The significance of CD105, TGF\(\beta\) and CD105/TGF\(\beta\) complexes in coronary artery disease

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Abstract

We have quantified levels of CD105, its ligand TGF\(\beta\) and receptor-ligand complexes in sera from healthy individuals \((n = 31)\), patients with triple vessel disease documented by coronary angiography (TVD; \(n = 36)\) and patients with chest pain and a positive exercise electrocardiogram but with normal coronary angiogram (NCA; \(n = 30)\). Both active TGF\(\beta\)\textsubscript{1} and active plus acid-activatable TGF\(\beta\)\textsubscript{1} [(a + l)TGF\(\beta\)\textsubscript{1}] were significantly depressed in patients with TVD compared with the other two groups \((P \leq 0.04)\). CD105 levels in TVD patients were also diminished but elevated in NCA patients. In contrast, patients with TVD had more CD105/TGF\(\beta\)\textsubscript{1} complex in their sera than the other two groups, suggesting that this may be the reason why TVD patients had low levels of receptor and ligand. TGF\(\beta\)\textsubscript{3} levels were similar in the three groups, but elevated CD105/TGF\(\beta\)\textsubscript{3} levels were noted in patients with NCA compared with those with TVD and healthy individuals \((P \leq 0.02)\). CD105 was correlated with both active TGF\(\beta\)\textsubscript{1} and (a + l)TGF\(\beta\)\textsubscript{1} \((P = 0.02)\). CD105 also strongly correlated with TGF\(\beta\)\textsubscript{3} and CD105/TGF\(\beta\)\textsubscript{3} complexes \((P = 0.001)\) in both cases. The changes in levels of CD105, TGF\(\beta\)\textsubscript{1} and the receptor-ligand complexes in sera of patients with atherosclerosis suggest that these molecules may be important in the pathobiology of the atherosclerotic disease. Further studies on sequential samples from a larger cohort of patients are needed to define a causal relationship between these molecules and the disease progression. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Atherosclerosis; CD105; TGF\(\beta\); Receptor–ligand complexes

1. Introduction

The role of endothelial dysfunction in initiating atherosclerosis is an active area of investigation, because it may reflect the global functional state of vascular disease and thereby help define subjects at increased coronary risk. Circulating markers of endothelial damage, such as von Willebrand factor (vWF) \cite{1}, vascular cell adhesion molecule-1 (VCAM-1) \cite{2} and plasminogen activator inhibitor-type 1 (PAI-1) \cite{3} have been shown to be altered in patients with vascular disease. While these factors may be significant through being involved in atherosclerosis, there is little definitive evidence to support a correlation with the progression of the disease. CD105 (endoglin) is a homodimeric transmembrane receptor for TGF\(\beta\)\textsubscript{1} and TGF\(\beta\)\textsubscript{3}, which is particularly abundant in vascular endothelial cells (ECs) \cite{4}. Markedly increased expression of this receptor has been demonstrated in activated endothelial cells following irradiation \cite{5} and in tissues undergoing angiogenesis \cite{6–10}. These observations suggest that CD105 may be a novel potential marker for endothelial damage/repair and activation. Although no signal sequence has been identified in CD105 protein, the constitutive phosphorylation of the cytoplasmic domain and the formation of the heteromeric complex of CD105 with the TGF\(\beta\) RI-RII signal complex in endothelial cells implies the involvement of CD105 in modulating TGF\(\beta\) signalling \cite{11}.

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The role of TGFβ in the pathobiology of atherosclerosis is being increasingly recognised. Its functional regulation of the vessel wall is by its direct effect on the vascular smooth muscle cells (VSMCs), endothelial cells and extracellular matrix (ECM). In addition, TGFβ has a number of other functions including suppression of immune responses [12] and reduction of superoxide anions [13], which are considered to be important in atherogenesis. TGFβ1 and TGFβ3, although share 70% homology, exhibit distinct functions in a range of pathobiological situations, such as wound healing, radiation-induced tissue damage and tumour development [14–16]. What functional role the two isoforms play in lation-induced tissue damage and tumour development pathobiological situations, such as wound healing, radi- homology, exhibit distinct functions in a range of

