Transglutaminase 2 Deficiency Decreases Plaque Fibrosis and Increases Plaque Inflammation in Apolipoprotein-E-Deficient Mice

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Key Words

Atherosclerosis • Extracellular matrix • Transforming growth factor β • Transglutaminase 2

Abstract

Aim: Transglutaminase 2 (TG2) is important for the deposition and stability of the extracellular matrix via effects on cross-linking of matrix proteins and transforming growth factor β (TGF β) activity. The purpose of this study was to investigate the effect of TG2 deficiency on the composition of atherosclerotic plaques. **Methods:** Apolipoprotein E (ApoE)^{-/-} mice were crossbred with TG2^{-/-} mice to obtain ApoE^{-/-}TG2^{-/-} mice. ApoE^{-/-} and ApoE^{-/-}TG2^{-/-} mice were fed a Western-type diet for 16 or 30 weeks to determine the effect of TG2 deficiency on early and advanced atherosclerosis, respectively. **Results:** Atherosclerotic plaques of ApoE^{-/-}TG2^{-/-} mice showed decreased cross-linking of matrix proteins, as well as decreased nuclear staining for phospho-Smad2/-Smad3, indicative of decreased TGF β activity. Compared to ApoE^{-/-} mice, plaque area was decreased by 45 and 48% in ApoE^{-/-}TG2^{-/-} mice after 16 and 30 weeks, respectively. Sirius red staining showed a significant decrease

in collagen content in early and advanced atherosclerotic plaques of ApoE^{-/-}TG2^{-/-} mice. Furthermore, there was a significant increase in macrophages in advanced atherosclerotic plaques of ApoE^{-/-}TG2^{-/-} mice. **Conclusion:** TG2 deficiency resulted in a decreased collagen content and increased inflammation, which are features of a more unstable plaque.

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Introduction

Most acute coronary syndromes are caused by rupture of thin cap fibroatheromas [1]. These lesions are characterized by a thin fibrous cap overlying a large necrotic core. Progressive thinning of the fibrous cap increases the risk of rupture, which leads to exposure of the necrotic core to the blood and luminal thrombosis. Two mechanisms contribute to the process of plaque rupture. First, matrix synthesis is diminished because of decreased smooth muscle cell content. Secondly, matrix breakdown is increased as a result of overproduction of proteases by inflammatory cells. Therefore, the extracellular matrix

(ECM) plays a crucial role in the stability of the atherosclerotic plaque.

Transglutaminase 2 (TG2) belongs to the family of transglutaminases, of which nine members have been identified [2]. TG2 catalyzes the Ca^{2+} -dependent formation of isopeptide linkage between the γ -carboxamide group of a glutamine residue and the ϵ -amino group of a lysine residue. These $\text{N}^{\epsilon}(\gamma\text{-glutamyl})$ lysine bonds are highly resistant to proteolytic and chemical degradation. Cross-linking of matrix proteins, such as fibronectin and collagen, leads to increased deposition and stability of the ECM. TG2 is also important for targeting of latent transforming growth factor β (TGF β) to the ECM. Inhibition of TG2 has been associated with impaired TGF β activity [3, 4]. TGF β regulates a wide variety of cellular processes, including ECM production. Binding of active TGF β to TGF β type I and type II receptors induces the phosphorylation of Smad2 and Smad3. Phospho-Smad2 and -Smad3 associate with Smad4 and translocate to the nucleus, where they interact with different transcription factors to mediate the effects of TGF β [5]. TG2 can therefore contribute to ECM accumulation, either directly through the cross-linking of matrix proteins or indirectly by addressing latent TGF β to the ECM. Indeed, increased TG2 expression has been associated with a number of fibrotic diseases of the lung, kidney and heart [6–8].

Previous studies have shown that active TG2 is present in human atherosclerotic plaques, especially in the shoulder regions and the fibrous cap [9, 10]. TG2 might have an important role in plaque stability through effects on matrix cross-linking and TGF β activity. Therefore, the aim of this study was to investigate the effect of TG2 deficiency on atherosclerotic plaque composition in apolipoprotein E (ApoE)-deficient mice.

