Homocysteine Induces Endothelial Cell Detachment and Vessel Wall Thickening During Chick Embryonic Development

Marit J. Boot, Régine P.M. Steegers-Theunissen, Robert E. Poelmann, Liesbeth van Iperen, Adriana C. Gittenberger-de Groot

Abstract—Homocysteine affects the migration and differentiation of neural crest cells in vitro and can result in neural tube defects in vivo. Furthermore, homocysteine has been described as an important determinant in vascular disease in human adults. However, little is known about the effects of homocysteine on the development of embryonic vessels. In this study, we injected homocysteine (30 μmol/L) into the neural tube lumen of chick embryos at the time point of neural crest cell emigration, and analyzed the effects on the neural crest–derived pharyngeal arch arteries, like the brachiocephalic arteries, and the mesoderm-derived arteries, such as the dorsal aorta. By stage HH35, we observed detachment of the endothelium, decreased expression of the extracellular matrix proteins fibrillin-2, and fibronectin in the pharyngeal arch arteries, whereas the dorsal aorta was identical in homocysteine-neural tube–injected and control embryos. No effect of homocysteine on endothelin-1 mRNA expression was observed. By stage HH40, the brachiocephalic arteries of homocysteine-neural tube–injected embryos displayed a decreased lumen diameter, an increased intima- and media-thickness, and an increased number of actin layers compared with the brachiocephalic arteries in control embryos. We propose that homocysteine affects the neural crest–derived smooth muscle cells and their extracellular matrix proteins in the pharyngeal arch arteries, resulting in an abnormal smooth muscle to endothelial cell interaction, leading to endothelial cell detachment. We suggest that, as in adult life, increased homocysteine concentrations lead to vascular damage in the embryo. This prenatal damage might increase the susceptibility to develop vessel pathology later in life. (Circ Res. 2004;94:542-549.)

Key Words: neural crest ■ cardiovascular development ■ pharyngeal arch arteries ■ extracellular matrix proteins

Hyperhomocysteinemia has been associated with premature coronary, cerebral, and peripheral vascular disease in adults.1 A moderate to high homocysteine concentration in blood appears to be a significant factor in the pathogenesis of arteriosclerosis.2 Mechanisms suggested through which homocysteine induces vascular disease are endothelial dysfunction, smooth muscle cell proliferation, and extracellular matrix modification.3 In asymptomatic hyperhomocysteinemic adults, the endothelium-dependent dilation of the brachial artery is significantly decreased compared with control subjects, whereas the endothelium-independent dilation is not affected.4 The endothelium-dependent dilation is mainly due to nitric oxide release by the endothelium, suggesting that the endothelial dysfunction observed in hyperhomocysteinemic adults might be due to reduced or abnormal release or action of nitric oxide.5,6 Studies on baboons have demonstrated that 3 months infusion of high homocysteine concentrations resulted in patchy endothelial desquamation and smooth muscle cell proliferation.7 Furthermore, homocysteine promotes endothelial cell detachment8 and vascular smooth muscle cell proliferation9 in vitro.

The relation between homocysteine and adult vascular disease has been studied in detail; however, the effects of homocysteine on embryonic vessels have not been described. Several clinical studies have shown that high homocysteine concentrations in the blood or amniotic fluid of pregnant women have profound consequences on the developing embryo, especially an increased risk for neural tube defects in the offspring has been observed.10–12 So far, no emphasis has been placed on the vessel wall morphology in offspring of hyperhomocysteinemic mothers. We hypothesize that offspring of hyperhomocysteinemic mothers might have affected vessel walls at the time of birth, making them more susceptible to vessel pathology later in life. We tested the plausibility of this hypothesis in chick embryos. Previous studies have shown that administration of high homocysteine...
concentrations (100 mmol/L, namely 5 μmol in 50 μL) on chick embryos resulted in neural tube defects and cardiac outflow tract defects, and 2.5 mol/L homocysteine (20 μmol in 8 μL) resulted in dorsal root ganglia malformations. However, the malformations were not limited to the nervous and cardiovascular system, whereas these embryos also possessed several external abnormalities including ventral midline closure defects. These previous studies did not describe any vessel wall abnormalities; therefore, our study is the first to focus on the relation between homocysteine and embryonic vessel wall morphology.

The pharyngeal arch arteries give rise to the brachiocephalic arteries and common carotids (third pharyngeal arch arteries), the ascending aorta (fourth pharyngeal arch artery), and both ductus arteriosus segments (sixth pharyngeal arch arteries). Their vessel walls are made up of several cell populations. The inner layer lining the lumen consists of endothelial cells, surrounded by a smooth muscle layer that arises largely from cardiac neural crest cells as shown by cardiac neural crest cell tracing studies. In contrast, the smooth muscle cell layers of the dorsal aorta, the subclavian arteries, the pulmonary arteries, and the coronary arteries completely arise from local mesoderm or the epicardium.

