Identification of integrin αMβ2 as an adhesion receptor on peripheral blood monocytes for Cyr61 (CCN1) and connective tissue growth factor (CCN2): immediate-early gene products expressed in atherosclerotic lesions


Cysteine-rich 61 (Cyr61, CCN1) and connective tissue growth factor (CTGF, CCN2) are growth factor–inducible immediate-early gene products found in blood vessel walls and healing cutaneous wounds. We previously reported that the adhesion of endothelial cells, platelets, and fibroblasts to these extracellular matrix–associated proteins is mediated through integrin receptors. In this study, we demonstrated that both Cyr61 and CTGF are expressed in advanced atherosclerotic lesions of apolipoprotein E–deficient mice. Because monocyte adhesion and transmigration are important for atherosclerosis, wound healing, and inflammation, we examined the interaction of THP-1 monocytic cells and isolated peripheral blood monocytes with Cyr61 and CTGF. THP-1 cells and monocytes adhered to Cyr61- or CTGF-coated wells in an activation-dependent manner and this process was mediated primarily through integrin αMβ2. Additionally, expression of αMβ2 on human embryonic kidney 293 cells resulted in enhanced cell adhesion to Cyr61. Consistent with these data, a GST-fusion protein containing the I domain of the integrin αM subunit bound specifically to immobilized Cyr61 or CTGF. We have also investigated the requirement of cell surface heparan sulfate proteoglycans (HSPGs) as coreceptors for monocyte adhesion to Cyr61. Pretreatment of monocytes with heparin or heparinase I resulted in partial inhibition of cell adhesion to Cyr61. However, monocytes, but not fibroblasts, were capable of adhering to a Cyr61 mutant deficient in heparin binding activity. Collectively, these results show that activated monocytes adhere to Cyr61 and CTGF through integrin αMβ2 and cell surface HSPGs. However, unlike fibroblast adhesion to Cyr61, cell surface HSPGs are not absolutely required for this adhesion process.

Introduction

Cysteine-rich 61 (Cyr61) and connective tissue growth factor (CTGF) belong to the Cyr61/CTGF/nephroblastoma-overexpressed (CCN) family of matricellular signaling molecules capable of mediating diverse biologic functions. Other members in this protein family include Nov, WISP-1, WISP-2, and WISP-3. These proteins are characterized by the presence of an N-terminal secretory signal, followed by 4 conserved structural domains, which include: (1) an insulinlike growth factor–binding protein homology domain, (2) a von Willebrand factor type C domain, (3) a thrombospondin type 1 repeat homology domain, and (4) a C-terminal domain with sequence similarities to growth factor cysteine knots. It has been suggested that the C-terminal domain may mediate protein-protein interaction. Moreover, 2 putative heparin-binding motifs are present in the C-terminal domain of Cyr61 and mutations of the basic amino acid residues in these motifs abolish heparin-binding affinity of Cyr61. WISP-2 and its human homolog are unique in that they lack the C-terminal domain.

Both Cyr61 and CTGF have been identified as products of immediate-early genes that are transcriptionally induced in fibroblasts in response to serum growth factors. On synthesis, they are secreted and become associated with the cell surface and the extracellular matrix, suggesting that these proteins may mediate cell-matrix interaction. In functional studies, both Cyr61 and CTGF have been shown to support cell adhesion, induce cell migration, and augment growth factor–induced cell proliferation in vitro and induce angiogenesis in vivo. Consistent with the adhesive properties of these proteins, 2 other family members, WISP-2 and Nov, have also been found to promote the adhesion of osteoblasts and vascular smooth muscle cells, respectively. Recently, we have identified several integrins, namely α1β1, α1β3, α1β5, α1β6β3, and α5β1, as cellular receptors mediating interaction of different cell types with Cyr61 and CTGF. In addition, cell surface heparan sulfate proteoglycans (HSPGs) are also required for α5β1-mediated adhesion of human skin fibroblasts to Cyr61. Identification of integrins as signaling receptors for Cyr61 and CTGF accounts for most, if not all, of the cellular and biologic activities of these extracellular matrix–associated proteins.

