Endothelial progenitor cell dysfunction in patients with progressive chronic kidney disease

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Endothelial progenitor cell dysfunction in patients with progressive chronic kidney disease. These impairments were already observed at an early stage of CKD, which may hamper physiological vascular repair and can add to the increased risk for cardiovascular diseases observed in CKD. In conclusion, EPC number and function decrease with disease progression.

CIRCULATING ENDOTHELIAL PROGENITOR cells (EPC) play a role in the maintenance and regeneration of the cardiovascular system (reviewed in Refs. 10 and 44). EPC are a self-renewing cell population present in the bone marrow and circulation, which can differentiate into functional endothelial cells in vitro and in vivo (15). We and others have identified two distinct EPC subsets, the CD34+ EPC (1, 27) and the CD14+ EPC (19, 29), in the peripheral blood of healthy subjects. These EPC subsets have been proposed and applied as tools in, among others, cell therapy for ischemic diseases (18, 22), and the generation of bioartificial tissues, such as endothelialized antithrombogenic hemodialysis access (24) or replacement blood vessels (17, 20).

In patients with chronic kidney disease (CKD), the risk for cardiovascular diseases (CVD), as well as cardiovascular morbidity and mortality, is increased (30, 34). Therefore, in these patients, EPC may serve as a potential tool for cell therapy. However, we and others have described functional and numerical impairment of EPC in patients with various CVD (25, 40), including CKD (5, 28). The latter reports may explain why traditional CVD risk factors, such as hypertension and dyslipidemia, only partly account for the increase in CVD-associated morbidity and mortality in CKD patients. Surprisingly, EPC functionality has not been investigated in the early stages of CKD, nor during the progression of CKD. This information gap must be filled to make predictions about the suitability of EPC for physiological repair or their application in regenerative medicine of the kidney. Here, we hypothesized that EPC number and function are affected by CKD progression and asked whether dialysis treatment ameliorates EPC dysfunction.

We studied the number and endothelial outgrowth potential of circulating EPC derived from patients with CKD in relation to disease progression (CKD K/DOQI, stages 1–5) (26). Endothelial outgrowth was performed on fibronectin-coated supramolecular films of oligocaprolactone under angiogenic conditions to determine their angiogenic capacity and future use in regenerative medicine. CDK patients had normal numbers of circulating CD14+ EPC but reduced numbers of circulating CD34+ EPC. Furthermore, EPC from patients with CKD displayed functional impairments, i.e., hampered adherence, reduced endothelial outgrowth potential, and reduced antithrombogenic function. These impairments were already observed at stage 1 CKD and became more apparent when CKD progressed. Dialysis treatment only partially ameliorated EPC impairments in patients with CKD. In conclusion, EPC number and function decrease with advancing CKD, which may hamper physiological vascular repair and can add to the increased risk for cardiovascular diseases observed in CKD patients.

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We studied the number and endothelial outgrowth potential of circulating EPC derived from patients with CKD in relation to disease progression (CKD K/DOQI, stages 1–5) (26). Endothelial outgrowth was performed on fibronectin-coated supramolecular diuoro-pyrimidione-modified oligocaprolactone (PCLdiUPy) (7, 8) biomaterial to explore the use of patient-derived EPC in regenerative medicine. Adherence of EPC to the PCLdiUPy biomaterial was assessed, as well as the capacity for endothelial differentiation. Furthermore, cell function of endothelial outgrowth cells (EOC) was assessed by thrombin generation assays.

MATERIALS AND METHODS

Subjects. In a cross-sectional, prospective study, 50 patients with various stages of CKD and matched healthy controls (n = 10/group) were included from the outpatient renal clinic of the University Medical Center Groningen (The Netherlands) and the Dialysis Center Groningen (The Netherlands). Patients had various underlying causes of kidney disease, and we included 10 patients in each of the following CKD categories; stages 1 and 2 CKD [estimated glomerular filtration rate (eGFR; expressed as ml·min⁻¹·1.73m⁻² throughout) > 60], stage 3 CKD (30 < eGFR < 60), stages 4 and 5 CKD but not yet in dialysis (eGFR < 30), hemodialysis, and peritoneal dialysis. Patients with diabetes mellitus, vasculitides, acute infections, neoplasms, acute (within past 6 mo) cardiovascular events, or using immunosuppressive medication (including corticosteroids) were excluded. Patients maintained their regular medication. All participants provided informed consent, and the study was conducted according to the principles of the Declaration of Helsinki.

