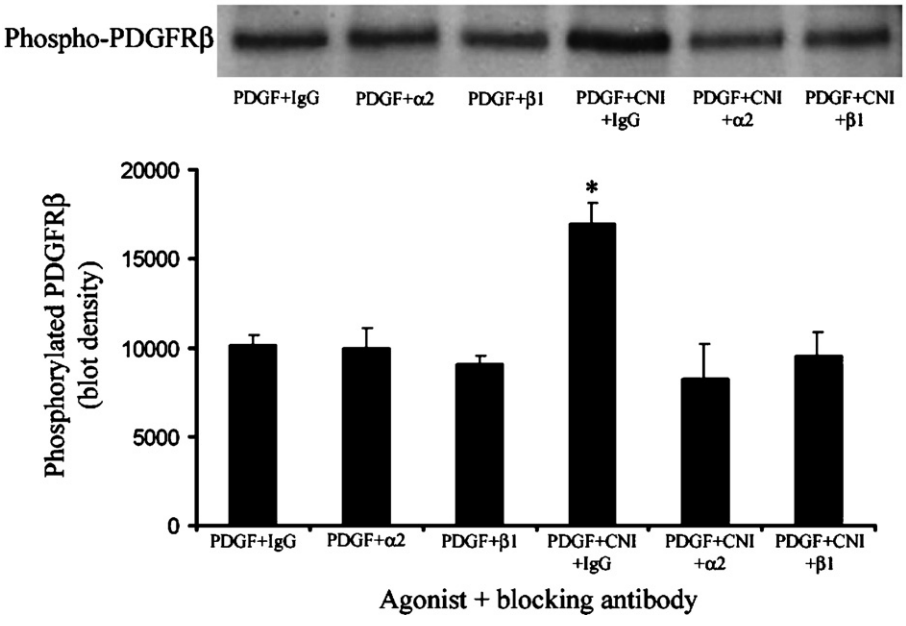

Fig. 4. Blocking antibodies to the integrin subunits α2 and β1 inhibit the synergistic effect of CNI and PDGF-BB on PDGFRβ phosphorylation. Human vascular SMCs were preincubated for 1 h with 5 μg/ml of either a blocking antibody to the α2 subunit, the β1 subunit, or mouse IgG and then stimulated with CNI (20 μg/ml) and/or PDGF-BB (5 ng/ml) for 15 min. PDGFRβ was immunoprecipitated and phosphorylated PDGFRβ was assessed with Western blotting using an anti-phosphotyrosine antibody. The upper figure is a representative Western blot from these experiments. Membranes were stripped and probed with an anti-PDGFRβ antibody and no changes in protein levels were observed. The graph represents a densometric analysis of the mean ± SD levels of PDGFRβ phosphorylation ($N = 3$, using cells from three different donors) (*$P < 0.05$, PDGF + CNI + IgG versus all other combinations).

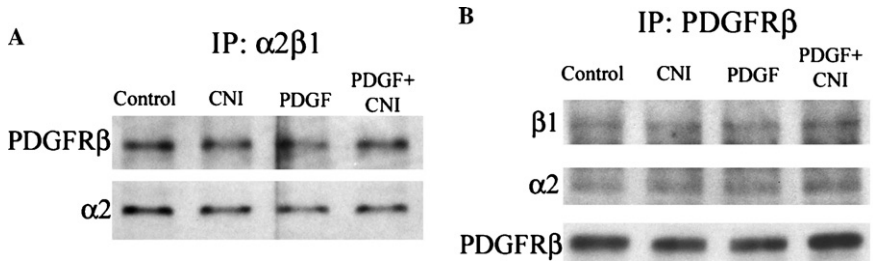

Fig. 5. Coassociation of the α2β1 and the PDGFR β. (A) The α2β1 integrin was immunoprecipitated and the PDGFRβ was assessed with Western blotting using an anti-PDGFRβ antibody, in human vascular SMCs stimulated with CNI (20 μg/ml) and/or PDGF-BB (5 ng/ml) for 15 min. Membranes were stripped and probed with anti-α2 integrin subunit antibody to establish equal loading. (B) The PDGFR β was immunoprecipitated, and β1 and α2 integrin subunits were assessed with Western blotting in human vascular SMCs stimulated with CNI (20 μg/ml) and/or PDGF-BB (5 ng/ml) for 15 min. Membranes were stripped and probed with anti-PDGFRβ antibody to establish equal loading. Figures are representative examples ($N = 3$, using cells from three different donors).

combination, however, did not further enhance the association between these proteins.