Immunohistochemical studies have localized both Cyr61 and CTGF in the cardiovascular system of developing mouse embryos and adult mice. Interestingly, CTGF has been shown to be...
overexpressed in human advanced atherosclerotic lesions as compared to normal blood vessels. High expression of CTGF has also been observed under diverse pathologic conditions, suggesting that it may play an important role in various diseases such as systemic sclerosis, renal fibrosis, and hepatic atresia. During cutaneous wound healing, the expression of Cyr61 and CTGF is up-regulated, and therefore these proteins may function downstream of transforming growth factor-β and fibroblast growth factor, strong inducers of Cyr61 and CTGF, in wound repair. Because monocyte adhesion and transendothelial migration play a central role in inflammation, atherosclerosis, and wound healing, we sought to examine the interaction of THP-1 cells and peripheral blood monocytes with Cyr61 and CTGF. In this study, we demonstrated that in addition to CTGF, Cyr61 is expressed in atherosclerotic lesions of apolipoprotein E-deficient (apoE−/−) mice. Moreover, monocytes adhere to both Cyr61 and CTGF, and this process is mediated through integrin α5β2 and cell surface HSPGs. Thus, these findings identified Cyr61 and CTGF as novel ligands for integrin α5β2, underscoring the importance of these proteins in the pathophysiological function of monocytes.

Materials and methods

Antibodies and peptides

The anti-α5β2 monoclonal antibodies 2LP19c and 44a were obtained from Dako (Carpinteria, CA) and Sigma (St. Louis, MO), respectively. The anti-α5β2 (LM609), anti-α6 (C8B-2), anti-β3 (6S6) and anti-β2 (YFC118.3) function-blocking monoclonal antibodies were purchased from Chemicon (Temecula, CA). An antithrombiline S-transferrase (anti-GST) polyclonal antibody raised in goats was from Amersham Pharmacia Biotech (Piscataway, NJ) and a rabbit antigoat IgG conjugated to horseradish peroxidase (HRP) was from Sigma. Polyclonal anti-Cyr61 and anti-CTGF (Piscataway, NJ) and a rabbit antigoat IgG conjugated to horseradish peroxidase (HRP) was from Sigma. Polyclonal anti-Cyr61 and anti-CTGF antibodies were raised in rabbits and affinity purified as described previously. An antipeptide antibody (anti-Cyr61 867-881), raised in rabbits against a peptide sequence corresponding to the extreme C-terminus of Cyr61 (FPST-PPYRLFNPDKHFRD89), single-letter amino acid codes) was affinity purified against the peptide. Echistatin, an RGD-containing snake fi, the region coding for the human Brie previously. An antipeptide antibody (anti-Cyr61 367-381), raised in rabbits and affinity purified as described previously.

Protein purification

Recombinant Cyr61 and CTGF, synthesized in a baculovirus expression system using Sf9 insect cells, were purified using a serum-free conditioned media by chromatography on Sepharose S as previously described. A Cyr61 mutant (Cyr61-DM) with alanine substitutions of the basic amino acid residues within the 2 putative heparin-binding motifs at residues 280-290 and residues 306-312 was constructed by splice-overlap extension mutagenesis as previously described.

Recombinant I domain of the integrin-α5 subunit was expressed as a fusion protein with GST (GST-α5I) and purified as previously described. Briefly, the region coding for the human α5 I domain sequence D102-A118 was amplified and inserted in the expression vector pGEX-4T-1 (Amersham Pharmacia Biotech). To express GST-α5I, BL-21 Escherichia coli cells were transformed with the above vector and protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 4 hours at 37°C. The GST-α5I fusion protein was affinity purified from cell lysates using glutathione-agarose (Sigma).

Binding assay of GST-α5I fusion protein to immobilized Cyr61 and CTGF

Microtiter wells were coated with Cyr61 or CTGF for 24 hours at 4°C and blocked with 0.1% polyvinyl alcohol (PVA, Sigma) for 30 minutes at 37°C and cell adhesion proceeded as described above. Adherent cells were quantified using the acid phosphatase assay by incubation with the substrate solution (0.1 M sodium acetate, pH 5.5, 10 mM p-nitrophenyl phosphate, and 0.1% Triton X-100; 100 μL/well) for 2 hours at 37°C. The reaction was stopped by the addition of 15 μL 1 N NaOH and A405 was measured. In inhibition studies, monocytes were preincubated with antibodies, peptides, or EDTA for 30 minutes at 37°C prior to addition to microtiter wells. The α5β2-expressing human embryonic kidney 293 cells used in the adhesion assay have been previously described. In cell adhesion experiments, microtiter wells were coated with 1 μg/mL Cyr61 and blocked with 0.5% polyvinylpyrrolidone (Sigma). The cells were labeled with Na235/CrO4 (0.5 μCi/mL [18.5 MBq/mL]), and the adhesion of 35/Cr-labeled cells to Cyr61-coated wells was performed as described.