bffect the bioavailability of TGFβ 1, the active TGFβ and the CD105/TGFβ complexes. VSMCs participate in the development and progression of atherosclerosis through their migration, proliferation and secretion of ECM. Each of these aspects of VSMC behaviour can be modified by TGFβ. A recent finding, which demonstrated the existence of CD105 mRNA and protein in human VSMCs [19], leads to the speculation that in addition to endothelial cells, it may modulate effects of TGFβ on VSMCs. Based on these findings, we hypothesised that the interaction of CD105 with TGFβ might affect the bioavailability of TGFβ and hence contribute to the progression of CAD. To examine this hypothesis, in this study we have examined circulating levels of CD105, TGFβ and the CD105/TGFβ complexes in healthy individuals, in patients with established triple vessel coronary artery disease and a group with normal angiograms but with continued chest pain and positive exercise electrocardiogram.

2. Materials and methods

2.1. Subjects

Two groups of patients were recruited for this study which had ethics committee approval. One group of patients had significant coronary artery disease in all three coronary arteries on coronary angiograms (TVD). Significant coronary artery disease was defined as a reduction of more than 50% in the intraluminal diameter of the coronary artery. The second group were patients who had continued chest pain suggestive of angina pectoris, a positive exercise electrocardiogram but normal coronary angiograms (NCA). Blood samples were withdrawn through the needle inserted into the femoral artery for angiography before heparin administration. Control samples were obtained from age matched healthy hospital staff and all were asymptomatic for vascular disease. Blood was allowed to clot in plastic tubes for 2 h at room temperature, and the serum was collected after centrifugation. All measurements were performed on aliquots of sera stored at –70°C.

2.2. Detection of active TGFβ1

The specific measurement for active TGFβ1 by ELISA was defined by the nature of the antibodies used in the assay. Both the coating antibody (mouse monoclonal anti-TGFβ antibody, Genzyme, MA) and the detecting antibody (chicken polyclonal anti-TGFβ1 antitbody, R&D Systems, Abingdon, UK) recognise ma- ture active TGFβ1 rather than latent forms. The validity of ELISA for active TGFβ1 was further verified by substituting the active TGFβ1 with the latent TGFβ1 (both from R&D Systems), indicating that the cross-reactivity with latent TGFβ1 was less than 1% (Fig. 1). The assay was therefore considered to detect active TGFβ1 in serum and the details of procedure are as follows.

A total of 96-well white plates (Dynatech Microfluor, VA) were coated at 4°C overnight with 100 μl/well mouse monoclonal antibody against TGFβ (Genzyme, MA) at 1μg/ml in PBS. After blocking with 1% BSA in 0.1 M PBS and 0.1% Tween 20 (PBS-Tween) for 2 h at room temperature, plates were washed three times with PBS-Tween and 100 μl serum was added to each well. A standard curve was generated using purified recombinant human TGFβ1 (R&D Systems). The plate was left at 4°C overnight in a humidified box. Subsequently, the wells were incubated with 100 μl polyclonal chicken anti-TGFβ1 antibody (R&D Systems), diluted 1/1000 (1 μg/ml) in PBS-Tween, for 3 h at 4°C. Three washes

![Fig. 1. Specificity of the ELISA for active TGFβ1. To evaluate the specificity of the ELISA for active TGFβ1, the active TGFβ1 was substituted with latent TGFβ1, wherein the signal was barely detectable. The calculated cross-reactivity with the latent TGFβ1 was less than 1%).](image-url)
with PBS-Tween were given between each procedure. After washing, the plates were incubated on a shaker with 100 μl per well horseradish peroxidase conjugated rabbit anti-chicken IgG (Jackson ImmunoResearch Laboratories Inc. PA), at 1/2000 dilution (0.2 μg/ml) in 1% BSA and PBS-Tween, for 30 min at room temperature. Finally, the plates were rinsed 3 times, 100 μl Amerlite signal reagent (Amersham UK) was added to each well and the plate was read immediately in an Amerlite plate reader. The procedures as for the active TGFβ1 were carried out between each of the procedures. Finally, 100 μl/well, Amerlite signal reagent (Amersham) was applied to each well and the light emission was immediately measured in an Amerlite plate reader.

