Enhanced Levels of Soluble and Membrane-Bound CD40 Ligand in Patients With Unstable Angina
Possible Reflection of T Lymphocyte and Platelet Involvement in the Pathogenesis of Acute Coronary Syndromes

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Background—The CD40 ligand (CD40L) on activated T cells and platelets may be activating matrix metalloproteinases, inducing procoagulant activity, and be involved in the pathogenesis of acute coronary syndromes by promoting plaque rupture in atheroma.

Methods and Results—To study the role of CD40L-CD40 interaction in coronary disease, we analyzed levels of soluble (s) and membrane-bound CD40L in the peripheral blood from 29 patients with stable angina, 26 with unstable angina, and 19 controls. Our main findings follow. (1) Patients with unstable angina had significantly raised serum levels of sCD40L when compared with patients with stable angina and controls. (2) Platelets could release large amounts of sCD40L when stimulated ex vivo with the thrombin receptor–agonist peptide SFLLRN in both patients and controls. (3) Platelets in patients with unstable angina were characterized ex vivo by decreased intracellular levels and decreased SFLLRN-stimulated release of sCD40L, which may possibly represent a higher percentage of degranulated platelets in these patients. (4) T cells in patients with unstable angina had enhanced surface expression of CD40L and increased release of sCD40L on anti-CD3/anti-CD28 stimulation in vitro when compared with patients with stable angina and controls. (5) Recombinant CD40L and serum from patients with unstable angina who had high sCD40L levels induced enhanced release of monocyte chemoattractant peptide-1 from mononuclear cells, a CC-chemokine involved in the pathogenesis of atherosclerosis.

Conclusions—This first demonstration of enhanced levels of soluble and membrane-bound forms of CD40L in angina patients, with particularly high levels in patients with unstable angina, suggests that CD40L-CD40 interaction may play a pathogenic role in both the long-term atherosclerotic process and in the triggering and propagation of acute coronary syndromes. (Circulation. 1999;100:614-620.)

Key Words: angina T cells immunology CD40 atherosclerosis

Increasing evidence shows that inflammatory and immunologic mediators may play a role in the pathogenesis of atherosclerosis and coronary artery disease. Elevated circulating levels of inflammatory substances, such as inflammatory cytokines and adhesion molecules, are found in patients with angina, particularly in those with unstable disease. Also, activated monocytes, T cells, and granulocytes occur in patients with unstable angina; enhanced activation occurs in cells isolated from the coronary sinus. Moreover, extensive infiltration of blood-derived macrophages and T cells into the vessel wall seems to be an important feature of the active stages of atherosclerosis.

The rupture of lipid-rich coronary plaques, with subsequent thrombosis, is an important mechanism underlying the sudden onset of acute coronary syndromes, and degradation of the connective tissue matrix protein by activated matrix metalloproteinases (MMPs) within the atherosclerotic plaque may play a major role in this process. Notably, inflammatory mediators, such as tumor necrosis factor-α (TNFα) and interleukin-1, upregulate MMP activity in macrophages, and this interaction may represent a pathogenic link between persistent immune activation and the development of plaque rupture.

CD40 ligand (CD40L), a transmembrane protein structurally related to TNFα, was originally identified on CD4+ T cells, but it was recently found also on activated platelets. Both membrane-bound and soluble (s) forms of this ligand may interact with CD40, which is constitutively expressed on
Enhanced CD40L Levels in Unstable Angina