Isolation of peripheral blood monocytes

Acid-citrate-dextrose anticoagulated human blood was collected from healthy donors and centrifuged at 200g for 20 minutes. After removal of the platelet-rich plasma, theuffy coat and packed red cells were diluted 2-fold with phosphate-buffered saline (PBS; 10 mM sodium phosphate, pH 7.35, 0.15 M NaCl) and 25 mL of the cell suspension was layered onto 20 ml Ficoll-Paque (Amersham Pharmacia Biotech). Mononuclear cells were isolated by centrifugation through Ficoll-Paque at 400g for 60 minutes at 4°C, diluted with an equal volume of PBS containing 2 mM EDTA, and sedimented at 400g for 10 minutes. To remove residual platelets, the mononuclear cells were washed twice with modified Tyrode buffer (10 mM Hepes, pH 7.35, 135 mM NaCl, 2.9 mM KCl, 12 mM NaHCO3, 1 mM MgCl2, 1 mM CaCl2, 0.1% dextrose and 0.2% bovine serum albumin (BSA)) by centrifugation at 130g for 10 minutes. To separate lymphocytes from monocytes, the mononuclear cells were resuspended in modified Tyrode buffer and subjected to discontinuous density gradient centrifugation on Percoll (Amersham Pharmacia Biotech). Peripheral blood monocytes were isolated between Percoll density of 1.047 and 1.050 g/mL, washed twice with modified Tyrode buffer, and resuspended to a final concentration of 2 to 3 x 106 cells/mL. The purity of the monocyte preparations was more than 80% as measured by anti-CD14 (Sigma) staining in flow cytometry, and cell viability was more than 98% as judged by trypan blue exclusion.

Cell culture and cell adhesion assay

The THP-1 cells (American Type Culture Collection, Rockville, MD) were maintained in RPMI 1640 media (Mediatech, Herndon, VA) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1% nonessential amino acid solution, 100 μM β-mercaptoethanol, 1.5 g/L NaHCO3, and antibiotics. Cells were grown to 1 x 106/mL and serum starved for 24 hours prior to cell adhesion experiments. Microtiter wells (Immulon 2 Removewell strips, Dynex Technologies, Chantilly, VA) were coated with 10 μg/mL Cyr61 or CTGF for 2 hours at 37°C and blocked with 1% BSA for 1 hour at 37°C. THP-1 cells, suspended in modified Tyrode buffer containing 0.1% BSA and 1 mM CaCl2, were added to the wells (100 μL/well) and incubated for 20 minutes at 37°C. Nonadherent cells were removed by washing and adherent cells were fixed and stained with 1% methylene blue. Cellular dye was extracted with acid ethanol and cell adhesion was quantified by A650 as described.

For the adhesion of peripheral blood monocytes, Cyr61-coated wells were blocked with 0.15% polyvinyl alcohol (PVA, Sigma) for 30 minutes at 37°C and cell adhesion proceeded as described above. Adherent cells were quantified using the acid phosphatase assay by incubation with the substrate solution (0.1 M sodium acetate, pH 5.5, 10 mM p-nitrophenyl phosphate, and 0.1% Triton X-100; 100 μL/well) for 2 hours at 37°C. The reaction was stopped by the addition of 15 μL 1 N NaOH and A405 was measured. In inhibition studies, monocytes were preincubated with antibodies, peptides, or EDTA for 30 minutes at 37°C prior to addition to microtiter wells. The α5β2-expressing human embryonic kidney 293 cells used in the adhesion assay have been previously described. In cell adhesion experiments, microtiter wells were coated with 1 μg/mL Cyr61 and blocked with 0.5% polyvinylpyrrolidone (Sigma). The cells were labeled with Na235/CrO4 (0.5 μCi/mL [18.5 MBq/mL]), and the adhesion of 35/Cr-labeled cells to Cyr61-coated wells was performed as described.