Src kinase activity is required for CNI enhancement of PDGF-BB-induced PDGFRβ phosphorylation

It is well established that the Src family of non-receptor tyrosine kinases plays an important role in signaling for both the PDGFRβ as well as the α2β1 integrin [22,23]. Thus, we explored whether Src kinase activity might be responsible for the observed synergy between α2β1 and PDGFRβ. To accomplish this, we measured tyrosine phosphorylation of the PDGFRβ following stimulation of SMC with CNI and/or PDGF-BB in cells pre-treated with or without the Src family specific inhibitor SU6656. Pretreatment of SMCs for 1 h with SU6656 (1 μM) did not alter the effect of PDGF-BB (5 ng/ml) on PDGFRβ tyrosine phosphorylation (Fig. 6). However, SU6656 completely eliminated the synergistic effect of CNI on PDGF-BB-induced PDGFRβ tyrosine phosphorylation. These data suggest that in vascular SMCs, the Src family of kinases plays a critical role in the crosstalk between α2β1 and PDGFRβ.

pp60^{src} (but not Fyn, Lyn or Yes) is the Src family member that coassociates with α2β1

The previous experiments imply that at least one member of the Src family is responsible for the crosstalk between α2β1 and the PDGFRβ. We postulated that the Src family member responsible for this interaction would coassociate with the α2β1 collagen receptor. SMCs were stimulated with CNI (20 μg/ml) and/or PDGF-BB (5 ng/ml), and lysates were immunoprecipitated with an antibody against the α2β1 integrin. These immunoprecipitates were then evaluated with Western blotting for pp60^{src}, Fyn, Lyn, Yes or PDGFRβ (Fig. 7). Only pp60^{src} was found to coassociate with the α2β1 integrin. This coassociation was present in unstimulated cells and the level of association did not change when SMCs were stimulated with PDGF-BB and or CN1. These data suggest that pp60^{src}, rather than

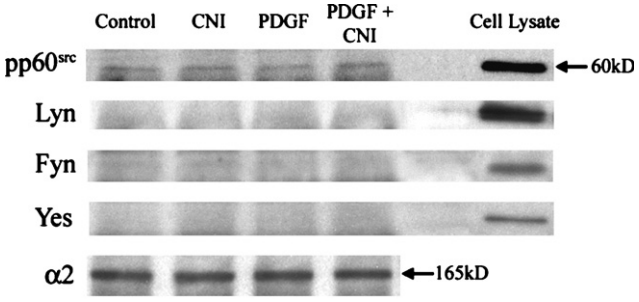

Fig. 7. CNI and PDGFRβ coassociate with pp60^{src} but not Lyn, Fyn or Yes. Human vascular SMCs were stimulated with CNI (20 μg/ml) and/or PDGF-BB (5 ng/ml) for 15 min followed by immunoprecipitation of cell lysates with an antibody to the α2β1 integrin. Western blotting of immunoprecipitates was then performed with the Src family tyrosine kinases; pp60^{src}, Lyn, Fyn, and Yes. Membranes were stripped and re-blotted with an antibody to the α2 subunit to establish equal loading. Cell lysates were used to demonstrate the presence of these various proteins in vascular SMC. The figure is a representative example ($N = 3$, using cells from 3 different donors).

Fig. 6. The Src kinase inhibitor (SU6656) inhibits CNI enhancement of PDGFRβ phosphorylation. Human vascular SMCs were pre-incubated for 1 h with or without SU6656 (1 μM) and stimulated with CNI (20 μg/ml) and/or PDGF-BB (5 ng/ml) for 15 min. PDGFRβ was immunoprecipitated followed by Western blotting with an anti-phosphotyrosine antibody. The figure is a representative example ($N = 3$, using cells from 3 different donors). Membranes were stripped and probed with an anti-PDGFRβ antibody and no changes in protein levels were observed.

Fyn, Lyn or Yes, is the Src family protein responsible for crosstalk between α2β1 and PDGFRβ.

Discussion

We have characterized a novel synergistic interaction between CNI and PDGF-BB in promoting human vascular SMC proliferation. Our data demonstrate that the signaling pathways activated by PDGF-BB that lead to proliferation of vascular SMC are enhanced by CNI via an interaction that involves the α2β1 integrin. In sum, the α2β1 integrin in an interaction mediated by $pp60^{src}$ produces synergistic enhancement of PDGFRβ tyrosine phosphorylation as well as activation of ERK2 and consequently enhanced SMC proliferation.