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Patients and Methods

Patients and Controls

Between January and June of 1998, patients undergoing clinically indicated diagnostic coronary angiography in our coronary care unit were consecutively registered. Among those fulfilling the criteria for unstable angina (see below), 26 patients were selected randomly for participation in the study (Table). All patients with unstable angina had experienced ischemic chest pain at rest within the preceding 48 hours (ie, Braunwald’s class IIIB17), but they had no evidence of myocardial necrosis by enzymatic criteria. Transient ST-T segment depression and/or T-wave inversion were present in all cases. For comparison, 29 sex- and age-matched patients with stable angina were randomly selected among those presenting to our department in the same period for diagnostic coronary angiography or percutaneous coronary angioplasty (PTCA). All these patients had long-term, stable, effort angina that had lasted at least 6 months and had a positive exercise test. The exclusion criteria in the study included a myocardial infarction within the previous month, the presence of any ECG abnormalities invalidating ST-segment analyses, elevated serum levels of cardiac-related enzymes, thrombotic therapy in the previous month, or body temperature >38.0°C. Furthermore, all patients with suspected or proven long-term or intercurrent inflammatory disease likely to be associated with short-term phase response (ie, patients with infection, malignancies, autoimmune disorders, diabetes mellitus, and pulmonary disease) and patients with liver or kidney disease were also excluded. Coronary angiography was performed by standard techniques within 2 days after admission, and the diagnosis of coronary artery disease was confirmed by at least 1 diseased vessel (>75% narrowing of luminal diameter) in all patients. Controls in the study were 19 sex- and age-matched healthy blood donors and health care workers (14 men and 5 women aged 53±15 years). Informed consent for participation in the study was obtained from all individuals.

Blood Sampling Protocol

Peripheral venous blood was drawn into pyrogen-free blood collection tubes without any additives (Becton Dickinson), immediately immersed in melting ice, and allowed to clot for 1 hour before centrifugation (1500g for 10 minutes). Serum was stored at −80°C until analyzed, and samples were thawed only once.

Release of sCD40L from Platelets in Platelet-Rich Plasma

Preparation and stimulation of platelet-rich plasma (PRP) was performed as previously described.18 Briefly, a volume of 475 µL of PRP was incubated by gentle tilting for 30 minutes at room temperature after the addition of 25 µL of the thrombin receptor-agonist peptide SFLLRN (Biotechnology Center of Oslo, Norway; final concentration, 100 µmol/L) or Tris-buffered saline only (unstimulated sample). At baseline and after 30 minutes, equal volumes of PRP were centrifuged at 11,000g and 4°C for 10 minutes, and platelet-free supernatant and a platelet pellet with 300 µL of Tris-buffered saline were stored separately at −80°C. The platelet pellets were lysed by freezing and thawing 3 times, and the concentration of sCD40L was analyzed in the lysates. The increase in sCD40L levels (nanograms per 10^6 platelets) in supernatants from unstimulated and SFLLRN-stimulated PRP was expressed as the concentration in the supernatant at the end of the experiment minus the concentration in the supernatant at baseline.

Isolation of Cells

Peripheral blood mononuclear cells (PBMCs) were obtained from heparinized blood by Isopaque-Ficoll (Lymphoprep, Nycomed Pharma AS) gradient centrifugation within 45 minutes. Negative selection of CD3 T cells from PBMCs was performed by monodisperse immunomagnetic beads as previously described.19 The negatively selected T cells consisted of >90% CD3 T cells (flow cytometry).

Stimulation of Cells

For examining the release of sCD40L from CD3 T cells (10^6 cells/mL; 200 µL/well), cells were incubated in flat-bottomed, 96-well microtiter trays (Costar) in serum-free medium alone (X-vivo 15; BioWhittaker) or with stimulants (anti-CD3 mAb [final concentration, 1.2 ng/mL]; clone SpvT,b combined with anti-CD28 mAb [final concentration, 50 ng/mL]; clone 15E8 [402]; from CLB) and monodisperse immunomagnetic beads coated with sheep anti mouse IgG (Dynal) at a cell-to-bead ratio of 1:1 [cross-linking]). For examining the biological effects of human sCD40L, PBMCs (10^6 cells/mL; 200 µL/well; Costar) were incubated in serum-free me-

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### Characteristics of the Study Group

