Soluble CD40 Ligand in Pulmonary Arterial Hypertension
Possible Pathogenic Role of the Interaction Between Platelets and Endothelial Cells

Jan K. Damås, MD, PhD; Kari Otterdal, MSc; Arne Yndestad, MSc; Halfdan Aass, MD, PhD; Nils O. Solum, PhD; Stig S. Frøiland, MD, PhD; Svein Simonsen, MD, PhD; Pål Aukrust, MD, PhD; Arne K. Andreassen, MD, PhD

Background—Inflammatory processes seem to be involved in pulmonary arterial hypertension (PAH). CD40 ligand (L) may promote inflammation and thrombus formation, and we hypothesized that CD40L could be involved in the pathogenesis of PAH.

Methods and Results—Several significant findings were revealed when examining the possible role of CD40L in PAH. (1) Patients with primary (n/13) and secondary (n/11) PAH but not those with chronic thromboembolic pulmonary hypertension (n/8) had increased plasma levels of soluble (s) CD40L compared with control subjects (n/8). (2) PAH patients using warfarin had markedly lower sCD40L levels than those without such therapy. (3) sCD40L levels were higher in arterial (femoral artery) compared with mixed venous blood (pulmonary artery), suggesting enhanced release or reduced clearance in the pulmonary vasculature. (4) Platelets from PAH patients showed enhanced spontaneous and SFLLRN-stimulated release of sCD40L compared with control subjects. (5) In vitro, recombinant sCD40L induced monocyte chemoattractant protein (MCP)-1 and interleukin-8 gene expression in endothelial cells, and plasma levels of these chemokines were raised in all PAH groups, significantly correlated to sCD40L and hemodynamic parameters. (6) Although prostacyclin therapy (3 months) showed clinical benefit, this therapy had no effect on sCD40L and increased MCP-1 levels in PAH patients, and prostacyclin enhanced MCP-1 in CD40L-stimulated endothelial cells.

Conclusions—Our findings suggest a role for CD40L in the pathogenesis of PAH, possibly operating through an interaction between platelets and endothelial cells involving chemokine-related mechanisms. (Circulation. 2004;110:999-1005.)

Key Words: inflammation ▪ platelets ▪ hypertension, pulmonary ▪ endothelium

Pulmonary arterial hypertension (PAH) is a severe condition characterized by raised pulmonary-artery pressure (ie, mean pulmonary artery pressure [MPAP] >25 mm Hg at rest), leading to progressive right-sided heart failure and ultimately death.1 PAH is a common complication of systemic inflammatory conditions, such as collagen vascular disease, liver cirrhosis, and human immunodeficiency virus (HIV) infection.1,2 PAH can also result from chronic thromboembolic disease and can occur as an idiopathic form (eg, primary pulmonary hypertension [PPH]).3,4

PAH is caused by chronic obstruction of small pulmonary arteries reflecting, at least partly, endothelial cell and vascular smooth muscle cell (VSMC) dysfunction and proliferation as well as thrombus formation.5,6 PPH occurs sporadically and in a familial form, in which mutations of bone morphogenetic protein receptor 2 have been identified,7 but although enhanced expression of growth factors, such as transforming growth factor-β may be involved,8 the mechanisms underlying PPH and other forms of PAH are far from clear. However, increasing amounts of evidence support the involvement of inflammation in the pathogenesis of PAH.9,10 Thus, elevated circulating levels of inflammatory cytokines (eg, interleukin [IL]-1 and IL-6), enhanced pulmonary expression of several chemokines (eg, macrophage inflammatory protein [MIP]-1α and monocyte chemoattractant protein [MCP]-1), and perivascular infiltration of inflammatory cells (eg, T cells and macrophages) have been found in patients with PAH.10–13 CD40 ligand (L), a transmembrane protein that may be solubilized, was originally identified on CD4+T cells but has recently also been found on activated platelets.14 Both membrane-bound and soluble (s) forms of this ligand may interact with CD40, which is constitutively expressed on B cells, macrophages, VSMCs, and endothelial cells, resulting in various inflammatory responses, matrix degradation, and...
TABLE 1. Characteristics of the Study Group