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Immunohistochemistry studies
Retired breeders of apoE<sup>−/−</sup> mice were obtained from Jackson Laboratory (Bar Harbor, ME) and killed by cervical dislocation under ether anesthesia. Their hearts with aortas were removed, fixed in 4% formaldehyde, frozen, and sectioned at a thickness of 10 μm on a cryostat. Sectioning began at the juncture of the aorta to the heart and continued toward the aortic arch covering a distance of 400 to 500 μm. The frozen sections were collected onto poly-L-lysine-coated coverslips and advanced atherosclerotic lesions were located by light microscopy. The sections were treated with 0.03% H<sub>2</sub>O<sub>2</sub> to inactivate endogenous peroxidase, blocked with 3% normal goat serum, and incubated with anti-Cyr61 and anti-CTGF antibodies for 1 hour at 22°C. After washing, bound antibodies were detected with a biotinylated goat antirabbit antibody followed by the avidin-biotin-peroxidase labeling system using diaminobenzidine as the substrate. The sections were counterstained with hematoxylin and eosin. Intense staining for Cyr61 was observed primarily in the subintimal regions of the lesion (Figure 1B, arrows). Similarly, CTGF was stained positively in the subintimal regions (Figure 1C, arrows) and also to a lesser degree just medial to the external elastic membrane (Figure 1C, arrowheads). The coronary artery also developed lesions with eccentric thickening of the intima and media, which were also stained positively with both antibodies (Figure 1E,F). By contrast, control sections incubated with normal rabbit IgG displayed minimal staining (Figure 1A,D).

To demonstrate further the specificity of Cyr61 expression in atherosclerotic lesions, immunohistochemistry was performed with an antipeptide antibody (anti-Cyr61<sub>367-381</sub>) directed against the C-terminus of Cyr61, a region of unique sequence identity in Cyr61. On immunoblots, anti-Cyr61<sub>367-381</sub> reacted with as little as 10 ng Cyr61 with no cross-reactivity with as much as 1 μg CTGF (data not shown). As expected, intense staining of the mouse lesions was observed with anti-Cyr61<sub>367-381</sub> (Figure 2B), and pretreatment of the antipeptide antibody with Cyr61 protein completely abolished this staining (Figure 2C). Together, these results indicate that both Cyr61 and CTGF are expressed in atherosclerotic lesions of apoE<sup>−/−</sup> mice.

Activation-dependent adhesion of THP-1 cells and peripheral blood monocytes to Cyr61 and CTGF
We previously reported that Cyr61 and CTGF induce cell adhesion and migration through interaction with integrin receptors. Because monocyte/macrophage-derived foam cells are present in subintimal regions of atherosclerotic lesions where the expression of Cyr61 and CTGF are prominent, we examined possible interaction of these mononuclear blood cells with Cyr61 and CTGF. In initial studies, cultured THP-1 mononuclear cells were used to establish optimal conditions for cell adhesion to these proteins. In these experiments, THP-1 cells were activated with 20 μM adenosine diphosphate (ADP) or 20 nM phorbol myristate acetate (PMA) and allowed to adhere to microtiter wells coated with recombinant Cyr61 or CTGF. As controls, cell adhesion to regions of these proteins. On immunoblots, anti-Cyr61 and anti-CTGF reacted specifically with Cyr61 and CTGF, respectively, and no cross-reactivity was detected. Intense staining for Cyr61 was observed primarily in the subintimal regions of the lesion (Figure 1B, arrows). Similarly, CTGF was stained positively in the subintimal regions (Figure 1C, arrows) and also to a lesser degree just medial to the external elastic membrane (Figure 1C, arrowheads). The coronary artery also developed lesions with eccentric thickening of the intima and media, which were also stained positively with both antibodies (Figure 1E,F). By contrast, control sections incubated with normal rabbit IgG displayed minimal staining (Figure 1A,D).

Results
Immunohistochemical localization of Cyr61 and CTGF in advanced atherosclerotic lesions in apoE<sup>−/−</sup> mice
It has been reported that CTGF is overexpressed in advanced atherosclerotic lesions in humans, which is likely due to growth factor stimulation of endothelial cells, aortic smooth muscle cells and fibroblasts at the lesion sites. Because Cyr61 is also transcriptionally induced in fibroblasts in response to serum growth factors, we examined whether the expression of Cyr61 is up-regulated in advanced atherosclerotic lesions. In these studies, we used the apoE<sup>−/−</sup> mice, which have been shown to develop advanced atherosclerotic lesions similar to those found in humans. Frozen sections of the mouse atherosclerotic lesions were taken through the midpoint of the aortic valve with the lesion occupying approximately 95% of the circumference of the aortic wall. As shown in Figure 1, panels A through C, both the subendothelial intimal space and the media were markedly thickened exhibiting characteristics of human atherosclerotic lesions. To examine the expression of Cyr61 and CTGF in the mouse lesions, immunohistochemical staining was performed with anti-Cyr61 and anti-CTGF polyclonal antibodies. These antibodies were raised against recombinant protein fragments corresponding to the central variable domain of the respective proteins.
BSA-coated wells was performed. The adherent cells were fixed, stained with methylene blue, and quantified by measuring A650 of the extracted dye. Figure 3A shows that THP-1 cells adhered to both Cyr61- and CTGF-coated wells, but not to control wells coated with BSA. Moreover, cellular activation with ADP resulted in an approximate 3-fold enhancement of cell adhesion to both proteins. An approximate 4-fold increase in cell adhesion was also observed with PMA stimulation. In 3 separate experiments, we consistently observed higher adhesion of THP-1 cells to Cyr61-than to CTGF-coated wells. Thus, in later experiments, we used Cyr61 as the prototype substrate to mediate cell adhesion.