<table>
<thead>
<tr>
<th></th>
<th>Stable Angina</th>
<th>Unstable Angina</th>
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<tbody>
<tr>
<td>Age, y</td>
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<td>59±13</td>
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<tr>
<td>Sex, M/F</td>
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<td>19/7</td>
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<td>Smokers, %</td>
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<td>Medication, %</td>
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<td>30</td>
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<tr>
<td></td>
<td>3</td>
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<td>4.8±1.0</td>
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<tr>
<td>Triglycerides, mmol/L</td>
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<td>1.6±0.9</td>
</tr>
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Data are mean±SD unless otherwise indicated. HMG CoA indicates hepatic hydroxymethylglutaryl coenzyme A; HDL, high-density lipoprotein; M, male; and F, female. *P<0.01 versus stable angina.
dium alone (X-vivo 15) or stimulated with trimeric human CD40 ligand/leucine zipper fusion protein (Immunex Corp, Seattle, Wash) or pooled serum from angina patients or controls; the pooled serum was processed as previously described. After 48 hours, cell-free supernatants were harvested and analyzed for levels of macrophage chemoattractant protein-1 (MCP-1) by enzyme immunoassay (EIA; R&D Systems). In some experiments, monoclonal mouse antibodies against human CD40L (clone M90, Immunex Corp; final concentration, 50 μg/mL) or control mouse IgG1 (final concentration, 50 μg/mL; R&D Systems) were also added to cell cultures.

Detection of Membrane-Bound CD40L on T-Cell Subsets by Flow Cytometry
Peripheral venous blood was drawn into sterile tubes containing EDTA (Becton Dickinson). Within 1 hour, 300 μL of whole blood was mixed with 5 to 20 μL of the appropriate monoclonal antibody conjugates for 30 minutes (4°C in darkness). The following antibodies were used for staining: anti-CD3 (clone SK7) phycoerythrin, anti-CD4 (clone SK3) and anti-CD8 (clone SK1) peridin chlorophyll protein (all from Becton Dickinson), and anti-CD40L (clone 24 to 31) fluorescein isothiocyanate (Ancell Corp). Isotype-matched fluorescein isothiocyanate– and phycoerythrin-conjugated mouse IgG (Pharmigen) were used as negative controls. After incubation, each sample was treated with 2 mL of a lysing solution of fluorescence-activated cell sorter (Becton Dickinson), washed once with cold PBS, resuspended in PBS with 1% paraformaldehyde, and stored at 4°C before examination in a fluorescence-activated cell sorter scanning flow cytometer (Becton Dickinson) within 24 hours. A total of 10 000 cells were acquired and analyzed by CellQuest software (Becton Dickinson). The boundaries between stained and unstained populations were set using the isotype control settings, such that 1% of the events in the control tube were scored as positive.

Detection of sCD40L by EIA
Levels of sCD40L were determined by EIA (detection limit, 0.095 ng/mL; Bender Medsystems) according to the manufacturer’s instructions.

Statistical Analysis
When comparing 3 groups of individuals, 1-way ANOVA was followed by Scheffe’s post hoc test for statistical significance. The CD40L data were not normally distributed, and the data were, therefore, subjected to logarithmic transformation before performing the ANOVA analysis. For comparisons within the same individuals over time, the Wilcoxon matched pairs test was used. The calculations were performed using the Statistical Package for Social Sciences (SPSS, version 7.5) software package. If not otherwise stated, data are given as medians and 25th to 75th percentiles. P<0.05 (2-sided) was considered significant.

Results
Serum Levels of sCD40L
Both patients with stable and unstable angina showed raised levels of sCD40L compared with controls; particularly high levels occurred in patients with unstable disease (Figure 1A). In 5 patients with unstable angina, blood samples were also available from a time before the development of the unstable disease (median time between blood samplings, 10 weeks; range, 4 to 15 weeks). Notably, all these patients had a rise in sCD40L levels concomitant with the development of unstable angina (Figure 1B). Furthermore, serum levels of sCD40L were analyzed in 5 patients with stable angina before and 16 hours after PTCA and, as can be seen in Figure 1C, PTCA induced a marked rise in sCD40L levels in all patients.

Medications used by angina patients may influence the serum level of sCD40L. However, we found no association between the concentration of sCD40L and the use of the actual medication (Table), including drugs with known effects on platelet function (ie, aspirin). Furthermore, no associations were found between sCD40L levels and sex, smoking status, or lipid status in patients with either stable or unstable angina (data not shown).