Material and Methods

Mice

TG2^{-/-} mice (mixed Svj129-C57Bl/6 background, kind gift of G. Melino, University of Rome, Tor Vergata, Italy) were crossbred with ApoE^{-/-} mice (C57Bl/6 background, Charles River). The resulting heterozygous ApoE^{+/-}TG2^{+/-} mice were crossbred with each other to obtain ApoE^{-/-} and ApoE^{-/-}TG2^{-/-} mice, both on a mixed Svj129-C57Bl/6 background. The genotype was determined by PCR, as previously described [11]. Female ApoE^{-/-} and ApoE^{-/-}TG2^{-/-} mice (4 weeks old) were fed a Western-type diet (Harlan Teklad, TD88137) for 16 or 30 weeks. Mice were euthanized by an overdose of pentobarbital. One day before sacrifice, phenylephrine (8 $\mu\text{g}/\text{kg}$ body weight, i.v., Sigma) was administered in a limited number of mice (4 ApoE^{-/-} and 4 ApoE^{-/-}

TG2^{-/-} mice) after 30 weeks of Western-type diet to investigate the effect of hemodynamic stress on atherosclerotic plaque integrity. All experiments were approved by the Ethical Committee of the University of Antwerp.

Plasma Total Cholesterol and Triglyceride Concentrations

At the time of euthanasia, blood samples were obtained by puncture of the retro-orbital plexus for the determination of total cholesterol and triglycerides by commercially available kits (Randox).

Histological Analysis of Atherosclerotic Plaques

The heart and the brachiocephalic artery were fixed in 4% formaldehyde (pH 7.4), dehydrated overnight and embedded in paraffin. Serial cross-sections (5 μm) throughout the entire aortic valve area were prepared for histological analysis. Total plaque area in the aortic root (mean of 3 sections per mouse at 75- μm intervals, encompassing the lower, middle and upper parts of the aortic valve cusps) was measured on hematoxylin-eosin-stained slides. Plaque composition of aortic atherosclerotic plaques was analyzed by immunohistochemistry with the following primary antibodies: anti- α -smooth muscle actin (Sigma), anti-Mac-3 (Pharmingen), anti-phospho-Smad2/-Smad3 (Ser465–467, Cell Signaling), anti-factor XIII (AbD Serotec) and anti-TG2 (Lab Vision). After primary antibody incubation, specimens were incubated with species-appropriate HRP-conjugated secondary antibodies (Vector Laboratories) followed by 60 min of reactive ABC (Vector Laboratories). Immunocomplexes were detected using 3,3'-diaminobenzidine or 3-amino-9-ethyl-carbazole. The $\text{N}^{\epsilon}(\gamma\text{-glutamyl})$ lysine cross-links were visualized by a primary antibody against $\text{N}^{\epsilon}(\gamma\text{-glutamyl})$ lysine (Abcam), using the ARK peroxidase kit (Dako). The association between TG2 and TG2-mediated cross-links was examined in adjacent sections of atherosclerotic plaques in ApoE^{-/-} mice. For the detection of oligonucleosomal DNA cleavage, a stringent TUNEL (terminal deoxynucleotidyl transferase end labeling) technique was used [12]. Sirius red staining was used for collagen detection. The necrotic core was defined as a hypocellular plaque cavity devoid of collagen and containing necrotic debris and cholesterol clefts. All plaque components were expressed as percent of total plaque area.

The number of buried fibrous caps was counted on sections of the proximal brachiocephalic artery, immunostained for α -smooth muscle actin. Buried fibrous caps were defined as smooth muscle cell-rich layers, covered by overlying plaque [13]. Acute plaque ruptures were defined as a visible defect in the cap, accompanied by intrusion of erythrocytes into the plaque [13]. All images were analyzed using a color image analysis system (Image Pro Plus 4.1, Media Cybernetics).

In vitro Phagocytosis Assay

Resident peritoneal macrophages were obtained by lavage of ApoE^{-/-} (n = 8) and ApoE^{-/-}TG2^{-/-} (n = 9) mice with PBS containing 10 U/ml heparin. Macrophages were cultured in RPMI 1640 supplemented with 10% FBS for 24 h. Non-adherent cells were removed by washing. To generate apoptotic cells, U937 monocytes were incubated with etoposide (50 μM) for 4 h and subsequently labeled with CellTracker CMTPX Red (10 μM , Molecular Probes). Macrophages were then incubated with apoptotic cells, and phagocytosis was allowed to proceed for 1 h. The phagocytosis capacity was determined by flow cytometry [14].