To further characterize the adhesion of THP-1 cells to Cyr61, we examined dose and time dependency of this process. As shown in Figure 3B, the adhesion of ADP-stimulated THP-1 cells to Cyr61 was dose dependent with saturable adhesion occurring at a coating concentration of approximately 15 μg/mL. Time-course studies in Figure 3C show that the adhesion process was transient, peaking at 20 minutes and declining thereafter. These results suggest that the adherent cells became detached from immobilized Cyr61 in a time-dependent manner. This transient nature of leukocyte adhesion has also been observed for T-lymphoblastoid Jurkat cell adhesion to vascular cell adhesion molecule-1 and to the CS-1 peptide of fibronectin.33

Having established conditions for the adhesion assay, we proceeded to examine the adhesion of human peripheral blood monocytes to Cyr61. Unless otherwise indicated, isolated monocytes were added to microtiter wells coated with 10 μg/mL Cyr61 and adhesion proceeded for 20 minutes at 37°C. Because of the limited recovery of peripheral blood monocytes from the isolation procedure, cell adhesion was quantified using a more sensitive method by measuring the acid phosphatase activity of the adherent cells. Figure 4 shows that unactivated monocytes adhered poorly to Cyr61-coated wells. However, cellular activation with 20 μM ADP or 1 μM formyl-Met-Leu-Phe (fMLP)34 resulted in a dramatic increase in monocyte adhesion to Cyr61-coated wells. Quantitation...
of acid phosphatase activities of adherent versus added cells indicated that approximately 40% to 50% of input monocytes adhered to Cyr61, and this level of cell adhesion was comparable to those using vitronectin or fibronogen as the adhesive substrates (Figure 5A,C). Preincubation of the cell suspension with 2 mM EDTA completely abolished the adhesion of unactivated and activated monocytes to Cyr61 (Figure 4). These results indicate that the adhesion process is activation and divalent cation dependent, consistent with the involvement of integrin receptors.

Identification of αMβ2 as the major integrin mediating monocyte adhesion to Cyr61

In previous studies, we demonstrated that the adhesion of endothelial cells, platelets, and fibroblasts to Cyr61 and CTGF is mediated through interaction with integrins α5β1, α5β2, and α6β1, respectively.14,15 It has been reported that human monocytes express a small quantity of functional α5β1,15 By flow cytometry analyses, we also found that ADP-activated monocytes stained positively with 2 anti-α5β1 monoclonal antibodies, LM609 and anti-VnR1 (results not shown). Thus, we evaluated the role of this integrin in mediating monocyte adhesion to Cyr61. Figure 5A shows that LM609 (anti-α5β1) caused little inhibition of the adhesion of ADP-activated monocytes to Cyr61. Likewise, echistatin, a high-affinity RGD-containing snake venom peptide,28 blocked monocyte adhesion to Cyr61 by only 20%. However, in parallel samples, both compounds effectively inhibited monocyte adhesion to vitronectin by more than 85%. These results suggest that integrin α5β2 is not the major adhesion receptor on monocytes mediating interaction with Cyr61.

