REVIEW

C-Peptide in the Vessel Wall

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■ Abstract

Patients with insulin resistance and early type 2 diabetes exhibit an increased sensitivity to develop a diffuse and extensive pattern of arteriosclerosis leading to a remarkable increase in vascular complications, including myocardial infarction and stroke. The accelerated atherosclerosis in these patients is likely to be multifactorial. In this review, we introduce the new hypothesis that C-peptide could play a role as a mediator of lesion development. Patients with type 2 diabetes show increased levels of the proinsulin cleavage product C-peptide, and in the past few years, various groups have examined the effect of C-peptide in vascular cells as well as its potential role in lesion development. Recent data suggest that C-peptide deposits in the vessel wall could promote the recruitment of monocytes and CD4-positive lymphocytes in early arteriosclerotic lesions. Furthermore, C-peptide induces proliferation of vascular smooth muscle cells, a critical step in atherogenesis and restenosis formation. The present review summarizes this new pathophysiological aspect and discusses the potential relevance for lesion development.

Keywords: diabetes · C-peptide · vascular cell · arteriosclerosis · inflammation · reactive oxygen species · smooth muscle cell · lipoprotein

Introduction

Patients with diabetes and insulin resistance exhibit an increased sensitivity to develop arteriosclerosis with its sequelae acute myocardial infarction and stroke [1]. These patients temporarily demonstrate elevated levels of C-peptide, the cleavage product of proinsulin (Figure 1). For a long time, C-peptide has been considered to be a biologically inert substance without any physiological function. Recent work in kidney cells suggests that C-peptide can activate intracellular signaling pathways such as Na^+-K^+-ATPase [2]. In addition, C-peptide activates various signaling pathways in different cell-types [3, 4] suggesting that C-peptide may be biologically active.

Some groups have shown that C-peptide administration in type 1 diabetes results in amelioration of diabetes-induced renal and nerve dysfunction. A C-peptide treatment for 6 months improved sensory nerve function [5, 6]. Furthermore, beneficial effects by C-peptide replacement on renal function and structure in type 1 diabetes were reported [7, 8]. Given that C-peptide increases capillary blood flow in type 1 diabetic patients, Wallerath *et al.* demonstrated *in vitro* that C-peptide stimulates the release of NO from endothelial NO synthase in endothelial cells, and that this effect is mediated by induction of Ca^{2+} influx into the cells [9]. In the kidney, C-peptide supplementation suppresses diabetes-induced abnormal renal eNOS expression. This downregulation can be an explanation for C-peptide's beneficial effects on diabetic nephropathy [10].

The present review focuses on C-peptides effects in vascular cells and discusses the potential relevance of these effects on atherogenesis in patients with type 2 diabetes.
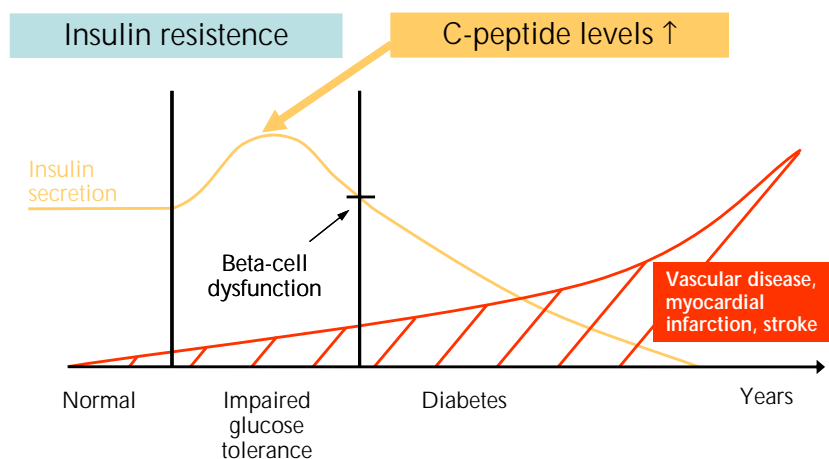
Figure 1. Development of arteriosclerosis in diabetic patients. Patients with diabetes exhibit an increased risk to develop vascular diseases with their sequelae myocardial infarction and stroke. During the "prediabetic" state of insulin resistance these patients temporarily demonstrate elevated levels of insulin and C-peptide. Recent experimental data suggest that C-peptide may play a causal role in early atherogenesis in these patients.