Figure 1. Serum levels of sCD40L in patients with stable and unstable angina pectoris (AP). A, Concentration of sCD40L in 26 patients with unstable angina, 29 patients with stable angina, and 19 healthy controls. B, Levels of sCD40L in 5 angina patients before (stable AP) and after developing unstable angina (median time between blood samplings, 10 weeks; range, 4 to 15 weeks). C, Levels of CD40L in 5 patients with stable angina before and 16 hours after PTCA.
Release of sCD40L from Unstimulated and SFLLRN-Stimulated PRP

Henn et al recently demonstrated that activated platelets express CD40L on their surface. We, therefore, examined whether (1) activated platelets could release sCD40L extracellularly and (2) any difference existed in the release of sCD40L between patients and control subjects. This was studied in 9 patients with unstable angina, 9 with stable angina, and 13 controls. As shown in Figure 2, the platelets provided large amounts of sCD40L after lysis and released large amounts of sCD40L into the supernatant on SFLLRN stimulation in both patients and controls. However, platelets from patients with unstable angina had markedly decreased intracellular sCD40L levels and decreased SFLLRN-stimulated release of this ligand when compared with both controls and patients with stable angina (Figure 2). As for the spontaneous release of sCD40L from platelets (30 minutes of incubation of PRP), the levels were low in both patients and controls (~1/50 of levels after SFLLRN stimulation), but raised concentrations existed in patients with unstable angina compared with controls and patients with stable angina (data not shown).

CD40L in CD4+ and CD8+ T Cells

Activated T cells express membrane-bound CD40L. Therefore, we examined the expression of CD40L in CD4+ and CD8+ T cells by flow cytometry in the same individuals in whom sCD40L in PRP had been studied. Although no differences existed in the percentage of CD4+ or CD8+ T cells out of the total numbers of lymphocytes when comparing patients with both kinds of angina and controls (data not shown), patients with unstable disease had a higher percentage of both CD4+ and CD8+ cells expressing CD40L compared with the 2 other groups of individuals (Figure 3). Compared with controls, patients with stable angina also had an increased percentage of CD8+, but not CD4+, T cells expressing CD40L (Figure 3).

In a separate experiment, we examined whether T cells from patients with unstable (n=5) and stable (n=5) angina and healthy controls (n=5) could release sCD40L on activation. Interestingly, T cells stimulated with anti-CD3/anti-CD28 released considerable amounts of sCD40L into the supernatant in both controls and angina patients, with the highest levels in patients with unstable disease (Figure 4).
unstable angina may potentiate the stimulatory effect of inflammatory mediators present in serum from patients with unstable angina, no MCP-1-inducing protein comparable to the sCD40L level in pooled serum was found (data not shown). Note inhibitory effect of blocking monoclonal anti-CD40L. No effect of isotype-matched control antibody was found (data not shown). Note different scaling in y-axis on left (sCD40L) and right (pooled serum). Data are given as mean ± SD.

**Biological Effects of sCD40L**

Several studies suggest that sCD40L, in its soluble form may elicit biological activity, eg, enhanced MMP synthesis, in vascular SMCs. To further elucidate the possible effects of raised sCD40L levels in patients with unstable angina, additional experiments were performed using PBMCs from 4 healthy controls. MCP-1 seems to play a pathogenic role in atherosclerosis, and we hypothesized that sCD40L, known as a potent monocyte activator, would enhance MCP-1 production in these cells. As shown in Figure 5, sCD40L (given as trimeric human CD40L/leucine zipper fusion protein) induced a dose-dependent and specific increase in the release of MCP-1 from PBMCs. Also, pooled serum from the 4 patients with unstable angina who had the highest sCD40L levels (mean, 8.1 ng/mL), but not pooled serum from the 4 healthy controls (Ctr.) (mean sCD40L levels, 0.9 ng/mL) are also shown. Note different scaling in y-axis on left (sCD40L) and right (pooled serum). Data are given as mean ± SD.