<table>
<thead>
<tr>
<th></th>
<th>CTEPH (n=8)</th>
<th>sPAH (n=11)</th>
<th>PPH (n=13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>51±15</td>
<td>44±14</td>
<td>39±15</td>
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<tr>
<td>Sex, male/female</td>
<td>2/6</td>
<td>4/7</td>
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<tr>
<td>RAP, mm Hg</td>
<td>8.3±4.5</td>
<td>6.0±3.9</td>
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<tr>
<td>MPAP, mm Hg</td>
<td>45.8±8.9</td>
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<td>CO, L/min</td>
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<td>3.4±0.7</td>
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<td>PCWP, mm Hg</td>
<td>7.8±4.3</td>
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<td>PVR, Wood units</td>
<td>10.8±4.3</td>
<td>13.3±4.0</td>
<td>17.1±6.0</td>
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<tr>
<td>High-sensitivity CRP, mg/L</td>
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<td>10.4±2.6</td>
<td>5.7±1.6</td>
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<tr>
<td>Prothrombin fragments, nmol/L</td>
<td>0.75±0.11</td>
<td>1.00±0.12</td>
<td>0.82±0.11</td>
</tr>
<tr>
<td>SVo2, %</td>
<td>63.8±11.9</td>
<td>58.6±6.6</td>
<td>56.3±7.3</td>
</tr>
</tbody>
</table>

Medication, %
- Prostacyclin      0          36         53
- Calcium antagonists 0        0          23
- Warfarin          100       45         54

Data are presented as mean±SD. RAP indicates right atrial pressure; and SVo2, mixed venous oxygen saturation. Other abbreviations as in text.

thrombus formation.14 Recently, the CD40L–CD40 interaction has been implicated in the pathogenesis of several inflammatory disorders, such as autoimmune diseases, cardiac allograft rejection, and atherosclerosis.14,15 We hypothesized that CD40L also could be involved in the inflammatory processes observed in PAH, possibly contributing to the progressive vascular remodeling characterizing this disorder. This hypothesis was investigated by different experimental approaches in vivo and in vitro, focusing in particular on the interaction between sCD40L and chemokines.

Methods

Patients and Control subjects
We studied 32 patients with severe PAH in NYHA functional classes III and IV (Table 1). Patients were divided into 3 groups according to the type of PAH: (1) patients with PPH (n=13), diagnosed by the criteria of the National Institutes of Health registry of PPH; (2) patients with secondary PAH (sPAH) (n=11) associated with collagen vascular disease (n=4), liver cirrhosis (n=3), HIV infection (n=3), and congenital heart disease with right-to-left shunt (n=1); and (3) patients with chronic thromboembolic pulmonary hypertension (CTEPH) (n=8) verified with pulmonary angiograms. Nine patients (5 with PPH and 4 with sPAH) were treated with continuous infusion of prostacyclin (ie, epoprostenol) for 3 months. Control subjects in the study were 8 sex- and age-matched individuals (4 men and 4 women, 50±15 years old) undergoing right-sided heart catheterization during electrophysiological examination. All control subjects had supraventricular arrhythmias with normal hemodynamic parameters, and none were using warfarin or any other anticoagulants. For comparison, we also included an additional control group (n=20) consisting of sex- and age-matched healthy blood donors. The regional ethics committee approved the study, and informed consent was obtained from each subject.

Hemodynamic Studies
During right-sided heart catheterization, MPAP and pulmonary capillary wedge pressure (PCWP) were obtained at the end of expiration. Cardiac output (CO) was determined by the thermodilution method. Pulmonary vascular resistance (PVR) was calculated by the formula PVR=(MPAP−PCWP)/CO (Wood units). The diagnosis of PAH was defined as MPAP >25 mm Hg at rest, with a normal PCWP (≤12 mm Hg), indicating precapillary pulmonary hypertension.