Atherogenesis

Atherogenesis is currently understood as an inflammatory process in the vessel wall with different phases and stages [11]. The early phase of atherogenesis is characterized by endothelial dysfunction caused by various risk factors like smoking, diabetes, hypertension or hypercholesterolemia. In addition, the endothelium is activated in this phase, releasing chemotactic proteins such as MCP-1, a monocyte chemoattractant, or RANTES and IP-10, both chemoattractants for lymphocytes. These chemokines attract inflammatory cells, mainly monocytes and CD4-positive lymphocytes, to adhere to endothelial adhesion molecules, with subsequent migration of these cells into the vessel wall. Monocytes, once migrated into the subendothelial space, become macrophages, expressing scavenger receptors on their surface. These macrophages mediate the uptake of lipoprotein particles such as oxidized low-density lipoprotein (LDL). Through lipid uptake, these cells differentiate to foam cells, the classic cellular substrate of arteriosclerotic lesions. The macrophage foam cells then express additional proinflammatory and proatherogenic mediators such as cytokines, matrix metalloproteinases, and reactive oxygen species. They also secrete procoagulant molecules such as tissue factor (Figure 2). All these factors act on other cells in the vessel

wall and thus promote lesion development [12, 13].

The second inflammatory cell type recruited during endothelial dysfunction is the CD4-positive lymphocyte. These cells enter the vessel wall as naïve Th0 cells [14]. In the subendothelial space, these cells then encounter antigens like oxidized LDL and differentiate towards Th1 cells, which release pro-inflammatory cytokines such as IFNγ, TNFα and IL-2. Some of these cytokines then enhance endothelial expression and release of T cell-specific chemokines, creating a vicious cycle of cell activation and cell recruitment [15]. In addition, these Th1 cytokines activate other cells in the vessel wall, namely macrophages and smooth muscle cells (SMCs), which orchestrates the inflammatory response in the vessel wall. With increased recruitment of these inflammatory cells, fatty streaks develop and SMCs from the media start to proliferate and migrate into the intima. As lesion formation progresses, advanced and potentially complicated lesions are formed. These lesions may form plaque and lead to a progressive narrowing of the vessel lumen, which potentially creates persistent angina if located in the coronary artery. Alternatively, the plaques may become vulnerable and upon rupture can cause an acute coronary artery syndrome [16].

C-peptide is present in the vascular wall of atherosclerotic lesions

Patients with metabolic syndrome or type 2 diabetes mellitus exhibit an increased propensity for the development of a scattered and extensive pattern of arteriosclerosis [1]. Typically, these insulin resistant patients demonstrate increased serum levels of insulin, and C-peptide which is a cleavage product of proinsulin. C-peptide is released into the blood stream in amounts equimolar to insulin.

Over the past few decades, the role of insulin in atherogenesis has been examined by various groups. It has been suggested that insulin may promote lesion development, for example, by in-

ducing proliferation of vascular SMCs [17]. Others demonstrated that insulin exhibits anti-inflammatory and antiatherogenic properties in vascular cells such as monocytes [18, 19]. Overall, the role of insulin in atherogenesis remains unclear. Presently and most important, we lack clinical and epidemiologic evidence to show that insulin may have harmful effects with respect to macrovascular events.

For a long time, C-peptide has been considered to be biologically inert, but recent data suggest that C-peptide binds to specific yet unidentified, cell surface receptors. Recently, our group hypothesized that C-peptide might deposit in the vessel wall in these patients in early atherogenesis [20]. Immunohistochemical analyses of early arteriosclerotic lesions of patients with diabetes from the PDAY Study (Patho-biological Determinants of Atherosclerosis in Youth) revealed that C-peptide deposition takes place mainly in the subendothelial space and the intima. Some of the examined diabetic patients also exhibited C-peptide deposition in the media. In contrast, only very little C-peptide deposition has been found in early arteriosclerotic lesions of non-diabetic subjects. Computer-assisted analyses revealed significantly higher C-peptide deposition in lesions from diabetic individuals compared with lesions of non-diabetic subjects matched by age, sex and risk-factors. Interestingly, in both diabetic and non-diabetic subjects, deposition of insulin or pro-insulin was not detectable.