2.5. Immunoassay of CD105/TGFβ1 complex

The measurement of soluble CD105/TGFβ1 complex was performed using untreated serum. The procedures were the same as described in the assay for active TGFβ1, with the exception that the coating anti-TGFβ antibody was substituted by Mab E9 at 1 μg/ml in order to capture the complex from the serum [23]. A serum sample with a high level of CD105/TGFβ1 complex (100 arbitrary units/ml) was serially diluted from 1/2 to 1/512 to generate a standard curve on each plate. The assay showed no cross-reaction with CD105/TGFβ3 complex and exhibited a wide range of detection from 0.05 to 100 units/ml.

2.6. Immunodetection of TGFβ3 in serum

The procedure to measure serum levels of TGFβ3 was as follows: 96-well white plates were coated with mouse monoclonal antibody against TGFβ at 100 μl/well (1 μg/ml diluted in PBS), and incubated in a humidified box overnight at 4°C. The coated plates were subsequently blocked using 1% BSA and PBS-Tween for 2 h at room temperature. Test samples diluted 1/2 in PBS-Tween were added to the plates in duplicate. A standard curve was generated on each plate using purified recombinant human TGFβ3 (rTGFβ3; R&D Systems). After incubation at 4°C, 100 μl/well, goat anti-TGFβ3 antibody (R&D Systems) (1 μg/ml) diluted in PBS-Tween, were added to the plates and incubated for 3 h at 4°C. After washing, the wells were incubated with agitation with 100 μl/well horseradish peroxidase conjugated donkey anti-goat antibody (Jackson ImmunoResearch Laboratories Inc., PA) at 0.2 μg/ml diluted in 1% BSA in PBS-Tween for 30 min at room temperature. Following washing, Amerlite signal reagent, was added to each well, 100 μl/well, and the light emission immediately measured in an Amerlite plate reader. The measured values of light emission were converted into absolute concentrations using a TGFβ3 standard curve.

2.7. Measurement of CD105/TGFβ3 complex

In the assay for CD105/TGFβ3 complex, Mab E9 was used as coating antibody to capture the complex from the serum. The ELISA procedure was the same as described in the assay for TGFβ3, with the exception that mouse monoclonal antibody to TGFβ was replaced by Mab E9 (100 μl/well) at 1 μg/ml diluted in PBS and subsequently detected using goat anti-TGFβ3
antibody. Thus the molecules determined in this system contain both CD105 and its ligand TGFβ3. The standard curve was generated using a serum sample containing 50 units/ml of the complex on each plate.

2.8. Statistical analysis

Data are presented either as mean (when normally distributed) or median (when skewed) for the various parameters in each group. The logged data were first analysed by the Kruskall–Wallis test. Further comparisons between groups were conducted using the Mann–Whitney U test. To determine associations between two variables, the Spearman’s correlation test was performed. For all the tests, the significance level was set at $P \leq 0.05$.

3. Results

3.1. Clinical data of the subjects

A summary of data related to the clinical tests and some risk factors are presented in Table 1. Compared to normal individuals, both groups of patients with ischemic heart disease (IHD) had higher total and LDL-cholesterol and triglyceride concentration, and lower HDL-cholesterol levels ($P$ in all cases was $\leq 0.01$). Patients with triple vessel disease had lower levels of HDL-cholesterol compared to patients with normal coronary angiograms ($P \leq 0.01$).

3.2. Quality assessment of the ELISAs

The quality of the immunoassays was assessed by measuring the sensitivities, intra-and inter-coefficients of variation (CV) and is illustrated in Table 2. The cross-reactivity between active TGFβ1 and latent TGFβ1 was less than 1% as determined by the inter-replacement assays (Fig. 1). The Mab E9 is specific to CD105 protein and recognises epitopes encoded by exons 7 and 8 [21].

3.3. Active TGFβ1 in serum

The TGFβ1 measured in native serum is assumed to represent the free mature TGFβ1 as defined by the characteristics of the antibodies. Data for active TGFβ1 in serum from the 36 TVD patients, 30 NCA patients and 31 healthy controls are shown in Fig. 2(a) and Table 3. Active TGFβ1 levels in TVD patients were significantly depressed compared with NCA and healthy controls. Seventy-eight% (28/36) TVD patients had TGFβ1 levels below 100 pg/ml. In contrast, only 13% (4/30) NCA and 58% (18/31) controls showed TGFβ1 levels less than 100 pg/ml. No significant difference in active TGFβ1 was seen between NCA and healthy controls, but its level was significantly higher in these two groups compared with TVD patients (Table 3).