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Isolation and quantification of EPC. Mononuclear cells (MNC) were isolated from peripheral blood by density-gradient centrifugation of Lymphoprep (Nycomed Pharma) as previously described (19, 21). Aliquots of $1 \times 10^9$ MNC were subsequently labeled using monoclonal antibodies to the EPC markers CD34 (BD Pharmingen, San Jose, CA) and CD14 (IQ Products, Groningen, The Netherlands), and EPC were quantified by flow cytometry (BD Biosciences). Peripheral blood MNC were cultured on fibronectin (1 μg/ml) at 37°C in 5% CO2 in a humidified atmosphere, and used when the number of adherent cells reached 50,000 cells/cm². After 24 h, nonadherent cells were removed and adhering cells were washed with PBS. Next, cells were incubated with fibrinogen-depleted plasma under normal culture conditions, activating the intrinsic coagulation cascade. After 15 min, a mixture of 30 mM CaCl2 and phospholipids was added, which results in the formation of thrombin. Samples (5 μl) were taken at regular intervals and added to ice-cold 25 mM Tris·HCl to prevent further formation of thrombin. Finally, the diluted samples were incubated with 3 mM thrombin substrate S2238 that results in a change of color, which was measured at 405 nm with 540 nm as the reference wavelength in a microtiter plate reader (Bio-Rad, Hercules, CA). A calibration curve of known thrombin concentrations was used to quantify thrombin formation in the experimental samples. Human umbilical vein endothelial cells and bare PCLdiUPy were included as negative and positive controls, respectively.

Statistical analysis. Subject data are expressed as the mean (line) of the individual values (dots) and as means ± SE within the text. To analyze mean differences between patient groups and healthy controls, data were analyzed using a one-way Kruskall-Wallis test followed by a post hoc Dunn’s multiple comparison test. To analyze mean differences between prehemodialysis data and posthemodialysis data, the Mann-Whitney U-test was performed. Univariate association studies were performed using Pearson’s correlation, followed by multivariate ANCOVA. Probabilities of $P < 0.05$ were considered to be statistically significant.

RESULTS

Subject characteristics. We studied 50 patients in various stages of CKD ($n = 30$), dialysis therapy ($n = 20$), and 10 healthy controls. Patients had various underlying causes of kidney disease. Some classic risk factors for cardiovascular disease were present, particularly hypertension ($n = 46$) and past cardiovascular events ($n = 9$). All patients used medication, including RAAS-blockade ($n = 50$), multivitamin supplements ($n = 14$), statins ($n = 17$), or erythropoietin ($n = 18$). Medication was unchanged during the study. Subject characteristics are summarized in Table 1.

Advancing CKD (higher CKD stage or lower eGFR) was associated with higher serum creatinin levels ($r = 0.95; P < 0.0001$), lower creatinin clearance ($r = 0.85; P < 0.0001$), higher serum urea levels ($r = 0.81; P < 0.0001$), and higher serum phosphate levels ($r = -0.73; P < 0.0001$; Supplement 1; all supplemental material for this article is available on the journal web site). The number of white blood cells, mononuclear cells, granulocytes, and thrombocytes did not vary between the different stages of CKD (Supplement 1), but the hemoglobin content decreased with advancing CKD ($r = 0.63; P = 0.0001$). Total serum protein, total serum cholesterol, and HDL- and LDL-cholesterol and serum calcium were not different between the stages of CKD (Supplement 1).