To identify which integrin(s) on monocytes may be involved in this adhesion process, we examined the effect of monoclonal antibodies directed against different integrin subunits. As shown in Figure 5B, monocyte adhesion to Cyr61 was completely blocked by an anti-β2 antibody (YFC118.3), but not by mouse IgG or an anti-β1 antibody (6S6). The lack of effect of 6S6 was not due to the inaccessibility of the antibody to β1 integrins on monocytes because it inhibited monocyte adhesion to fibronectin by about 60% in control samples (results not shown). In addition to the anti-β2 antibody, 2 monoclonal antibodies directed against the αM subunit, 2LPM19c (Figure 5B) and 44a (results not shown), also caused complete inhibition of monocyte adhesion to Cyr61. These results suggest that integrin αMβ2 serves as an adhesion receptor on monocytes for interaction with Cyr61. Because integrins αMβ2 and α5β2 share some common ligand specificity,26 we examined possible interaction of Cyr61 with αMβ2 on monocytes. As shown in Figure 5C, an anti-αM antibody (CBR-p150/4G1) had no significant effect on monocyte adhesion to Cyr61, whereas an anti-α5 antibody (2LPM19c) completely blocked this process. In control samples, both anti-αM and anti-α5 antibodies exerted partial inhibition on monocyte adhesion to fibronectin as expected. Thus, these findings indicate that monocyte adhesion to Cyr61 is mediated specifically by integrin αMβ2.

To substantiate the ability of αMβ2 to support cell adhesion, we used human embryonic kidney 293 cells genetically engineered to express the αMβ2 integrin. Figure 6A shows that mock-transfected 293 cells adhered poorly to Cyr61; however, the expression of αMβ2 on 293 cells caused an approximate 5-fold enhancement of cell adhesion to Cyr61. The specificity of αMβ2-mediated cell adhesion was further demonstrated by the capacity of 2 anti-αM antibodies, 2LPM19c and 44a, to inhibit the adhesion of αMβ2-expressing 293 cells to Cyr61 (Figure 6B).

Role of cell surface HSPGs on monocyte adhesion to Cyr61

We previously showed that αMβ2-mediated fibroblast adhesion to Cyr61 requires cell surface HSPGs to serve as coreceptors.3 In this regard, 2 putative heparin-binding motifs are present in the C-terminal domain of Cyr61 to mediate interaction with cell surface HSPGs. To investigate whether HSPGs on monocytes are also required for cell adhesion to Cyr61, we examined the effect of heparin, which binds Cyr61 with high affinity. As we previously reported,4 heparin dose dependently inhibited fibroblast adhesion to Cyr61 and complete inhibition was attained at concentrations of 1 μg/mL or higher (Figure 7A). In parallel samples, we found that heparin also inhibited monocyte adhesion to Cyr61. However, it was much less effective in blocking monocyte adhesion, and only partial inhibition was observed at a heparin concentration as high as 10 μg/mL. When cells were treated with heparinase to remove cell surface HSPGs prior to addition to Cyr61-coated wells, fibroblast adhesion was inhibited by about 75%, whereas monocyte adhesion was reduced by only about 45% (Figure 7B). To further investigate the role of cell surface HSPGs on monocyte adhesion to Cyr61, we examined the ability of monocytes to adhere to Cyr61-DM with alanine substitutions of the basic amino acid residues in both heparin binding motifs of Cyr61 (Figure 7C). Cyr61-DM was shown to have less than 10% of the adhesion activity of Cyr61.
deficient in heparin binding and incapable of supporting fibroblast adhesion.4 Figure 7C shows that monocytes adhered to both wild-type Cyr61 and Cyr61-DM, but higher coating concentrations of Cyr61-DM were required for cell adhesion to occur. These results suggest that cell surface HSPGs are also involved in α3β2-mediated monocyte adhesion to Cyr61; however, the interaction of HSPGs on monocytes with Cyr61 is not absolutely required for this adhesion process.

### Binding of the I domain of α3 to immobilized Cyr61 and CTGF

The cell adhesion data above identify Cyr61 and CTGF as novel adhesive ligands for α3β2 on monocytes. The integrin α3 subunit contains an inserted I domain that is important for ligand interaction.37 Thus, we examined direct binding of a GST fusion protein containing the I domain of the α3 subunit (GST-α3I) to Cyr61 and CTGF in a solid phase binding assay. In these experiments, microtiter wells were coated with Cyr61 or CTGF and the binding of GST-α3I was detected with an anti-GST antibody in an ELISA system. As shown in Figure 8, panels A and B, GST-α3I bound to wells coated with either protein in a dose-dependent and saturable manner. In control samples, GST itself did not bind to either Cyr61- or CTGF-coated wells. To further demonstrate binding specificity, we tested the inhibitory effect of 2LPM19c, a monoclonal antibody directed against the I domain of α3.38 Consistent with the cell adhesion data, preincubation of GST-α3I with 2LPM19c completely inhibited GST-α3I binding to Cyr61 and CTGF-coated wells (Figure 8C). As expected, the anti-β2 antibody (YFC118.3) had no effect on GST-α3I binding even though it blocked monocyte adhesion to both proteins.