We have recently demonstrated that homocysteine directly stimulates neural crest cell outgrowth and inhibits normal differentiation of neural crest cells in vitro. Using 300 μmol/L homocysteine, we observed a 30% increase in neural crest cell outgrowth and a nearly absent differentiation of neural crest cells in vitro. Neural crest cell outgrowth and differentiation of neural crest cells into smooth muscle cells. Using 30 μmol/L homocysteine, the effect on neural crest cell outgrowth and differentiation was less profound. The in vitro findings gave rise to the question whether homocysteine also affects cardiac neural crest cell behavior in vivo. For the present study, we examined the effects of both 300 and 30 μmol/L homocysteine. The normal homocysteine concentration in human and chick embryos has been described to be between 5 to 15 μmol/L. The 30 μmol/L concentration is comparable to mild to moderate elevation in homocysteine concentrations.

In this study, we analyzed the effects of homocysteine on the cardiac neural crest–derived pharyngeal arch arteries compared with the effects on mesoderm-derived arteries, like the dorsal aorta in chick embryos. Focus was placed on the morphology of the endothelium and smooth muscle cell layers, as well as on the adhesion of the endothelium to the smooth muscle layers. Furthermore, we studied the distribution of matrix proteins fibrillin-2, fibronectin, and elastin in the vessel walls and analyzed the mRNA expression pattern of endothelin-1, a gene involved in vascular tone regulation and patterning of the pharyngeal arch arteries. We analyzed the lumen diameters, vessel wall thickness, thickness of the intima-like layer and the media, and determined the number of actin-positive layers. As a control for the local homocysteine exposure, we also studied general homocysteine exposure by injecting homocysteine (300 and 30 μmol/L) into the circulatory system.

Materials and Methods

Homocysteine Concentration

L-homocysteine thiolactone hydrochloride solutions with concentrations of 30 and 300 μmol/L in M199 medium (Gibco, Invitrogen Corporation) were used in vivo. Indigo carmine blue (0.25 g/mL) was added for visualization of the solution during in vivo injection at Hamburger and Hamilton (HH) stage HH9–10 and HH14.

Injection of Homocysteine Into Chick Embryos

Fertilized specified pathogen-free White Leghorn eggs (local egg supplier) were incubated at 37°C for 33 to 40 hours and windowed at HH9–10. The homocysteine solution with indigo carmine was injected into the lumen of the neural tube at somite level 4 to 6, filling the neural tube in anterior direction until the solution reached the otic placode level as described before. These embryos were called the homocysteine-neural tube–injected (NT) embryos. Previous experiments by our group have shown that injection of an indigo carmine solution in M199 medium does not result in cardiovascular malformations, so the vessel abnormalities we describe in this study can be attributed to homocysteine exposure.

As a control for the local exposure to homocysteine in the neural tube, we also studied general exposure by injecting homocysteine into the circulatory system at HH14 (eggs incubated at 37°C for 54 to 56 hours). Homocysteine (30 or 300 μmol/L) solutions with indigo carmine were injected into the marginal sinus of the yolk sac vasculature, and approximately 1/8 of the yolk sac vasculature and the intraembryonic circulatory system was filled with the homocysteine solution within 3 seconds. The homocysteine solution mixed quickly with the blood present in the circulatory system and was therefore diluted approximately eight times. These embryos were named the homocysteine-circulatory system–injected (CS) embryos.

The neural tube injections were performed at HH9–10 just before cardiac neural crest cell emigration from the neural tube takes place. The circulatory system injections were performed at HH14, the first stage when the circulatory system is a closed circuit, the pharyngeal arch artery system is just established, and the endothelial cells are not yet surrounded by differentiated neural crest cells. After injection, the eggs were sealed with Scotch tape and returned to the incubator for further development.

Tissue Preparation and Immunohistochemistry

We analyzed the following number of embryos: NT (300 μmol/L) HH30–31 (n = 3), HH35 (n = 4); NT (30 μmol/L) HH30–31 (n = 10), HH35 (n = 7), HH40 (n = 6); CS (30 μmol/L) HH35 (n = 4); and CS (300 μmol/L) HH35 (n = 3). The same numbers of control embryos per stage were studied.

The HH30–31 embryos were immersion fixed in 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4). The HH35 and HH40 embryos were first perfused with PFA through the liver, subsequently the thorax was isolated and immersion fixed overnight. The embryos were dehydrated in graded ethanol and embedded in paraffin.

Sections of 5 μm were distributed on glass slides. Alternate sections were immunohistochemically stained using overnight incubation with HHF35 (diluted 1:1000, anti-muscle specific actins, DAKO Denmark), JB3 (diluted 1:2, anti-fibrillin-2, a gift from Dr R.R. Markwald, Medical University of South Carolina, Charleston, SC), or anti-fibronectin (diluted 1:5000, a gift from Dr B.N. Bachra, Leiden University Medical Center, Leiden, The Netherlands) as described before. The sections were microwave-processed to enhance JB3 and fibronectin staining. After the DAB-procedure, the sections were counterstained with Mayer’s hematoxylin, dehydrated, and mounted in Entellan. Sections were stained for elastic fibers using a standard resorcin/fuchsine staining. Sections were stained with Mayer’s hematoxylin for analysis of cell death as described previously.