Number of circulating EPC. Circulating CD34+ EPC and CD14+ EPC were detectable by flow cytometry in all patient groups and healthy controls. The mean number of circulating CD34+ EPC in healthy subjects was $2.6 \times 10^3$ CD34+ EPC/ml peripheral blood (range $1.3–3.7 \times 10^3$ CD34+ EPC/ml). Already in patients with an eGFR > 60, the number of CD34+ EPC was lower (mean 0.8; range 0.2–2.6 $\times 10^3$ CD34+ EPC/ml peripheral blood; 68% reduction; $P < 0.001$) compared with healthy controls. Patients with 30 < eGFR < 60 had even lower numbers of CD34+ EPC (mean 0.7; range 0.11–1.9 $\times 10^3$ CD34+ EPC/ml peripheral blood; 73% reduction; $P < 0.001$). CKD patients in the eGFR < 30 group
showed the highest reduction in CD34+ EPC number (mean 0.4; range 0.08–0.9 × 10³ CD34+ EPC/ml peripheral blood; 89% reduction; \( P < 0.001 \); Fig. 1A).

In univariate analysis, the reduction in CD34+ EPC numbers was associated with CKD stage (eGFR; \( r = 0.44; P = 0.008 \); Fig. 1B). Interestingly, the number of circulating CD34+ EPC increased during hemodialysis treatment (0.3 ± 0.07 vs. 1.0 ± 0.29 × 10³ CD34+ EPC/ml peripheral blood; pre- vs. post-hemodialysis; 208% increase; \( P = 0.02; \) Fig. 1C), but not to the level of healthy controls (1.0 ± 0.29 vs. 2.6 ± 0.21 × 10³ CD34+ EPC/ml peripheral blood; post-hemodialysis vs. healthy control; 62% decrease; \( P < 0.001 \)). Similarly, patients receiving peritoneal dialysis had higher CD34+ cell numbers than patients in stage 5 CKD who did not receive renal replacement therapy (0.8 ± 0.24 vs. 0.4 ± 0.08 × 10³ CD34+ EPC/ml peripheral blood; peritoneal dialysis vs. eGFR < 30, 132% increase; \( P = 0.04 \)). However, the CD34+ EPC number did not reach...
normal levels (0.8 ± 0.24 vs. 2.6 ± 0.21 × 10^3 CD34^+ EPC/ml peripheral blood; peritoneal dialysis vs. healthy control; 68% decrease; P < 0.001).

In untreated CKD patients, with the use of multivariate analysis, the number of CD34^+ EPC no longer correlated with eGFR (r = 0.18; P = 0.34) but was correlated with serum urea levels (r = 0.28; P = 0.03), serum phosphate levels (r = 0.88; P < 0.01), and the presence of concomitant risk factors for cardiovascular disease, such as age (r = -0.50; P = 0.02), LDL-cholesterol (r = 0.28; P < 0.05) (Table 2), and a history of cardiovascular disease (P = 0.03), but not with body mass index, blood pressure, HDL-cholesterol, total cholesterol levels, or the presence of vascular comorbidity. There was no association with gender or the use of erythropoietin (Supplement 2). Statin use increased the number of circulating CD34^+ EPC (P < 0.01; Fig. 1D).

In contrast to CD34^+ EPC, the numbers of CD14^+ EPC were similar in patients and healthy controls (Fig. 2A) and did not associate with increasing CKD stage (Fig. 2B). No significant association was found between the number of circulating CD14^+ EPC and known cardiovascular risk factors or the use of statins or erythropoietin by CKD patients (Supplement 3). Also, hemodialysis (Fig. 2C) and peritoneal dialysis did not alter the number of circulating CD14^+ EPC.

**Adherence and apoptosis of EOC.** Peripheral blood MNC were plated on fibronectin-coated PCLdiUPy at a density of 5.0 × 10^3 cells/mm^2 and cultured according to standard pro-tocols for the culture of EOC (2, 14). Cell adhesion in patients with mild CKD (eGFR > 60) was lower than in healthy controls (2.13 ± 0.15 vs. 2.86 ± 0.21 × 10^3 cells/mm^2, respectively; 25% reduction; P < 0.05), and declined further in advancing CKD stages [0.84 ± 0.19 × 10^3 cells/mm^2 (30 < eGFR < 60); 71% reduction; P < 0.001]. Cell adhesion was lowest in patients with eGFR < 30 (0.63 ± 0.11 × 10^3 cells/mm^2; 78% reduction; P < 0.001; Fig. 3, A and B). Reduced cell adhesion was not caused by apoptosis, because patients and controls had comparable percentages of apoptotic cells (14.5 ± 2.5 vs 9.7 ± 2.4% respectively; P = 0.09; Fig. 3C). Also, there was no correlation between the percentage of apoptotic cells and the number of adhered cells. Interestingly, in CKD patients, the number of circulating CD34^+ EPC was associated with cell adhesion (r = 0.62; P < 0.001; Fig. 3D) and reduced apoptosis (r = -0.36; P = 0.01; Fig. 3E). The reduction in cell adhesion observed in CKD patients was not ameliorated by hemodialysis, nor by peritoneal dialysis (Fig. 3A), nor did dialysis treatment augment apoptosis of cells in culture (Fig. 3C). Notably, adhesion of MNC isolated directly before hemodialysis adhered less than cells isolated directly after hemodialysis (0.44 ± 0.13 vs. 0.79 ± 0.16 × 10^3 cells/mm^2 respectively; 80% increase; P < 0.01; Fig. 3F).