Table 1. Characteristics of ApoE^{-/-} and ApoE^{-/-}TG2^{-/-} mice after 16 and 30 weeks of Western-type diet

Characteristics	16 weeks		30 weeks	
	ApoE ^{-/-} (n = 9)	ApoE ^{-/-} TG2 ^{-/-} (n = 9)	ApoE ^{-/-} (n = 13)	ApoE ^{-/-} TG2 ^{-/-} (n = 20)
Body weight, g	34.2 ± 1.0	32.6 ± 1.5	34.7 ± 1.4	33.6 ± 1.0
Spleen weight, mg	179 ± 32	190 ± 30	265 ± 33	232 ± 20
Total cholesterol, mg/dl	1,910 ± 260	2,207 ± 246	1,597 ± 240	1,762 ± 220
Triglycerides, mg/dl	514 ± 37	567 ± 43	421 ± 71	360 ± 70
Plaque area, × 10 ³ μm ²	344 ± 32	190 ± 15***	616 ± 22	322 ± 15***
N ^ε (γ-glutamyl) lysine cross-links positive area, %	9.3 ± 2.2	0.9 ± 0.4**	12.9 ± 3.3	1.9 ± 0.6***
Factor XIII-positive area, %	0.9 ± 0.3	1.0 ± 0.3	1.8 ± 0.5	2.0 ± 0.5
Sirius red-positive area, %	70.2 ± 2.1	48.1 ± 2.2***	71.8 ± 1.6	58.8 ± 1.9***
α-Actin-positive area, %	2.4 ± 0.2	2.4 ± 0.3	2.1 ± 0.3	2.0 ± 0.3
Mac-3-positive area, %	7.4 ± 1.1	7.8 ± 1.5	7.8 ± 0.8	16.6 ± 1.5***
TUNEL-positive area, %	0.18 ± 0.15	0.27 ± 0.18	0.18 ± 0.09	0.29 ± 0.17
Necrotic core area, %	5.7 ± 1.4	7.4 ± 2.5	7.2 ± 1.0	8.9 ± 1.7

Data are expressed as means ± SEM. ** p < 0.01, *** p < 0.001.

Statistical Analysis

Results are presented as means ± SEM. For comparison of group means, an unpaired, 2-tailed Student's t test was performed. Discontinuous data (number of buried fibrous caps) were analyzed by the Mann-Whitney test. The relationship between the expression of TG2 and cross-links was assessed using crosstabulation and χ^2 test (SPSS). p < 0.05 was considered significant.

Results

Characteristics of ApoE^{-/-}TG2^{-/-} Mice

To investigate the effect of TG2 deficiency on early and advanced atherosclerosis, ApoE^{-/-} and ApoE^{-/-}TG2^{-/-} mice (both on a mixed Svj129-C57Bl/6 background) were fed a Western-type diet for 16 and 30 weeks, respectively. In the early atherosclerosis groups, there were no premature deaths of ApoE^{-/-} or ApoE^{-/-}TG2^{-/-} mice. In the advanced atherosclerosis groups, 1 of 14 ApoE^{-/-} mice and 2 of 22 ApoE^{-/-}TG2^{-/-} mice died prematurely. The characteristics of ApoE^{-/-} and ApoE^{-/-}TG2^{-/-} mice are shown in table 1. ApoE^{-/-} and ApoE^{-/-}TG2^{-/-} mice had a similar body weight at 16 and 30 weeks. Also, plasma concentrations of total cholesterol and triglycerides were not affected by TG2 deficiency. There were no signs of autoimmunity (e.g. lymphadenopathy or splenomegaly) at the time of euthanasia.

Because TG2 is important for the phagocytosis of apoptotic cells, we compared the phagocytosis capacity of apoptotic cells by peritoneal macrophages of ApoE^{-/-} mice and ApoE^{-/-}TG2^{-/-} mice. After incubation with fluorescently labeled apoptotic cells, macrophages of ApoE^{-/-}TG2^{-/-} mice showed decreased mean fluorescence compared to macrophages of ApoE^{-/-} mice (154 ± 12 for ApoE^{-/-}TG2^{-/-} mice vs. 230 ± 31 for ApoE^{-/-} mice, p < 0.05). The decreased mean fluorescence indicates impaired phagocytosis of apoptotic cells in ApoE^{-/-}TG2^{-/-} mice.