3.4. (a + l)TGFβ1 in serum

Data for (a + l)TGFβ1 concentrations in sera from the normal individuals, NCA and TVD patients are shown in Table 3 and Fig. 2(b). (a + l)TGFβ1 levels were markedly lower in the TVD group compared with the control and NCA group ($P = 0.04$ and $P = 0.003$, respectively). The (a + l)TGFβ1 levels in NCA were

Table 1

<table>
<thead>
<tr>
<th>Parameters</th>
<th>TVD</th>
<th>NCA</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male: female</td>
<td>31:5</td>
<td>10:20</td>
<td>20:11</td>
</tr>
<tr>
<td>Smokers</td>
<td>12</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>Age (years)</td>
<td>63 ± 7</td>
<td>56 ± 9</td>
<td>56 ± 7</td>
</tr>
<tr>
<td>Total cholesterol, mmol/l</td>
<td>6.2 ± 0.9</td>
<td>6.0 ± 1.1</td>
<td>5.3 ± 1.1*</td>
</tr>
<tr>
<td>Triglycerides, mmol/l</td>
<td>1.66 (0.7–4.4)</td>
<td>1.2* (0.5–3.7)</td>
<td>1.25* (0.7–4.1)</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/l</td>
<td>0.8 ± 0.2</td>
<td>1.1 ± 0.2*</td>
<td>1.5 ± 0.4*</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/l</td>
<td>4.5 ± 0.8</td>
<td>4.3 ± 1.1</td>
<td>3.1 ± 1.0*</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>138 ± 21</td>
<td>143 ± 23</td>
<td>134 ± 22</td>
</tr>
<tr>
<td>Diastolic blood pressure</td>
<td>74 ± 15</td>
<td>78 ± 11</td>
<td>78 ± 9</td>
</tr>
</tbody>
</table>

*a Data represented as mean ± SD or as median and range. Comparisons between groups were made by the Mann–Whitney U test. TVD, triple vessel disease; NCA, normal coronary angiogram; HDL, high density lipoprotein; LDL, low density lipoprotein.

*Signifies $P \leq 0.01$.
lower than in normal, but the difference was not statistically significant \( (P > 0.05) \).

3.5. Soluble CD105 in serum

The results of soluble CD105 in the three groups are presented in Table 3 and Fig. 2(c). Although values for CD105 in TVD patients were lower compared to both control and NCA group, the difference was only statistically significant against the latter group.

3.6. CD105/TGFβ1 complex in serum

By using the optimised conditions, the complex levels were measured and data are shown in Table 3 and Fig. 2(d). Markedly increased CD105/TGFβ1 complex levels were noted in TVD group \( (P = 0.002) \) compared with normal subjects, but no significant difference was found between NCA and TVD or NCA and controls \( (P > 0.05) \).

3.7. TGFβ3 and CD105/TGFβ3 complex in serum

Data for TGFβ3 and the receptor-ligand complex levels are presented in Table 3 and Fig. 2(e and f). Although mean TGFβ3 level was nearly threefold higher in both TVD and NCA compared to controls, this difference was not statistically significant. In contrast, significant increase in the CD105/TGFβ3 complex levels was noted in the NCA group compared with TVD and normal controls \( (P = 0.02 \text{ and } P = 0.002, \text{ respectively}) \).