C-peptide colocalizes with inflammatory cells in the vessel wall

Staining of parallel sections as well as immunofluorescence techniques demonstrated colocalization of C-peptide with intimal monocyte/ macrophages and CD4-positive lymphocytes in some of the diabetic

individuals [20, 21]. In the studies cited above, C-peptide deposition has been found in 100% of the 21 diabetic individuals examined, while monocyte infiltration was only present in 77%, and CD4-positive lymphocyte infiltration only in 57%. These data suggested that C-peptide deposition may precede monocyte and T cell migration into the vessel wall. Based on this observation, the hypothesis was raised that C-peptide may deposit in the vessel wall during early atherogenesis and then—through chemotactic effects—promote the recruitment of monocytes and CD4-positive lymphocytes.

C-peptide - a peptide with chemotactic activity?

In vitro migration assays, employing a modified Boyden chamber, revealed that C-peptide induces the migration of both monocytes and CD4$^+$ lymphocytes. Interestingly, insulin did not have such an effect. Stimulation of monocytes with C-peptide-induced cell migration in a concentration-dependent manner with a maximal augmentation of 2.3 ± 0.4 at 1 nmol/l. The extent of C-peptide-induced monocyte migration resembled the effect of the established monocyte chemokine MCP-1,
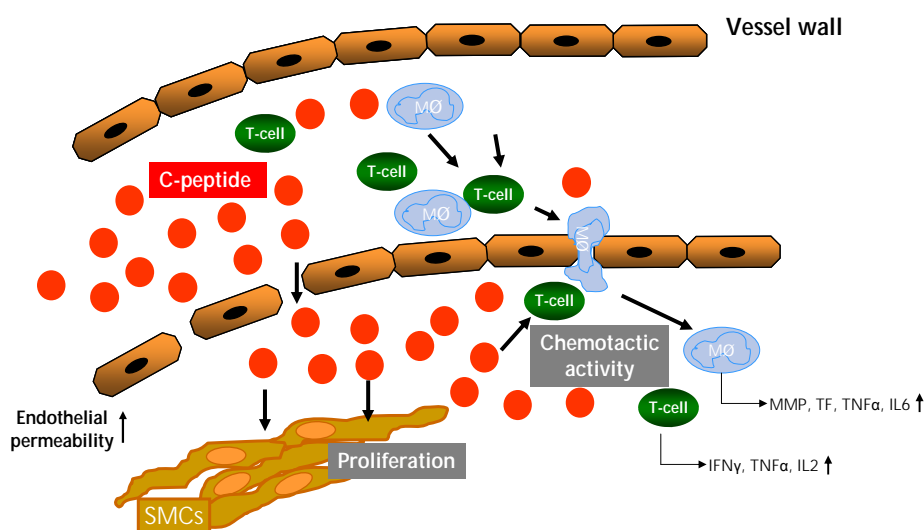

Figure 2. Potential role of C-peptide in early atherogenesis in patients with insulin resistance and early type 2 diabetes mellitus. C-peptide deposits in the intima and through its chemotactic activity on monocytes and CD4$^+$ lymphocytes during endothelial dysfunction with increased endothelial permeability. This facilitates the recruitment of inflammatory cells into the vessel wall and perpetuate the proinflammatory process. Furthermore, C-peptide can activate smooth muscle cell proliferation. T-cell: CD4-positive lymphocytes. Mø: monocytes/macrophages. SMC: smooth muscle cell. MMP: matrix metalloproteinase. TF: tissue factor.

which increased monocyte migration by 2.9 ± 0.2 fold. Stimulation of CD4$^+$ cells with C-peptide induced cell migration in a concentration-dependent manner with a maximal induction of 2.1 ± 0.5 at 10 nmol/l. The extent of C-peptide-induced CD4$^+$ cell migration was similar to the effect of an established T cell chemokine (RANTES), which led to a 2.1 ± 0.6 fold increase in cell migration. It is interesting that the combined stimulation of CD4$^+$ cells with C-peptide and RANTES did not have an additive migratory effect. In addition, checkerboard analyses showed that C-peptide induces chemotaxis rather than chemokinesis [20, 21].