TG2 Deficiency Leads to Decreased Cross-Linking of ECM Proteins

TG2 was strongly expressed in atherosclerotic plaques of ApoE^{-/-} mice, but was absent in atherosclerotic plaques of ApoE^{-/-}TG2^{-/-} mice (fig. 1). In plaques of ApoE^{-/-} mice, TG2 expression was strongly associated with the presence of TG2-mediated cross-links (Fisher's exact test, p < 0.05). The absence of TG2 resulted in a significant decrease in N^ε(γ-glutamyl) lysine cross-links in atherosclerotic plaques of ApoE^{-/-}TG2^{-/-} mice (fig. 1, table 1). The expression of another transglutaminase, factor XIII, was examined because a residual amount of N^ε(γ-glutamyl) lysine cross-links was present in ApoE^{-/-}TG2^{-/-} mice. Factor XIII was expressed in atherosclerotic plaques of both ApoE^{-/-} and ApoE^{-/-}TG2^{-/-} mice (fig. 1, table 1) and colocalized with macrophages (fig. 2).

Fig. 1. Immunohistological analysis of TG2, N ϵ (γ -glutamyl) lysine cross-links and factor XIII in atherosclerotic plaques of ApoE $^{-/-}$ and ApoE $^{-/-}$ TG2 $^{-/-}$ mice after 30 weeks of Western-type diet. TG2 is strongly expressed in atherosclerotic plaques of ApoE $^{-/-}$ mice. In atherosclerotic plaques of ApoE $^{-/-}$ TG2 $^{-/-}$ mice,

the absence of TG2 is associated with decreased staining of N ϵ (γ -glutamyl) lysine bonds. The deficiency in TG2 in atherosclerotic plaques of ApoE $^{-/-}$ TG2 $^{-/-}$ mice is partially compensated by the expression of factor XIII. Scale bar = 100 μ m.

TG2 Deficiency Leads to Decreased TGF β Activity in Atherosclerotic Plaques

Because TG2 is important for the regulation of TGF β activity, the expression of phospho-Smad2/-Smad3, a marker of TGF β activity, was studied. Compared to ApoE $^{-/-}$ mice, there was a significant decrease in phospho-Smad2/-Smad3-positive nuclei in early and advanced atherosclerotic plaques of ApoE $^{-/-}$ TG2 $^{-/-}$ mice (fig. 3), indicative of a decreased TGF β activity in atherosclerotic plaques of mice lacking TG2.

TG2 Deficiency Is Associated with Decreased Plaque Fibrosis and Increased Macrophage Content in Advanced Atherosclerotic Plaques

To investigate the effect of decreased cross-linking and decreased TGF β activity on the atherosclerotic process, the area and composition of atherosclerotic plaques at the aortic valves were compared between ApoE $^{-/-}$ and ApoE $^{-/-}$ TG2 $^{-/-}$ mice. Total plaque area was decreased by 45 and 48% in ApoE $^{-/-}$ TG2 $^{-/-}$ mice after 16 and 30 weeks of Western-type diet, respectively (table 1). TG2 deficiency

Fig. 2. Colocalization of macrophages and factor XIII in atherosclerotic plaques of ApoE^{-/-}TG2^{-/-} mice. Immunofluorescence double staining shows colocalization (arrowheads) between macrophages and factor XIII, indicating that macrophages are the main source of factor XIII. Elastic lamellae of the media exhibit green autofluorescence.

Fig. 3. Number of phospho-Smad2/-Smad3-positive nuclei in atherosclerotic plaques of ApoE^{-/-} mice (n = 9 at 16 weeks; n = 13 at 30 weeks) and ApoE^{-/-}TG2^{-/-} mice (n = 9 at 16 weeks; n = 20 at 30 weeks). Compared to ApoE^{-/-} mice, the percentage of phospho-Smad2/-Smad3-positive nuclei was significantly decreased in early and advanced atherosclerotic plaques of ApoE^{-/-}TG2^{-/-} mice. *** p < 0.001. Scale bar = 100 μ m.