Blood Sampling Protocol
During heart catheterization, blood samples were collected from the pulmonary and femoral arteries into pyrogen-free blood collection tubes with EDTA as anticoagulant. The tubes were immediately immersed in melting ice and centrifuged at 2500g for 25 minutes within 20 minutes to obtain platelet-poor plasma. All samples were stored at −80°C and thawed only once.

Stimulation of Platelet-Rich Plasma
Preparation and stimulation of citrated platelet-rich plasma (PRP) with the thrombin receptor (PAR-1) agonist SFLLRN or Tris-buffered saline only (unstimulated) was performed as described previously.16 The increase in sCD40L levels (ng/10^9 platelets) was expressed as the concentration in platelet-free plasma at the end of the experiments minus the concentration at baseline.

Endothelial Cell Culture
Primary human umbilical vein endothelial cells (HUVECs) were obtained from umbilical cord veins and cultured as described previously.16 HUVECs were stimulated with different concentrations of trimeric recombinant soluble CD40L (rsCD40L, R&D Systems). In some experiments, different concentrations of prostacyclin (PGE_1, Sigma) were added to the HUVEC cultures 15 minutes before rsCD40L stimulation. Cells were harvested after 6 and 20 hours.

RNase Protection Assay
Total RNA was extracted from HUVECs by use of RNasy columns (Qiagen) and stored in RNA storage solution (Ambion) at −80°C. RNase protection assay was performed with the chemokine (hCK5) multiprobe (Pharmingen). Gene expression of the housekeeping gene GAPDH was used for normalization.

Miscellaneous
Levels of sCD40L (Bender MedSystems), MCP-1, IL-8 (R&D Systems), and the prothrombin fragments F1+2 (Dade Behring GmbH) were determined by enzyme immunoassays.

Statistical Analysis
For comparison of 2 groups of individuals, the Mann-Whitney U test was used. When comparing more than 2 groups, 1-way ANOVA was followed by Scheffé’s post hoc test for statistical significance. The data were subjected to logarithmic transformation before the ANOVA was performed. For comparisons within the same individuals, the Wilcoxon matched-pairs test was used. Coefficients of correlation were calculated by the Spearman rank test. Probability values (2 sided) were considered significant at a value of P<0.05.

Results
sCD40L Levels in Patients With PAH
When analyzing plasma concentrations of sCD40L in mixed venous blood (pulmonary artery), we found that patients with PPH and sPAH but not those with CTEPH had significantly increased levels of sCD40L compared with control subjects (Figure 1). Interestingly, patients using warfarin had markedly lower sCD40L levels than those without such therapy (Figure 1), possibly contributing to the low levels of sCD40L in the CTEPH group. Moreover, in the patient group as a whole, we found a significant positive correlation between sCD40L and the prothrombin fragment F1+2 (r=0.48, P<0.01), suggesting an association between raised sCD40L and ongoing thrombus formation.

The increased sCD40L levels in sPAH could potentially reflect the primary disease condition. However, although
those with sPAH had raised high-sensitivity C-reactive protein (hsCRP) levels compared with those with PPH (Table 1), there was no difference in sCD40L levels between these groups of patients (Figure 1). Furthermore, there was no significant correlation between hsCRP and sCD40L in the patient group as a whole (r/H11005 0.05). Moreover, the 3 sPAH patients with the highest sCD40L levels (Figure 1) were all HIV infected, and notably, HIV-infected patients (n/H11005 7) with similar CD4+ T-cell counts and viral load, but without PAH, had much lower sCD40L levels (480±83 pg/mL). Finally, 3 of the sPAH patients were taking antiinflammatory medications (prednisolone <10 mg/d), but sCD40L levels in these patients were similar to the concentrations in the other sPAH patients. Thus, although we do not have disease control subjects for all patients, these findings suggest that the raised sCD40L levels in sPAH do not merely reflect their primary disease condition.