**Endothelial phenotype of cultured EOC.** To investigate the endothelial outgrowth potential of cultured cells, cells were examined for the coexpression of endothelial cell markers CD31 and vWF or CD144 and eNOS. Differentiation of cells into macrophages was determined by assessing CD163 expression (Table 3). Endothelial cell markers were not present at day 0 of culture, but high numbers of endothelial cells had mobilized by 10.22 ± 0.33.5 on October 14, 2017 http://ajprenal.physiology.org/ Downloaded from
A and B), but cells from hemodialysis patients isolated directly after hemodialysis showed higher endothelial outgrowth compared with cells isolated directly before hemodialysis ($P < 0.01$, both CD31$^{+}$vWF$^{+}$ cells and CD144$^{+}$eNOS$^{+}$ cells; Fig. 4, G and H). Furthermore, the use of statins increased endothelial outgrowth in patients with CKD (both CD31$^{+}$vWF$^{+}$ cells and CD144$^{+}$eNOS$^{+}$ cells $P < 0.001$; Fig. 4, I and J).

**Cell proliferation and endothelial function.** We assessed the proliferative potential of cultured endothelial cells by determining the expression of the nuclear proliferation marker Ki67 at day 21. Proliferation was reduced in EOC from CKD patients (average 70% reduction), independently of the disease stage ($P < 0.01$ vs. healthy controls) (Fig. 5A). In CKD patients who did not receive dialysis treatment, the percentage of proliferating cells was associated with the number of circulating CD34$^{+}$ EPC ($r = 0.59; P < 0.001$; Fig. 5B), but this did not correlate with the number of CD14$^{+}$ EPC ($r = -0.22; P = 0.08$). Hemodialysis and peritoneal dialysis had no effect on the number of proliferating cells, nor did the number of proliferating cells change following hemodialysis (prehemodialysis vs. posthemodialysis).

We asked whether EOC from CKD patients exerted antithrombogenic behavior, an endothelial cell function. Using a modified thrombin generation assay (21), we examined the ability of cells to inhibit thrombin formation in an in vitro coagulation assay. EOC from healthy controls inhibited the formation of thrombin, and maximum thrombin concentration did not exceed thrombin formation by human umbilical cord endothelial cells (data not shown). Cells from patients with an eGFR $< 30$ were also able to inhibit the formation of thrombin [maximum thrombin concentration 47.46 ± 6.16 mU/ml (30 $<$ eGFR $< 60$) vs. 34.41 ± 5.11 mU/ml (healthy controls); $P > 0.10$]. Thereafter, the antithrombogenic property of EOC decreased, resulting in increased thrombin formation.
EPC DYSFUNCTION IN CKD PATIENTS