Fig. 4. Collagen content in atherosclerotic plaques of ApoE^{-/-} mice (n = 9 at 16 weeks; n = 13 at 30 weeks) and ApoE^{-/-}TG2^{-/-} mice (n = 9 at 16 weeks; n = 20 at 30 weeks). Sirius red staining showed a decrease in the relative amount of collagen in early and advanced atherosclerotic plaques of ApoE^{-/-}TG2^{-/-} mice after 16 and 30 weeks of Western-type diet, respectively. *** p < 0.001. Scale bar = 100 μ m.

cy was also associated with a significant decrease in collagen content in both early and advanced atherosclerotic plaques of ApoE^{-/-}TG2^{-/-} mice (fig. 4). The relative amount of macrophages was not different in early atherosclerotic plaques after 16 weeks of Western-type diet, but increased significantly in advanced plaques of ApoE^{-/-}TG2^{-/-} mice after 30 weeks of Western-type diet (fig. 5). The relative amount of smooth muscle cells, TUNEL-positive apoptotic cells and necrotic core was not affected by TG2 deficiency, both in early and advanced atherosclerotic plaques (table 1).

TG2 Deficiency Does Not Affect the Number of Buried Fibrous Caps

The brachiocephalic artery has been reported to be a site of predilection for the development of vulnerable atherosclerotic plaques in ApoE^{-/-} mice [13]. At this site, the thickness of the fibrous cap was significantly decreased in ApoE^{-/-}TG2^{-/-} mice, both at 16 and 30 weeks (fig. 6a). However, there was no difference in the number of buried fibrous caps between ApoE^{-/-} and ApoE^{-/-}TG2^{-/-} mice (fig. 6b). In addition, we did not observe any acute plaque ruptures, neither spontaneously nor after intravenous administration of phenylephrine.

Fig. 5. Macrophage content in atherosclerotic plaques of ApoE^{-/-} mice (n = 9 at 16 weeks; n = 13 at 30 weeks) and ApoE^{-/-}TG2^{-/-} mice (n = 9 at 16 weeks; n = 20 at 30 weeks). The relative amount of Mac-3-positive macrophages was not different in early atherosclerotic plaques after 16 weeks of Western-type diet, but increased significantly in advanced atherosclerotic plaques of ApoE^{-/-}TG2^{-/-} mice after 30 weeks of Western-type diet. ***p < 0.001. Scale bar = 100 μm.

Discussion

Our results demonstrate that TG2 deficiency in ApoE^{-/-} mice was associated with a decreased amount of ECM in early and advanced atherosclerotic plaques. This effect can be explained by a decreased number of cross-links, which renders the ECM more susceptible to proteolytic breakdown by matrix metalloproteinases. In addition,

lack of TG2 was also associated with a decreased number of phospho-Smad2/-Smad3-positive nuclei, which is suggestive of decreased TGFβ activity. TGFβ can stimulate ECM production in smooth muscle cells [15, 16]. Therefore, decreased TGFβ activity might also have contributed to the decrease in collagen content in ApoE^{-/-}TG2^{-/-} mice.

Fig. 6. Histological analysis of atherosclerotic plaques in the brachiocephalic artery of ApoE^{-/-} mice (n = 9 at 16 weeks; n = 8 at 30 weeks) and ApoE^{-/-}TG2^{-/-} mice (n = 12 at 16 weeks; n = 9 at 30 weeks). **a** Fibrous cap thickness was significantly decreased in atherosclerotic plaques of ApoE^{-/-}TG2^{-/-} mice. **b** The number of buried fibrous caps was not different between ApoE^{-/-} and ApoE^{-/-}TG2^{-/-} mice at 16 and 30 weeks of Western-type diet. *** p < 0.001.

Furthermore, deficiency in TG2 led to a significant increase in macrophage content in advanced atherosclerotic plaques, which was probably related to the decreased TGFβ activity in ApoE^{-/-}TG2^{-/-} mice. TGFβ has important anti-inflammatory properties due to its immunomodulating effects on endothelial cells, smooth muscle cells, macrophages and T cells [5]. Inhibition of TGFβ signaling, either systemically [17, 18] or specifically in T lymphocytes [19, 20], promotes inflammation in atherosclerotic plaques.