We also compared plasma sCD40L levels in mixed venous (pulmonary artery) and arterial (femoral artery) blood. Although both control subjects (≈1.3-fold, P/H11021 0.05) and PAH patients (≈1.7-fold, P/H11021 0.01) had increased sCD40L levels in arterial compared with mixed venous blood, the increase was more evident in PAH patients (P/H11021 0.05 versus control subjects), suggesting enhanced sCD40L release or reduced clearance in the pulmonary vasculature in these patients. Moreover, the difference in sCD40L levels between arterial and mixed venous blood was significantly correlated with PVR in the PAH patients (r/H11005 0.57, P/H11021 0.05).

**CD40L Release From Platelets in PAH**

Because platelets seem to be the major source of CD40L in the circulation,15 we next examined the release of CD40L in unstimulated and SFLLRN-stimulated PRP from patients with PAH (n/H11005 9) and healthy control subjects (n/H11005 9). As shown in Figure 2, platelets from PAH patients showed enhanced spontaneous and SFLLRN-stimulated release of sCD40L, with highest levels of sCD40L in lysed platelet pellets compared with control subjects. Three of the PAH
patients were taking warfarin, and notably, these patients also showed enhanced release of sCD40L (data not shown).

Effect of rsCD40L on Chemokine Expression in HUVECs

Chemokines seem to be of particular importance in the pathogenesis of PAH.\textsuperscript{11–13} To map any possible pathogenic consequences of increased sCD40L levels in PAH, we therefore examined the effect of rsCD40L on chemokine expression in HUVECs. Using RNase protection assay, we screened 8 different CC- and CXC-chemokines, and as shown in Figure 3, rsCD40L induced MCP-1 and IL-8 gene expression in a dose-dependent manner; this enhancing effect was also verified at the protein level. In contrast, rsCD40L had no effect on gene expression of the other chemokines examined (Figure 3).

MCP-1 and IL-8 Levels in PAH

Because rsCD40L seems to be a potent inducer of MCP-1 and IL-8 in endothelial cells, we next examined plasma levels (pulmonary artery) of these chemokines in PAH patients. As shown in Figure 4, MCP-1 and IL-8 were significantly increased in all PAH groups (ie, PPH, sPAH, and CTEPH) compared with control subjects, significantly correlated with PVR ($r$=0.61, $P<0.01$ and $r$=0.54, $P<0.01$ for MCP-1 and IL-8, respectively). Moreover, in PAH patients not using warfarin, both MCP-1 ($r$=0.64, $P<0.05$) and IL-8 ($r$=0.59, $P<0.05$) were significantly correlated with sCD40L, further suggesting a link between sCD40L and these chemokines in PAH. Finally, and similar to sCD40L, plasma levels of both IL-8 and MCP-1 were higher in arterial compared with mixed venous blood (≈1.5-fold, $P<0.05$ and ≈1.7-fold, $P<0.01$, respectively), suggesting increased production or reduced clearance in the pulmonary vasculature.

| TABLE 2. Hemodynamic and Biological Parameters in PAH Patients ($n=9$) at Baseline and After 3 Months of Prostacyclin Infusion |
|-------------|-----------------|---------------|
|            | Baseline  | 3 Months  | $P$     |
| RAP, mm Hg | 6.1±1.1 | 4.2±1.0 | 0.19 |
| MPAP, mm Hg | 60.1±6.2 | 43.4±3.3 | $<0.01$ |
| CO, L/min | 3.0±0.2 | 5.3±0.4 | $<0.01$ |
| VO\textsubscript{max}, mL·kg\textsuperscript{-1}·min\textsuperscript{-1} | 10.8±1.0 | 15.8±1.0 | $<0.01$ |
| PVR, Wood units | 17.3±2.2 | 7.8±1.2 | $<0.01$ |
| SiO\textsubscript{2}%, | 56.8±2.2 | 69.0±2.4 | $<0.01$ |
| Pro-BNP, pmol/L | 499±138 | 148±56 | $<0.01$ |