Because heparin caused partial inhibition on monocyte adhesion to Cyr61 (Figure 7A), we examined its inhibitory effect on GST-α3I binding to wild-type Cyr61 and to the heparin binding-deficient Cyr61-DM protein. Figure 9 shows that GST-α3I bound equally well to both wild-type Cyr61 and Cyr61-DM, but not to BSA, indicating that the α3β2 binding site(s) in Cyr61 is not dependent on the 2 heparin-binding motifs in its C-terminal domain. In the presence of heparin, partial inhibition of GST-α3I binding to wild-type Cyr61, but not to Cyr61-DM, was observed. Thus, heparin binding to wild-type Cyr61 may impose steric hindrance to the α3β2 binding site(s) in Cyr61. Furthermore, these results eliminate the possibility that heparin exerts its inhibitory effect by binding to the GST-α3I fusion protein.
Discussion

Cyr61 and CTGF are extracellular matrix–associated signaling molecules that support cell adhesion, promote cell migration, and augment growth factor–induced cellular proliferation by interacting with specific integrins on different cell types. In this study, we demonstrated that both Cyr61 and CTGF are expressed in advanced atherosclerotic lesions of apoE−/− mice. Furthermore, we found that THP-1 monocyctic cells and peripheral blood monocytes adhere to these proteins in an activation-dependent manner, and identified integrin α5β2 as the major adhesion receptor mediating monocyte adhesion to Cyr61. In support of these findings, we showed that expression of α5β2 in human embryonic kidney 293 cells resulted in α5β2-mediated cell adhesion to Cyr61. Additionally, the I domain of α5 binds specifically to immobilized Cyr61 and CTGF in a solid phase binding assay. Finally, we found that cell surface HSPGs are also involved in, but not absolutely required for, monocyte adhesion to Cyr61. These observations establish Cyr61 and CTGF as novel adhesive ligands for integrin α5β2 and suggest a role for these proteins in the pathophysiologic function of monocytes.

Both Cyr61 and CTGF are expressed in the blood vessels and in the vasa vasorum of the plaque lesions. Using the apoE−/− mouse model, we extended these observations and showed that both Cyr61 and CTGF are highly expressed in mouse atherosclerotic lesions. It should be noted that both Cyr61 and CTGF are products of growth factor–inducible immediate-early genes that are rapidly activated on the transcriptional level. Also, it is well established that the levels of growth factors and cytokines such as platelet-derived growth factor, transforming growth factor-β, basic fibroblast growth factor, and interleukin 1β are elevated in atherosclerotic lesions. Thus, interaction of these growth regulatory molecules with endothelial cells, smooth muscle cells, and fibroblasts at the lesion sites would undoubtedly result in the up-regulation of Cyr61 and CTGF expression. Although the functional significance of these proteins in atherosclerosis remains to be determined, their ability to interact with different cell types in the vasculature suggests that they may participate in lesion development as well as the consequences of plaque rupture. In this regard, both Cyr61 and CTGF are angiogenic factors, and thus they may induce neovascularization in the fibrous plaques. Moreover, we previously demonstrated that activated platelets adhere to Cyr61 and CTGF through integrin α5β2. On plaque rupture, the exposure of these proteins in the underlying subendothelial matrix would likely lead to platelet adhesion and the formation of occluding thrombi.

Leukocyte adhesion and migration are essential for their recruitment to atherosclerotic lesions and to areas of extravascular inflammation. These adhesive interactions are mediated primarily by leukocyte-specific β2 integrins. Cyr61 and CTGF are expressed in advanced atherosclerotic lesions and in the granulation tissue of the healing cutaneous wounds; therefore, we investigated the interaction of monocytes with these proteins. The results of our studies show that isolated peripheral blood monocytes adhere to Cyr61 following cellular activation with ADP or fMLP. The observation of activation-dependent adhesion, coupled with the divalent cation dependency of this process, suggests the involvement of integrin receptors. Indeed, monoclonal antibodies directed against the α5 or β2 subunit specifically blocked monocyte adhesion to Cyr61, indicating that α5β2 is the major integrin on activated monocytes mediating the adhesion event. Activation of monocytes with fMLP, ADP, and PMA causes a conformational change of α5β2 through inside-out signaling mechanisms. Our findings that ADP- and fMLP-activated monocytes adhere strongly to Cyr61 as opposed to resting monocytes suggest that activated α5β2 binds Cyr61 with a higher affinity as compared to the unactivated receptor. An alternative explanation is that activation of monocytes increases cell surface expression of α5β2.
thereby facilitating monocyte adhesion to Cyr61. However, this possibility is unlikely because increased α5β2 expression is not sufficient to promote α5β2-dependent cell adhesion to other adhesive ligands such as fibronectin and intercellular adhesion molecule–1 (ICAM-1).40