**Discussion**

The present study shows, for the first time, signs of abnormal CD40L-CD40 interaction in patients with unstable angina; these patients had significantly higher levels of sCD40L compared with both controls and patients with stable angina. The patients with unstable angina were also characterized by enhanced expression of membrane-bound CD40L on both CD4+ and CD8+ T cells. Finally, although endothelial cells, vascular SMCs, tissue-derived macrophages, and B cells may be involved, our findings suggest that T cells and, particularly, platelets are major contributors to the elevated serum levels of sCD40L in patients with unstable angina.

Both soluble and, in particular, membrane-bound CD40L may have biological effects on a number of cell types, and the increased CD40L levels in patients with unstable angina may reflect important pathogenic aspects in these patients. The rupture of an atherosclerotic plaque, which again triggers thrombosis, is an important pathogenic event in the development of acute coronary syndromes, and enhanced CD40L-CD40 interaction may be involved in this process. The MMP-enzyme family probably plays a crucial role in underlining the integrity of the tissue in an atherosclerotic plaque, which then favors plaque rupture. Interestingly, it was recently demonstrated that both sCD40L and membrane-bound CD40L are potent inducers and activators of MMPs in macrophages and vascular SMCs but do not affect the expression of their inhibitors, tissue inhibitors of MMPs. Furthermore, enhanced CD40L-CD40 interaction may promote thrombotic activity by enhancing tissue-factor expression in macrophages and through the direct regulation of endothelium-procoagulant activity. Thus, although not specific for angina and without being the ultimate cause of this disease, CD40 activation may lead to procoagulant responses and MMP activation which, in patients with an preexisting atherosclerotic lesion, ultimately may lead to plaque rupture and the development of an acute coronary syndrome. However, other factors not necessarily related to enhanced CD40L-CD40 interaction also seem to be involved in this process (eg, enhanced apoptosis of vascular SMCs and levels of “rupture-inhibitory” mediators such as tissue inhibitors of MMPs), and the exact contributory role of CD40L will have to further elucidated.

We believe that the increased serum levels of sCD40L in patients with unstable angina are not only a marker of immune activation, but may also be involved in pathogenic processes in these patients. sCD40L in the circulation may pass through damaged atherosclerotic endothelium and come into direct contact with cells inside the lesion. However, even more importantly, sCD40L may activate circulating leukocytes to enhance the release of proinflammatory cytokines, increase the expression of adhesion molecules, and as demonstrated in the present study, enhance the release of CC-chemokines (ie, MCP-1) in mononuclear cells. Such an activation of the circulating leukocytes may be facilitated at the endothelium outside an atherosclerotic plaque, with upregulation of adhesion molecules and tethering of circulating cells. These inflammatory responses, possibly mediated by sCD40L, may further promote the infiltration of activated leukocytes into the atherosclerotic lesion, which in turn, may directly activate SMCs, macrophages, and T cells inside the vessel wall.

In addition to increased levels of sCD40L, patients with unstable angina also had enhanced expression of membrane-bound CD40L on both CD4+ and CD8+ T cells in peripheral blood. Advanced human atheromata contain numerous T cells, and relevant to the process of plaque rupture, T cells seem to account for almost 20% of the cells in the shoulder region of a plaque. These T cells are predominantly of the CD4+ subset and, interestingly, we found increased per-
centages of CD4+ T cells expressing CD40L only in patients with unstable angina, further supporting a role for CD4+ T cells in the development of acute coronary syndromes. However, although CD40L+ T cells have been found within human atherosclerotic plaques and not in nonatherosclerotic human arteries,13 forthcoming studies will have to clarify whether this increase in the numbers of CD40L+ T cells also exists within atherosclerotic lesions in the coronary arteries of patients with unstable angina.