Data are presented as mean±SEM. VO\textsubscript{max} indicates maximum rate of oxygen consumption; BNP, brain natriuretic peptide. Other abbreviations as in Table 1 and text.
Effects of Prostacyclin on sCD40L, MCP-1, and IL-8

Nine of the PAH patients received continuous prostacyclin infusion (mean epoprostenol dose, 28 ± 6 ng · kg⁻¹ · min⁻¹), and after 3 months, all patients showed hemodynamic and functional improvement (Table 2). However, this beneficial effect of prostacyclin was not accompanied by any decrease in plasma levels of sCD40L, IL-8, and MCP-1 in arterial or venous blood. In fact, surprisingly, prostacyclin therapy increased MCP-1 levels in 8 of 9 of these patients (Figure 5).

Because prostacyclin therapy seems to enhance MCP-1 in vivo, we finally examined the effect of prostacyclin on chemokine expression in endothelial cells in vitro. As illustrated in Figure 6, prostacyclin enhanced MCP-1 gene expression in rsCD40L-stimulated HUVECs in a dose-dependent manner. However, no effect was seen when prostacyclin was given alone, suggesting an enhancing effect of this prostaglandin on rsCD40L-stimulated MCP-1 production.

Discussion

In the present study, we demonstrate significantly increased plasma levels of sCD40L in PAH patients, and our compartment studies suggest enhanced release or decreased clearance of this ligand in the pulmonary vasculature. Moreover, rsCD40L dose-dependently induced the production of MCP-1 and IL-8 in endothelial cells, and in PAH patients, plasma levels of sCD40L were significantly correlated with these chemokines. Although we have no data on the possible role of CD40L in pulmonary vascular remodeling, our findings may suggest the involvement of CD40L–CD40 interaction in the pathogenesis of PAH, possibly involving chemokine-related mechanisms.

Several reports have described perivascular infiltrates composed of macrophages and lymphocytes in lung biopsies from PAH patients, possibly involving chemokine-related mechanisms. Here, we show enhanced plasma levels of MCP-1 and IL-8, significantly correlated to hemodynamic measures in PAH. Moreover, our findings suggest that enhanced CD40L–CD40 interaction could contribute to the increased chemokine level in these patients. Thus, plasma levels of sCD40L were significantly correlated with IL-8 and MCP-1 in PAH patients. Even more importantly, we show that rsCD40L is a potent inducer of IL-8 and MCP-1 in endothelial cells, as shown at both the gene and the protein levels. Enhanced MCP-1 expression has previously been reported in PAH, and it has recently been shown that anti–MCP-1 therapy inhibits the progression of monocrotaline-induced PAH in rats. In contrast, the potential role of IL-8 in PAH is at present unknown, but notably, IL-8 may enhance tissue factor expression in monocytes, and it is tempting to hypothesize that IL-8 could contribute to the pathogenic loop between thrombus formation and inflammation in PAH. However, although MCP-1 and IL-8 were upregulated by rsCD40L in endothelial cells, we found no effects on other chemokines, such as MIP-1α, MIP-1β, and RANTES. Together with MIP-1α, RANTES has been shown to contribute to pulmonary inflammatory cell recruitment in PAH, and our findings suggest that these chemokines may not necessarily be regulated by CD40L–CD40 interaction.