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**Table 3**

Data for CD105, TGFβ1, TGFβ3 and CD105/TGFβ complexes for patients with triple vessel disease, normal coronary angiograms and controls

<table>
<thead>
<tr>
<th>Group</th>
<th>Active TGFβ1 (pg/ml)</th>
<th>(a + l) TGFβ1 (pg/ml)</th>
<th>CD105 (ng/ml)</th>
<th>CD105/TGFβ1 (units/ml)</th>
<th>TGFβ3 (ng/ml)</th>
<th>CD105/TGFβ3 (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TVD</td>
<td>75.40 ± 9.60</td>
<td>138.00 ± 14.76</td>
<td>1.97 ± 0.80</td>
<td>1.94 ± 0.30</td>
<td>1.78 ± 0.69</td>
<td>1.39 ± 0.63</td>
</tr>
<tr>
<td></td>
<td>52.70</td>
<td>119.00</td>
<td>0.40</td>
<td>1.60</td>
<td>0.22</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(22.1–255.0)</td>
<td>(54.4–544.0)</td>
<td>(0–26.7)</td>
<td>(0–5.8)</td>
<td>(0.1–18.1)</td>
<td>(0–15.9)</td>
</tr>
<tr>
<td>NCA</td>
<td>134.20 ± 10.50</td>
<td>241.30 ± 35.20</td>
<td>2.76 ± 0.60</td>
<td>1.14 ± 0.20</td>
<td>2.07 ± 0.69</td>
<td>3.12 ± 1.06</td>
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<td></td>
<td>136.00</td>
<td>187.00</td>
<td>1.71</td>
<td>1.50</td>
<td>0.24</td>
<td>0</td>
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<tr>
<td></td>
<td>(27.2–272.0)</td>
<td>(68.0–935.0)</td>
<td>(0–12.5)</td>
<td>(0–3.4)</td>
<td>(0.1–15.0)</td>
<td>(0–25.0)</td>
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<tr>
<td>Control</td>
<td>275.00 ± 100.90</td>
<td>434.96 ± 158.0</td>
<td>2.41 ± 1.10</td>
<td>1.26 ± 0.34</td>
<td>0.62 ± 0.24</td>
<td>0.08 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>75.10</td>
<td>118.60</td>
<td>0.91</td>
<td>0.20</td>
<td>0.31</td>
<td>0</td>
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<tr>
<td></td>
<td>(20.5–2678.4)</td>
<td>(50.8–4237.3)</td>
<td>(0–29.5)</td>
<td>(0–5.70)</td>
<td>(0–6.6)</td>
<td>(0–1.1)</td>
</tr>
<tr>
<td>TVD versus</td>
<td>*****</td>
<td>****</td>
<td>***</td>
<td>NS</td>
<td>NS</td>
<td>**</td>
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<td>NCA</td>
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<tr>
<td>TVD versus</td>
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</table>

* Data shown are mean ± SEM and median (range). TVD, triple vessel disease; NCA, normal coronary angiograms. Kruskall–Wallis test and Mann–Whitney U test were used for comparisons and \( P \) values are indicated as: \( *P = 0.04; **P ≤ 0.02; ***P = 0.01; ****P = 0.003; *****P = 0.001 \).
Fig. 2.
3.8. Correlation analysis

Spearman’s correlation test was performed to examine the relationship among the various parameters in TVD and NCA groups. CD105 was weakly but positively correlated with active TGFβ1 (r = 0.28, P = 0.02) and (a + l)TGFβ1 (r = 0.26, P = 0.03). Active TGFβ1 and (a + l)TGFβ1 were strongly correlated with each other (r = 0.72, P = 0.001). A positive correlation was observed between CD105 and TGFβ3 (r = 0.43, P < 0.001), CD105 and CD105/TGFβ3 complex (r = 0.67, P < 0.001). In addition, TGFβ3 and CD105/TGFβ3 were also significantly correlated (r = 0.70, P < 0.001). None of the other correlations was significant.

4. Discussion

The observation that patients with coronary artery atherosclerotic disease have significantly depressed levels of TGFβ1 is consistent with the report by Grainger et al. [18], who have suggested that it is a potential cardiovascular protector. The elevated levels of CD105/TGFβ1 complex seen in the present study in patients with TVD indicate an increased interaction between CD105 and TGFβ1. This could be the reason why CD105 and TGFβ1 levels were decreased in these patients. The lack of a significant difference in TGFβ3 levels in this cohort of patients does not mean that this isoform is not important in atherosclerosis, since it might have been masked by the formation of CD105/TGFβ3 complexes, which were markedly elevated in NCA group.