All of the α subunits complexes to β2 integrins contain an approximately 200–amino acid insert or domain in their N-terminal regions.37 Isolated I domains of several α subunits are capable of binding ligands,25,28,41-44 and their atomic resolution structures reveal a unique divergent cation coordination sphere designated the “metal-ion dependent adhesion site.”45 In a solid phase binding assay, we demonstrated that a soluble GST-α5-I fusion protein binds specifically to immobilized Cyr61 and CTGF, confirming that these proteins are direct ligands of α5β2. Unlike the other integrins (ie, α5β3, α5β6, α5β15, and α5β11) that have previously been reported to mediate cellular interaction with Cyr61 and CTGF,4,12,15,16 α5β2 is the first I domain–containing integrin that serves as an adhesion receptor for the CCN family of matrix-associated proteins. At present, the integrin binding site(s) on Cyr61 and CTGF are not known. However, analogous to the findings that α5β1 and α5β6 binding to different sites on fibronogen,28,46,47 we speculate that the I domain–containing α5β2 may bind to a unique site on Cyr61 and CTGF than those recognized by other integrins without the I domain.

Increasing evidence suggests that cell surface HS PGs such as syndecan-4 may act cooperatively with integrins to promote focal adhesion formation.48 Recently, we demonstrated that α5β1-mediated fibroblast adhesion to Cyr61 requires cell surface HS PGs to act as coreceptors for 2-hexamer-binding motifs in the C-terminal domain of Cyr61.4 By contrast, adhesion of endothelial cells to Cyr61 through α5β2 is independent of the heparin binding activity of Cyr61.5 In the present study, we found that heparin partially blocked monocyte adhesion to Cyr61. Heparin may exert its inhibitory effect by binding to the heparin binding motifs in Cyr61 or by interacting with α5β2 on monocytes. However, this possibility may have been excluded by the observation that heparin inhibited the binding of GST-Loα5 to wild-type Cyr61 but not to the heparin binding-deficient Cyr61-DM protein. Moreover, treatments of monocytes with heparinase I to remove heparan sulfates from the cell surface resulted in partial inhibition of cell adhesion. Together, these findings indicate that HS PGs on monocytes also participate in optimal cell adhesion to Cyr61. Nevertheless, unlike fibroblast adhesion, monocytes are capable of adhering to Cyr61-DM that is deficient of heparin binding activity. Thus, while the binding of cell surface HS PGs to Cyr61 is involved in monocyte adhesion, such interaction is not necessary for this process. The relative affinities of different integrins with Cyr61 may account for the differential requirement of cell surface HS PGs for the adhesion of different cell types to Cyr61.

Although the biologic significance of monocyte interaction with Cyr61 and CTGF remains to be established, the expression of these proteins in plaque lesions and healing wounds suggests that they may play an important role in atherosclerosis and inflammatory responses. In this regard, monocyte adhesion to activated endothelium and their emigration into the extravascular space are important for these processes. It has been reported that CTGF messenger RNA and protein are expressed in endothelial cells at the luminal site of atherosclerotic plaques,19 and therefore, it may act in concert with other adhesion molecules such as ICAM-1 to mediate monocyte adhesion through α5β2. Both Cyr61 and CTGF have been reported to stimulate cell migration through integrins,13,14,16 and it is likely that they would also induce transendothelial migration of monocytes. Once migrated, monocytes differentiate into macrophages; whether Cyr61 and CTGF play a regulatory role in this process is an intriguing possibility. Finally, these proteins are ligands of α5β2 on monocytes and would likely induce integrin-dependent outside-in signaling as has recently been shown in fibroblasts.50 In monocytes, integrin signaling leads to the expression of proinflammatory and proatherosclerotic substances such as interleukin 1β.51 Based on these considerations, the potential role of CTGF and Cyr61, and perhaps other members of the CCN family, in the pathophysiologic function of monocytes merits further investigation.

References

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