Table 3. Endothelial cell marker expression by endothelial outgrowth cells

<table>
<thead>
<tr>
<th>Healthy Controls</th>
<th>eGFR &gt;60 (stages 1-2 CRF)</th>
<th>60 &lt; eGFR &lt;30 (stage 3 CRF)</th>
<th>eGFR &lt;30 (stages 4-5 CRF)</th>
<th>Prehemodialysis</th>
<th>Posthemodialysis</th>
<th>Peritoneal Dialysis</th>
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</thead>
<tbody>
<tr>
<td>CD31</td>
<td>86.02±4.01*†</td>
<td>40.72±2.51*††</td>
<td>25.15±5.61*††</td>
<td>15.01±2.65*††</td>
<td>15.24±2.18*††</td>
<td>16.94±2.34*††</td>
</tr>
<tr>
<td>vWF</td>
<td>88.53±4.21*†</td>
<td>43.88±2.88*††</td>
<td>26.56±3.72*††</td>
<td>15.48±2.40*††</td>
<td>16.64±3.22*††</td>
<td>17.60±3.25*††</td>
</tr>
<tr>
<td>CD144</td>
<td>83.20±5.02*†</td>
<td>36.84±3.46*††</td>
<td>23.13±3.36*††</td>
<td>18.30±2.87*††</td>
<td>12.61±1.77*††</td>
<td>14.37±2.52*††</td>
</tr>
<tr>
<td>eNOS</td>
<td>91.76±5.27*†</td>
<td>37.28±4.18*††</td>
<td>26.92±3.89*††</td>
<td>16.97±2.22*††</td>
<td>13.58±2.43*††</td>
<td>15.59±3.23*††</td>
</tr>
<tr>
<td>CD163</td>
<td>7.83±1.08</td>
<td>4.47±0.61*††</td>
<td>3.90±0.77</td>
<td>6.03±0.88</td>
<td>5.12±1.09</td>
<td>3.71±0.67*</td>
</tr>
</tbody>
</table>
| Values are means ± SE expressed as the percentage of positive cells. vWF, von Willebrand factor; eNOS, endothelial nitric oxide synthase. *P < 0.05 vs. healthy controls. †P < 0.05 vs. eGFR >60. ‡P < 0.05 vs. prehemodialysis.

DISCUSSION

We have investigated the number and endothelial outgrowth capacity of circulating CD34+ EPC and CD14+ EPC in patients with CKD and found that 1) the numbers of circulating CD34+ EPC decreased with increasing kidney disease as reflected by increased serum urea and phosphate levels; 2) the numbers of circulating CD34+ EPC did not correlate with eGFR; 3) adherence and endothelial outgrowth of EPC from patients with CKD is progressively reduced during kidney disease, 4) the antithrombogenic function of EOC was hampered in patients with end-stage renal disease; and that 5) EPC dysfunction is not fully ameliorated by dialysis treatment. Taken together, EPC dysfunction in patients with progressive CKD may hamper physiological vascular repair, adding to increased risk for cardiovascular diseases observed in these patients. Furthermore, EPC dysfunction may challenge the use of patient-derived EPC for regenerative medicine therapies.

Although EPC dysfunction has been described for late-stage kidney disease (stages 4–5 CKD), we are the first to show a marked decrease in the number of circulating CD34+ EPC as early as stage 1 CKD, which worsened during CKD progression, indicated by increasing serum urea levels. Corroboratory, Sturiale et al. (38) and Choi et al. (5) have described reduced numbers of CD34+ and CD133+ EPC in patients with CKD that undergo hemodialysis treatment. Commonly, this implies that the production, or mobilization of CD34+ EPC from the bone marrow is impaired in patients with CKD.

This impairment may be explained by chronic uremia, since increases in plasma urea concentration are associated with decreased CD34+ EPC numbers. Similarly, others have described decreases in erythropoiesis (11, 13), increases in progenitor cell apoptosis (45) and impairment of EPC migration (6, 9) caused by uremic toxins present in serum from CKD patients. However, we cannot exclude interference of anti-hypertensive drugs as confounder in our analysis, since all CKD patients were kept on their regular medication. Interestingly, the number of CD34+ EPC increased following hemodialysis. Although, the CD34+ EPC mobilization may have increased by a (temporary) decrease in uremic toxins following hemodialysis, most patients (90%) received erythropoietin before hemodialysis. Since erythropoietin is a known attractant for CD34+ EPC (3, 12), we feel that the biological effects of erythropoietin, and not the decrease in uremic toxins, caused the observed increase in circulating CD34+ EPC. However, the true mechanism behind hemodialysis-induced CD34+ EPC mobilization remains to be elucidated.