Also, C-peptide did not induce the migration of human neutrophils—cells not present in arteriosclerotic lesions. This underscored the specificity of C-peptide's chemotactic effects on monocytes and lymphocytes. However, no migratory effect of C-peptide was seen on B cells, another subset of lymphocytes [22].

C-peptide activates intracellular signaling pathways in inflammatory cells

Inhibition migration assays in monocytes and CD4-positive lymphocytes, western blot analyses, and activity assays, all demonstrated that C-peptide mediates its chemotactic activity through an unidentified pertussis toxin-sensitive G-protein coupled receptor, with subsequent downstream activation of PI3-kinase γ.

Previous work from other groups has shown that Src-kinase is involved in interferon-gamma-inducible protein-10 (IP-10) mediated chemotactic response of lymphocytes [23]. In our experiments, PP2 (a specific inhibitor of Src-kinase) and transfection of Src siRNA, abolished C-peptide-induced T-cell migration, suggesting that C-peptide also signals through this pathway. Additional experiments demonstrated that PI3-kinase activation led to the involvement of small Rho-GTPases, like RhoA, Rac-1, and Cdc42 in these cells. Furthermore, C-peptide stimulated phosphorylation of p21 activated kinase (PAK), LIM domain-containing protein kinase (LIMK), and cofilin downstream of Rac-1 and Cdc42, leading to cofilin inactivation and actin filament stabilization. On the other hand, C-peptide activates Rho kinase (ROCK) and myosin light chain (MLC) phosphorylation downstream of RhoA, thereby stimulating myosin-mediated cell contraction [22]. These data support an active role of C-peptide in chemotaxis of inflammatory cells.

In summary, these data suggest that C-peptide may deposit in the arterial intima in patients with insulin resistance and early type 2 diabetes during endothelial dysfunction with increased endothelial permeability. Subsequently, this results in the attraction of monocytes and CD4-positive lymphocytes into the vessel wall. Such mechanisms may promote lesion development and potentially explain why patients with diabetes develop such a diffuse and extensive pattern of arteriosclerosis at a very early point in time.

C-peptide promotes the differentiation of monocytes/macrophages

In addition to the chemotactic effect, interesting data exists that in monocyte-like ThP1 cells C-peptide increases the expression of the PPAR$_γ$-regulated gene CD36, an important scavenger receptor for the macrophage uptake of oxidized LDL in arteriosclerotic lesions [24]. These data suggest that C-peptide may also promote the differentiation of monocyte/macrophages towards foam cells, thus representing another potential proatherogenic effect of C-peptide.

C-peptide is internalized in vascular cells

Recent reports showed that C-peptide is internalized in the cytoplasm of HEK-293 and Swiss 3T3 cells. In addition, Lindahl *et al.* demonstrated C-peptide transport into nuclei using a labeled peptide [25]. The internalization was followed at 37°C for up to 1 h, and was reduced at 4°C after preincubation with pertussis toxin. It is concluded that this occurs via an energy-dependent pertussis toxin-sensitive mechanism. Recently, C-peptide internalization was found in vascular cells, like endothelial cells and vascular smooth muscle cells. C-peptide was detected as punctuate intracellular structures and these structures were identified as early endosomes. From these results the authors concluded that endosomes could be a signaling station, through which C-peptide might achieve its cellular effects [26]. Similar results we found in monocytes and CD4-positive cells (Walcher *et al.*, unpublished data).