Platelet activation is regarded as an important contributing factor in pulmonary vascular remodeling and hypertension. Thus, enhanced levels of several platelet-derived...
vasoconstrictors and growth factors have been found in PAH.\textsuperscript{23,24} It is estimated that >95\% of the circulating sCD40L is derived from platelets,\textsuperscript{18} and our finding in the present study of raised plasma levels of sCD40L in PAH significantly correlated with the prothrombin fragment F1 + 2 further suggests platelet activation associated with ongoing thrombus formation in this disorder. Moreover, although the measurement of sCD40L in serum samples may be influenced by ex vivo release of sCD40L during the coagulation phase, measurement of sCD40L in platelet-poor plasma, as in the present study, is more likely to reflect the in vivo situation. Furthermore, our findings with enhanced release of sCD40L from platelets in PAH patients, both spontaneously and after SFLLRN-stimulation, are also in line with platelet activation in PAH. Recently, much attention has been focused on the role of platelet-derived CD40L in the inflammatory loop between platelets and endothelial cells.\textsuperscript{18} Recent evidence indicates that platelet-derived CD40L also displays prothrombotic properties by inducing tissue factor expression and by stabilizing arterial thrombi by an integrin-dependent mechanism.\textsuperscript{18,25} Our findings suggest that such platelet-driven mechanisms potentially promoting both inflammation and thrombosis also may be operating in PAH.

In the present study, we found, surprisingly, that patients receiving warfarin had significantly lower sCD40L than those without such treatment. The reason for this finding is at present unclear. However, coagulation factors (eg, thrombin and factor Xa) may augment inflammatory responses by activating one or more of the protease-activated receptors expressed on monocytes, platelets, and endothelial cells.\textsuperscript{26} Consequently, anticoagulants that inhibit these factors would be expected to dampen the inflammatory response by attenuating the interaction between coagulation factors and platelets within an inflamed and procoagulant endothelium. Anti-coagulant therapy has been widely used for PAH, but the evidence supporting this approach is limited.\textsuperscript{5} Although the antithrombotic properties of warfarin are believed to account for the favorable effects of this medication in PAH, our findings may suggest that warfarin also may exert antiinflammatory properties by reducing sCD40L levels, possibly contributing to its beneficial effect in PAH. However, although warfarin seems to suppress sCD40L levels in plasma, our ex vivo experiments showed enhanced spontaneous and SFLLRN-stimulated release of this ligand in PRP also in PAH patients who were taking this medication. Thus, even in those who received warfarin, enhanced CD40L–CD40 interaction may still occur at least to some degree. Moreover, our findings in this cross-sectional study, suggesting potential antiinflammatory effects of warfarin, will have to be confirmed during longitudinal testing as well, in which sCD40L levels before warfarin therapy will be available.

Recently, intravenous prostacyclin (ie, epoprostenol) has been documented to improve exercise tolerance, hemodynamic measures, and survival in PAH patients.\textsuperscript{22} However, despite these improvements, PAH is still a disorder with high mortality and morbidity, suggesting that important pathogenic mechanisms are unmodified by the current treatment modalities. Our findings in the present study suggest that enhanced CD40L–CD40 interaction, possibly leading to increased MCP-1 expression, may represent such an unmodified mechanism. On the basis of the potential antiplatelet properties of prostacyclin, the lack of effect on sCD40L levels may seem surprising. However, the clinical relevance of cAMP-induced antiplatelet activity of prostacyclin seems uncertain,\textsuperscript{24,28} and the beneficial effects of this medication are believed to be primarily because of vasodilation, positive inotropism, and antiproliferative properties. In addition, the regulation of sCD40L release from platelets is rather complex and seems not to involve α-granule–related mechanisms.\textsuperscript{18} Although warfarin and not prostacyclin appears to suppress plasma levels of sCD40L in PAH patients, this does not necessarily imply that warfarin is a better medication in these patients. However, our findings showing that potential pathogenic mechanisms (ie, sCD40L–CD40/chemokine interactions) are unmodified or even increased during prostacyclin therapy underscore the need for additional treatment modalities in PAH. These findings may also support the view of combined warfarin and prostacyclin therapy in PAH operating through different mechanisms of action, potentially leading to additive or even synergistic effects.

The present study suggests a role for CD40L in the pathogenesis of PAH, possibly operating through an interaction between platelets and endothelial cells involving chemokine-related mechanisms. Our findings may suggest that the CD40L–CD40 dyad could represent a new target for adjuvant therapy in this disorder.

References


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