In contrast to the reduction in CD34+ EPC numbers, there was no effect of CKD on the number of circulating CD14+ EPC. Therefore, we hypothesize that the chemoattractants for CD14+ EPC and CD34+ EPC are differentially expressed in patients with CKD. Although we did not include such analysis in our current study, there are multiple reports on the increased expression of MCP-1, a chemoattractant for CD14+ EPC during CKD, which seem to corroborate this hypothesis (16, 37). However, little mechanistic insight is known on the impaired mobilization of CD34+ EPC, and future research will have to elucidate this phenomenon.

When used in regenerative medicine, e.g., for endothelialized hemodialysis shunts, endothelial outgrowth on natural or synthetic biomaterial is key. Endothelial outgrowth potential is commonly used as a surrogate marker for the quality and function of circulating EPC. Here, we tested the ability of CKD patient-derived EPC to adhere to fibronectin-coated PCL-diaUPy and found a high reduction in the number of adhering cells (up to 78%) compared with EPC from healthy controls. This reduction in adherent EPC numbers was not caused by EPC apoptosis in CKD patients (45), since apoptosis frequencies were comparable in CKD patients and healthy controls. Moreover, endothelial outgrowth of adherent EPC was also highly reduced (up to 80%) in patients with CKD compared with healthy controls.

Endothelial outgrowth and proliferation of CD14+ EPC depend on paracrine signaling by CD34+ EPC (21). We and others have previously described that endothelial outgrowth of CD14+ EPC depends on interaction between CD34+ EPC and CD14+ EPC (21). Herein, CD34+ EPC secrete various proangiogenic cytokines and growth factors by CD34+ EPC that augment the behavior of CD14+ EPC (21, 39). Here, we found that adhesion of EOC correlated positively with CD34+ EPC numbers. Secretion of proangiogenic factors by CD34+ EPC may therefore explain the relationship between the number of CD34+ EPC and endothelial outgrowth and proliferation observed here.

We therefore postulate that there is a disturbed balance between CD34+ EPC and CD14+ EPC in the circulation of CKD patients, which may cause the observed impairments.

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Restoration of this balance between CD34⁺/EPC and CD14⁺/EPC numbers, by mobilization of CD34⁺/EPC from the bone marrow, may therefore restore endothelial outgrowth potential and vascular regeneration in vivo. We and others have described increased numbers of circulating CD34⁺/EPC with statin (41, 42) use or erythropoietin (4) and improved endothelial outgrowth percentage. Additionally, recent clinical results show improved cardiovascular outcome of high-dose statin use in patients with end-stage renal failure (31–33), which argues for prophylactic prescription of EPC-mobilizing agents for CKD patients.

Antithrombogenic behavior is a key property of endothelial cells, which, physiologically, maintain a balance between...
thrombolytic and thrombogenic activities, preventing the formation of blood clots. In this study, thrombin generation was efficiently prevented by EOc from healthy controls, but this property was lost during disease progression. We are the first to describe loss of in vitro hemostatic control in EOc from patients with end-stage renal disease, and this loss may contribute to the increased occurrence of thrombosis within this patient group (23, 43). Since the EOc in the in vitro coagulation assay were expressing endothelial markers CD31, VE-Cadherin, vWF, and eNOS, the loss of antithrombogenic behavior may also reflect improper maturation of EOc.

Remarkably, EPC dysfunction persists in the presence of nonuremic fetal calf serum, as was the case in our in vitro assays. This would argue for long-lasting changes in the EPC that may be elicited by uremic toxins, but do not need these toxins to remain in effect. Such changes may be introduced in the EPC epigenome (as reviewed in Refs. 35 and 36) but remain to be elucidated.

In conclusion, patients with CKD have reduced numbers of circulating CD34+ EPC, which decrease progressively with advancing disease severity and increasing serum urea levels. EPC dysfunction results in a functional impairment in cell adherence and endothelial outgrowth formation. Although diagnosis caused some improvement, functional impairments were not completely alleviated. These observations are compatible with the increased occurrence of cardiovascular disease in patients suffering from CKD. Moreover, decreased numbers and functional impairment of circulating CD34+ EPC pose a major limitation for their use in regenerative medicine. Proper mobilization and revitalization strategies, e.g., treatment with erythropoietins or statins, need to be investigated to prevent further decay of progenitor cell function. Early intervention may reduce cardiovascular morbidity in CKD patients through increased physiological vascular regeneration.

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