A major finding in the present study was that platelets could release large amounts of sCD40L on activation, and the marked and rapid release of sCD40L after SFLLRN stimulation and the complete inhibition of sCD40L by preincubation with prostaglandin E1 (N.O. Solum, PhD, and P. Aukrust, MD, PhD, unpublished data, 1998) suggest that this protein is stored in the α-granules within the platelets like other proteins secreted from these cells. Notably, it seems that platelets from patients with unstable angina are characterized by markedly decreased intracellular sCD40L levels as well as decreased release of sCD40L on SFLLRN stimulation. These findings probably reflect the fact that PRP in patients with unstable angina contains a higher percentage of degranulated platelets, resulting in decreased levels of sCD40L in platelet pellets and in stimulated supernatants, but with no change in the percent release from the nondegranulated platelets (data not shown). From time to time, circulating platelets in patients with unstable angina may well encounter a secretion-inducing “event,” eg, contact with “foreign surfaces” at an atherosclerotic lesion, and such stimuli may possibly be potentiated by enhanced levels of proinflammatory cytokines, eg, TNFα.18 Such an in vivo activation may induce a marked degranulation in a small but significant proportion of platelets, resulting in a persistently higher proportion of degranulated platelets in these patients. A similar pattern of decreased SFLLRN-stimulated platelet activation ex vivo, reflecting enhanced platelet activation in vivo, has recently been reported in patients with septic shock30 and AIDS.18 Whatever the mechanisms, these extensively activated platelets in patients with unstable angina may, by expressing and releasing CD40L, contribute to inflammatory reactions, resulting in MMP activation and procoagulant activity. This activation further destabilizes the atherosclerotic plaque which, in turn, may further enhance platelet activation and thrombus formation, causing a vicious circle to operate in acute coronary syndromes.

We found no association between the use of aspirin and serum levels of sCD40L. In fact, ≈90% of the patients with both stable and unstable angina used this medication, but only the latter group had indications of marked platelet activation in vivo. Thus, although aspirin is extensively used in angina patients and may inhibit some platelet functions, more potent platelet inhibitors may be required to inhibit the enhanced release of sCD40L from these cells. Interestingly, a recent study in patients with unstable angina demonstrated beneficial effects of a platelet glycoprotein Ib/IIa inhibitor, which is a potent inhibitor of platelet function, beyond that of aspirin.31

Although thrombin and contact with an atherosclerotic lesion may trigger the release of sCD40L from platelets, the triggers of T-cell activation in patients with unstable angina will have to be further elucidated. Interestingly, we found that anti-CD3/anti-CD28 stimulation was a potent inducer of sCD40L release from T cells, and such stimulation also enhances the expression of membrane-bound CD40L in these cells.32 The in vivo correlate to such stimulation may be T-cell activation induced by persistent stimulation of antigen-presenting cells. Some have suggested that long-term infections, eg, Chlamydia pneumonia, are involved in the pathogenesis of angina,33 and persistent stimulation by microbial antigens might well lead to enhanced CD40L expression on T cells. However, the “CD40-CD40L hypothesis” clearly does not depend on the “infectious hypothesis,” and several other factors may well lead to T-cell activation in these patients. For example, enhanced oxidative stress and oxidized-LDL might also lead to increased CD40L levels and T-cell activation through monocye activation or direct effects on T cells.34,35

One may argue that elevated levels of CD40L are a consequence rather than a cause of unstable angina, and our data do not permit any definitive conclusion on this important issue. The marked rise in sCD40L levels after mechanically induced plaque rupture by PTCA may suggest that raised sCD40L levels are a secondary phenomenon. However, the fact that plaque rupture may induce further elevation of sCD40L does not exclude a pathogenic role for CD40L in this process. In fact, several “minor plaque ruptures” may possibly precede the onset of an acute coronary event and, although not the ultimate cause of unstable angina, enhanced CD40L levels may, at least in a subgroup of patients, contribute to the progression and aggravation of this disease, leading to enhanced destabilization of the coronary plaque. Thus, elevated CD40L levels may be both a cause and a consequence of plaque rupture, possibly representing a vicious circle operating in patients with acute coronary syndromes, and therapeutic modalities that downregulate CD40L-CD40 interaction may represent a new therapeutic approach in these patients.

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References

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