Previous studies have shown that TG2 is required for efficient phagocytosis of apoptotic bodies by macrophages [21]. Using flow cytometry, we were able to confirm the impaired phagocytosis of apoptotic cells by peritoneal macrophages of ApoE^{-/-}TG2^{-/-} mice. However, the impaired phagocytosis of apoptotic cells was not associated with an increase in the TUNEL-positive area in atherosclerotic plaques of ApoE^{-/-}TG2^{-/-} mice. In other studies using mice with impaired phagocytosis of apoptotic cells (e.g. mice with a deficiency of lactadherin, Fas ligand and Fas), apoptotic debris accumulated in atherosclerotic plaques [22–24]. The discrepancy with our results suggests that TG2 has only a limited role in the phagocytosis of apoptotic bodies in atherosclerotic plaques. Although TG2 deficiency does not lead to accumulation of apoptotic debris, TG2 is still important for the stabilization of the structure of the dying cell by cross-linking of intracellular proteins [25]. TG2 thereby prevents the leakage of harmful cell content. A deficiency in TG2 can result in the release of proinflammatory components from apoptotic cells, thereby promoting tissue inflammation. These effects could have contributed to the

increase in macrophages in advanced atherosclerotic plaques of ApoE^{-/-}TG2^{-/-} mice. The release of nuclear and cellular components can also trigger autoimmune responses, which can accelerate the atherosclerotic process. TG2^{-/-} mice have been reported to develop autoimmune disease at an advanced age (>1 year) [21]. In our study, there were no signs of autoimmunity (e.g. lymphadenopathy or splenomegaly) in the ApoE^{-/-}TG2^{-/-} mice at the time of euthanasia, making an effect of autoimmunity on atherogenesis unlikely.

Although the plaque area was decreased in ApoE^{-/-}TG2^{-/-} mice, atherosclerotic plaques in mice lacking TG2 had a more vulnerable plaque composition. The occurrence of acute coronary syndromes is more closely related to plaque composition than to plaque size [26]. Both decreased plaque fibrosis and increased plaque inflammation in advanced atherosclerotic plaques of ApoE^{-/-}TG2^{-/-} mice are features of a more rupture-prone plaque. To determine the effect of TG2 deficiency on plaque vulnerability, the number of buried fibrous caps in atherosclerotic plaques of the brachiocephalic artery was counted. Atherosclerotic plaques in the brachiocephalic artery have a multilayered appearance. According to Jackson et al. [27], this appearance is suggestive of ruptured fibrous caps that have been incorporated into the growing lesion. Although the fibrous cap thickness of atherosclerotic plaques in the brachiocephalic artery was significantly reduced in ApoE^{-/-}TG2^{-/-} mice, the number of buried fibrous caps was not different, both at 16 and 30 weeks. There are two possible reasons for the lack of an effect on the number of buried fibrous caps. First, the decrease in fibrous cap thickness in TG2-deficient mice was insuf-

ficient to lead to an increase in plaque rupture. In this regard, it is important to note that other transglutaminases might partially compensate for the deficiency in TG2 [2, 28]. Indeed, factor XIII is expressed in atherosclerotic plaques of ApoE^{-/-} and ApoE^{-/-}TG2^{-/-} mice. Factor XIII is likely derived from macrophages and may be responsible for the limited amount of cross-links in ApoE^{-/-}TG2^{-/-} mice. It is possible that this residual transglutaminase activity is sufficient to prevent plaque rupture in ApoE^{-/-}TG2^{-/-} mice. Secondly, buried fibrous caps are not related to plaque rupture, but rather represent episodic plaque growth. Indeed, the concept of buried fibrous caps is still controversial [29].

In conclusion, our study showed that TG2 plays an important role in the composition of atherosclerotic plaques in ApoE^{-/-} mice. Deficiency in TG2 resulted in decreased plaque fibrosis in early and advanced atherosclerotic

plaques of ApoE^{-/-}TG2^{-/-} mice. In addition, there was an increase in macrophage content in advanced atherosclerotic plaques of ApoE^{-/-}TG2^{-/-} mice. These effects of TG2 deficiency can be explained by decreased cross-linking of matrix proteins and diminished TGFβ activity in atherosclerotic plaques of ApoE^{-/-}TG2^{-/-} mice.

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