There is substantial evidence that cellular functions can be highly regulated by surrounding matrix proteins. Furthermore, matrix composition may be altered during disease states and these changes can have a direct impact on cell signaling and consequently the ability of cells to proliferate, migrate or undergo apoptosis [13,24]. Following arterial balloon injury, medial SMCs undergo phenotypic modulation and migrate into the neointima [5]. This process is influenced by both growth factors and ECM proteins. There is evidence that CNI, present in arterial lesions, can exist in both soluble monomeric and insoluble fibrillar forms [7–9,25]. Within the media and neointima, synthetic SMCs produce soluble procollagen which undergoes proteolytic cleavage yielding monomeric collagen, the precursor of insoluble or fibrillar collagen [6,8,26]. In addition to CNI synthesis, SMCs produce interstitial collagenases which have the ability to degrade fibrillar collagen into smaller fragments [27,28]. The development of intimal hyperplasia is highly dependent on the function of these collagenases [29,30]. Thus, following arterial injury, SMCs are exposed to a variety of forms of CNI as well as its byproducts.

There is an increasing awareness that the state or form of CNI may determine its effect on SMC behavior [8]. In our studies we repeatedly found that soluble CNI enhanced proliferation of human saphenous vein SMCs. Similarly, Wilson et al. [31] demonstrated a 33% increase in basal DNA synthesis in rat aortic SMCs cultured on CNI compared to SMCs cultured on plastic or pronectin. CNI also appears to support proliferation of rat embryonic vascular SMCs subjected to mechanical strain [32]. Alternatively, Koyama et al. [24] have shown that human embryonic aortic SMCs cultured on insoluble fibrillar CNI remain in a quiescent state, even after the addition of growth factors. Importantly, the effect of CNI on SMCs appears to be dependent upon the composition of CNI, as Koyama also found that monomeric CNI stimulated SMC proliferation. In an attempt to more closely mimic the in vivo situation associated with arterial injury, we have added soluble CNI to adherent SMCs. We found, contrary to Koyama's observation with insoluble fibrillar CNI, that soluble CNI alone stimulates SMC proliferation and soluble CNI also greatly enhances the effect of PDGF-BB on SMC proliferation. In sum, these findings suggest that in the uninjured artery, SMCs are held in quiescence by organized fibrillar CNI. However, following arterial injury, soluble CNI is a very potent stimulus of SMC proliferation.

We did not explore the signaling mechanism by which CNI alone produces SMC proliferation. However, it is well established that integrins, independent of growth factors, can activate multiple cellular pathways. Pozzi et al. demonstrated that α1 integrin null fibroblasts following stimulation with collagen matrices were not able to activate MAPK. Moreover, compared to wild type controls, α1 null fibroblasts had a significant reduction in CNI-induced proliferation, suggesting a role for the α1 subunit in activation of mitogenic signaling [33]. In the present study, CNI alone did produce an increase in SMC proliferation, albeit minor compared to that of PDGF. However, activation of the α2β1 integrin by CNI alone did not increase MAPK activity. Thus, an alternative pathway must be used by CNI to stimulate proliferation of vascular SMCs. CNI and/or the α2β1 integrin have been shown in several cell types to activate a number of other signaling proteins associated with proliferation including the Src family of kinases, focal adhesion kinase, and the adaptor protein Shc [13]. Which of these pathways is required for CNI proliferation requires further exploration.

Interactions between integrins and growth factors can be complex. There are many examples where integrins have been found to be necessary for growth factor signaling. A well-characterized example of integrin/growth factor receptor interaction is the relationship between αVβ3 and PDGFRβ. In fibroblasts cultured on vitronectin, αVβ3 synergistically increases PDGF-BB-induced chemotaxis and proliferation [12,34]. Further defining a relationship between αVβ3 and PDGFRβ, Borges et al. found in endothelial cells that the αVβ3 integrin coassociates with the PDGFRβ. Furthermore, when cells plated on fibronectin were transfected with the β3 subunit, chemotaxis in response to PDGF-BB was further enhanced suggesting crosstalk between β3 and PDGFRβ [35]. Sundberg et al. have previously demonstrated in fibroblasts a relationship between the β1 integrin subunit and PDGFRβ. In the absence of PDGF, both CNI and an enhancing antibody to β1 were capable of inducing PDGFRβ tyrosine phosphorylation [36]. We have now identified in human vascular SMCs a relationship between α2β1 and PDGFRβ that requires the presence of both CNI and PDGF-BB. This relationship may have clinical significance in that CNI through this interaction can potentiate the already profound effect of PDGF-BB on SMC proliferation.