C-peptide's proatherogenic role in smooth muscle cell proliferation

Vascular smooth muscle cells play a critical role in the development of arteriosclerotic plaques

by proliferating and subsequently moving from the media into early lesions and fatty streaks [11]. VSMCs are also important in restenosis formation after coronary intervention. After vascular injury, these cells start to proliferate and then they migrate into the developing neointima. Thus, they become the major cellular substrate of the restenotic tissue [27]. Strategies to modulate VSMC proliferation after intervention, e.g. by the implantation of drug-eluting stents, reduce neointima formation and restenosis [28]. This underscores the pathophysiological relevance of VSMC proliferation in these processes. Several mechanisms, like platelet-derived growth factor (PDGF) release from activated platelets, secretion of cytokines, and growth factors from inflammatory cells, have all been shown to induce VSMC proliferation during atherogenesis and restenosis formation [11]. Since C-peptide also co-localizes with SMCs in the media of early arteriosclerotic lesions in some diabetic subjects, it has been suggested that C-peptide could also exhibit biological activity in these cells [15].

Conflicting data exist on the role of C-peptide in SMC proliferation. A recent report by Kobayashi *et al.* demonstrated that 3 days of treatment with human C-peptide under high glucose concentrations resulted in the inhibition of rat SMC proliferation [29]. These effects observed at high C-peptide concentrations (100 nmol/l) were mediated by an inhibition of PDGF-beta receptor expression and a reduction in p42/p44 MAP-kinase phosphorylation. In contrast, our own group demonstrated an induction of SMC proliferation upon C-peptide stimulation [30]. In our experimental setting, human C-peptide induced the proliferation of human aortic SMCs, while rat C-peptide exhibited similar effects in rat aortic SMCs.

Moreover, C-peptide stimulation of VSMCs activates Src- and PI-3 kinase and leads to downstream phosphorylation of the MAPK ERK1/2. Previous work has shown that Src-kinase is involved in LDL-induced VSMC proliferation [31]. Recently, activation of PI-3 kinase by C-peptide has been demonstrated in various cell types including vascular cells like monocytes and CD4-positive lymphocytes, suggesting that this signaling molecule plays a critical role in C-peptide-induced cell activation in different cell types. The downstream control of VSMC proliferation by extracellular stimuli takes place in the mid- to late G_1 phase of the cell cycle, where D-type cyclins promote G_1- to S-phase transition, which leads to Rb phosphorylation [32, 33]. Our data, showing an

increase in cyclin D1 expression as well as Rb phosphorylation suggest that C-peptide acts via similar signaling pathways [30].

In addition to its chemotactic effect on monocytes and CD4-positive lymphocytes, C-peptide could enhance VSMC proliferation in the vasculature. It would thus promote both the development of arteriosclerotic lesions and neointima formation after coronary intervention. Future studies in animal models will be needed to evaluate the pathophysiological relevance of C-peptide-induced VSMC proliferation in atherogenesis and neointima formation *in vivo*.

C-peptide: conflicting data in type 1 and type 2 diabetes?

The majority of data described above suggest that C-peptide may promote lesion development in patients with type 2 diabetes mellitus and insulin resistance, while the application of C-peptide in type 1 diabetic patients who lack C-peptide has been shown to improve diabetic microvascular complications such as diabetic neuropathy. The potential proatherogenic action of C-peptide is not in conflict with the clinical benefits of C-peptide treatment in patients with type 1 diabetes. From other clinical disorders, like hypo- and hyperthyroidism, we know that treatment with L-thyroxine in patients with hypothyroidism is beneficial, but elevated levels of L-thyroxine in those with hyperthyroidism can cause serious clinical manifestations. Comparing situations, supplementation of C-peptide in type 1 diabetic patients may be beneficial, whereas in patients with insulin resistance and type 2 diabetes, increased levels of C-peptide may be harmful.

Further studies in animal models of arteriosclerosis are warranted to examine whether the hypothesis of C-peptide's proatherogenic effects holds true *in vivo*. Moreover, additional work is needed to identify the C-peptide receptor, in order to modulate the physiological function of C-peptide.

Conclusions and perspectives

C-peptide might contribute to the initiation and progression of lesion development in diabetic subjects. Further understanding of these mechanisms may pave the way for future therapies to target vascular disease in patients with diabetes and as

such reduce cardiovascular morbidity and mortality in this high risk population.

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