An increasing body of evidence indicates that endothelial dysfunction is a key event in the pathogenesis of atherosclerotic lesions. Endothelial injury is said to initiate atherosclerosis through the proliferation and migration of VSMCs, platelet adherence and release of numerous vasoactive growth factors [24]. One such growth factor is TGFβ1, which is involved in the pathogenesis of atherosclerosis, through its pleiotropic effects. TGFβ1 stimulates the synthesis and deposition of extracellular matrix in the vessel wall and suppresses inflammatory processes, which is thought to have strong implications for atherogenesis [25]. TGFβ1 is potentially a negative growth modulator in a number of cell types. It inhibits the cell proliferation and migration of VSMCs [26, 27]. In addition, TGFβ1 may play a role in repairing ischemic injury by acting as a cardioprotective agent by reducing superoxide anions [13].

Unlike other TGFβ receptors, relatively little is known about the function of CD105. CD105 is capable of forming heteromeric complexes with the TGFβ receptor I and receptor II [11]. The observation that patients with peripheral vascular disease but not myocardial infarction had elevated serum CD105 levels [17] suggests that it may be important in only some instances of vascular injury. What could be a reason for this difference? Since the risk profiles (e.g., smoking, lipids, blood pressure, use of blood pressure modifying therapeutic agents) were similar in the two groups, therefore, it can probably be attributed to a difference in anatomy. Further studies on a larger group of patients are warranted, to resolve possible significance of these results and to determine what effect it had on TGFβ levels.

In the present study, we have therefore quantified the soluble CD105, TGFβ and the CD105/TGFβ complexes in controls and patients with NCA and TVD. In agreement with a previous study [18], active TGFβ1 was found to be markedly depressed in the TVD group compared with both NCA and controls. There was no significant difference between the NCA and control groups. TGFβ1 in the circulation exists mainly in latent forms. Active TGFβ1 can be released in vivo from the latent complexes after activation by plasmin [28] and thrombospondin [29] or conversion in vitro to active molecules by transient acidification using pH 2.0 buffer [20]. Quantification of the active plus acid-activatable TGFβ1 provided information about the proportion of the active form in sera. The proportion of active TGFβ1 in the TVD group was much lower than in either NCA or healthy controls, which indicated that the active TGFβ1 might be complexed with its binding molecules to a greater extent in patients with TVD. Indeed increased levels of CD105/TGFβ1 complex were observed in the TVD group. We propose that the soluble CD105/TGFβ1 complex may be responsible for lowering active TGFβ1 in TVD patients.

The assay for soluble CD105 utilising the same antibody as capture and detection reagent, specifically measures CD105 and cross-reaction with other proteins was undetectable. The presence of high levels of CD105 in NCA suggests that it may be an early event in atherosclerosis, whereas the occurrence of low levels of CD105 in TVD patients could partially be the result of increased formation of CD105/TGFβ1 complexes.

Physical insult, such as irradiation, can up-regulate CD105 expression in endothelial cells [5]. There is enhanced expression also in tissues undergoing angiogenesis [4]. TGFβ1 enhances CD105 expression in CD105-transfected leukocytes [30]. Injury to the endothelium invariably leads to deposition of platelets and leukocytes, releasing a cocktail of growth factors including TGFβ1. It is likely that transient over-expression of CD105 may be provoked at this stage in response to the primary vascular damage. Data presented in this study are consistent with this view since higher levels of CD105 were encountered in sera of patients with NCA compared to TVD group, which could have resulted following initial injury to the vessel wall, leading to release of growth factors.
The depression of TGFβ1 in TVD patients could have been caused by a number of factors, such as lipoprotein (a) [Lp(a)] and plasminogen activator inhibitor (PAI-1) [3,26]. As shown in this study, increased levels of CD105 in the NCA group and higher levels of CD105/TGFβ1 complex in TVD suggest that CD105 may make a considerable contribution to lowering TGFβ1 levels. This action appears to be operative at an early stage of an atherogenic process.

In conclusion, the data presented here suggest that CD105 may initiate its effects in atherosclerosis at an early stage through interaction with TGFβ. However, establishment of precise role for these molecules warrants further investigations using in vitro and in vivo animal models together with examination of sequential blood samples from a large number of patients with atherosclerotic and related cardiovascular diseases.

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