Two general mechanisms have been described by which integrins can regulate growth factors (collaborative and direct activation) [37]. Direct activation occurs when a ligand bound integrin directly phosphorylates a growth factor receptor, in the absence of the growth factor ligand, leading to receptor activation and the consequent physiologic response [37]. Alternatively, collaborative activation requires simultaneous binding of both the ECM and growth factor ligands, and is also characterized by enhanced growth factor receptor phosphorylation and activity. There are multiple examples of both mechanisms. Borges et al. [35] found in porcine aortic endothelial cells that chemotaxis and mitogenicity on vitronectin surfaces result from an interaction between αVβ3 and PDGFRβ that does not require PDGF-BB binding. Conversely, in fibroblasts, migration and proliferation are dependent upon an interaction between αVβ3 and PDGFRβ but this response is dependent upon costimulation with both vitronectin and PDGF-BB. In the case of human vascular SMCs, CNI bound α2β1 enhances PDGFRβ phosphorylation only in the presence of PDGF-BB, suggesting a collaborative activation. Stimulation of SMC with CNI alone did not activate the PDGFRβ.

The mechanism through which integrins and growth factor receptors interact may vary with the ECM protein, the growth factor as well as the specific cell studied. What appears to be a consistent finding in receptor crosstalk is the necessity of a physical association or co-clustering between two receptors. Co-clustering may occur transiently in response to ligands. In this setting, aggregation of receptors leads to conformational changes that stimulate intrinsic receptor kinase activity [13]. Moro et al. [38] have shown that β1 or αV activation can produce partial activation of the EGF receptor, independent of the EGF ligand, through intrinsic receptor kinase activity. In a similar fashion, Miagkova et al. [39] have demonstrated that CNI activated β1 integrins can alter the oligomerization of the RON receptor tyrosine kinase, initiating receptor autophosphorylation independent of ligand binding. Alternatively, integrin and growth factor receptors can coassociate under basal conditions without activation. In this circumstance, crosstalk is dependent upon ligand/receptor induced activation of intracellular kinases [13]. In our studies we have found that co-clustering between the α2β1 integrin and PDGFRβ occurs independent of either CNI or PDGF-BB and is not enhanced by ligand binding, suggesting that an intracellular kinase may be necessary for receptor crosstalk.

The Src family of non-receptor tyrosine kinases has been proposed as a mediator of collaborative receptor crosstalk. It is well established that following PDGF stimulation, Src family proteins associate with the PDGFRβ [40] as part of the signaling pathway that mediates PDGF-induced mitogenicity [41]. Moreover, Src activity has also be shown to be required for fibronectin-induced tyrosine phosphorylation of a number of focal adhesion proteins, indicating that Src is an important signaling protein for integrins [42]. The forgoing observations prompted us to examine the role of Src in the collaborative interaction between α2β1 and PDGFRβ. First, we found that the synergistic enhancement in tyrosine phosphorylation of PDGFRβ in response to CNI was completely eliminated by inhibition of Src family of kinases, suggesting that α2β1/PDGFRβ crosstalk is significantly dependent upon the activity of a member of the Src family. Human vascular SMCs express multiple Src family kinases including $pp60^{src}$, Fyn, Lyn, and Yes. We postulated that the Src kinase family member responsible for this crosstalk would coassociate with the α2β1 integrin. Of the four most prominently expressed Src family members, only $pp60^{src}$ was found to associate with the α2β1, strongly indicating that this particular Src kinase is responsible for α2β1/PDGFRβ crosstalk. Moro et al. [43] have described a similar role for the Src family of kinases in αVβ3/EGF receptor crosstalk, however, the specific Src family member that mediated this interaction was not identified.

Signaling proteins other than Src have been identified as mediators of integrin/growth factor crosstalk [44]. For example in fibroblasts, fibronectin enhanced the mitogenic effect of PDGF by increasing SHP-2 interaction with PDGFB [45]. Thus, there are several intracellular proteins that may serve as mechanisms of communication between integrins and growth factor receptors.

Evaluating the isolated effect of growth factors and integrins on cellular function is naíve since the in vivo environment is complex and cells are exposed simultaneously to multiple agonists. Following arterial injury, new matrix proteins are produced while these and existing structural proteins are degraded. Moreover, there are changes in the expression of growth factors and their receptors creating an environment that favors multiple integrin-growth factor receptor interactions. From our studies, it seems evident that the α2β1 integrin has a distinct role as a collaborator with the PDGFRβ allowing CNI to synergistically enhance the effect of PDGF-BB on SMC proliferation. Understanding this complex relationship may better guide attempts to inhibit the factors that lead to restenosis following angioplasty or arterial bypass.

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