Endothelin-1 In Situ

A linearized 619-bp chick-endothelin-1 fragment (ET-1, kindly provided by M. Yanagisawa, University of Dallas, Tex) was transcribed using 2.5 MBq 35S-UTP to make a 35S-labeled antisense RNA probe. The concentration of the probe was adjusted to 1×107 counts per minute. The hybridization, stringent wash steps, and coating of the sections with emulsion were performed as described previously.
tube resulted in external malformations, like ventral midline defects and craniofacial defects in 86% of the embryos at HH31–35 (n=7), whereas administration of 30 µmol/L homocysteine resulted in external malformations (ventral midline defect) in 8% of the embryos at HH31–40 (n=25). For this study on vessel development, we only describe the embryos without external malformations that received 30 µmol/L homocysteine solution into the neural tube; this group is called the NT embryos.

Endothelial Cell Detachment and Expression of Fibrillin-2, Fibronectin, and Endothelin-1
HH30–31 NT embryos displayed pharyngeal arch arteries with aberrant endothelial lining. Small areas with one or two detached endothelial cells were observed. By HH35, the areas of detachment of the endothelium in the NT embryos were strongly extended. In six out of seven embryos, severe detachment of the endothelial cell layer from the smooth muscle layer in the pharyngeal arch arteries was observed (Figure 2a). The left and right brachiocephalic arteries (III pharyngeal arch arteries), as well as the common carotid arteries, displayed multiple areas of detached endothelium in the segment between the bifurcation from the ascending aorta and the division into the inner and outer carotid arteries (Figure 2b). However, the adhesion of the endothelium in the subclavian arteries was normal in all embryos (Figure 2b).

Multiple areas of detached endothelium were observed in the ascending aorta, but not in the dorsal aorta (Figure 2c) and not in the coronary arteries. Furthermore, numerous patches of detached endothelium were noted in the proximal parts of the right and left ductus arteriosus (VI pharyngeal arch arteries) (Figures 2d through 2f), as well as in the pulmonary trunk, but not in the pulmonary arteries. The shape of the endothelial cells in detached areas was not squamous but more cuboidal; furthermore, the endothelial cells showed no signs of rupture and appeared to be intact (Figure 2e). We analyzed every 7th section for endothelial detachment and observed that patches of detached cells were on average 15 cells wide. The number of observed cells that were detached from the smooth muscle layer showed a large variation between the embryos (49 to 937 cells per embryo). The patches were distributed equally among the III, IV, and VI pharyngeal arch arteries. Also, 68% of the detached endothelial cells were located in the inner curvature (dorsal side) of the pharyngeal arch arteries and 32% was observed in the outer curvature (ventral side) of the arteries.

Immunostaining for fibrillin-2 was low in the pharyngeal arch artery vessel walls (Figure 2d) and almost absent within the subendothelial layer (Figure 2e) of NT embryos. In contrast, the fibrillin-2 protein expression was abundant in the smooth muscle layers (Figure 2g) and the subendothelium (Figure 2h) of the pharyngeal arch arteries of control embryos. No difference between the fibrillin-2 expression in the dorsal aorta of NT embryos and control embryos was observed.

Fibronectin protein expression was low in the subendothelium of the pharyngeal arch arteries of NT embryos (Figure 2f). In contrast, in the control embryos, a continuous, intensely stained fibronectin layer was observed adjacent to the endothelium of the pharyngeal arch arteries (Figure 2f).
elastin fibers of the pharyngeal arch arteries in control embryos and NT embryos appeared to have the same structure (not shown).

Endothelin-1 mRNA was expressed at similar levels in the endothelium of the pharyngeal arch arteries of control embryos (Figure 2j) and NT embryos (Figure 2k). In the NT embryos, endothelin-1 was expressed both in the attached and detached parts of the endothelium (Figure 2l).

By HH40, the vascular morphology of the pharyngeal arch arteries had changed drastically by the formation of a clearly discernible intima-like layer and the lamellar formation of actin in the media layer. At this stage only small patches of one to three endothelial cells that were detached from the smooth muscle layer were observed in the NT embryos.

As a control for the local exposure to homocysteine (neural tube injections), we also studied the effects of general exposure through the circulatory system. The circulatory system–injected (CS) embryos received 30 or 300 μmol/L homocysteine and were studied at HH35. Patches of endothelial cell detachment were never observed in the CS embryos. However, the endothelial cells seemed to be mildly affected. A few endothelial cells were cuboidal instead of squamous (Figures 3a and 3b). Furthermore the endothelial cell layer was slightly aberrant, whereas an irregular array of nuclei was visible (Figure 3c) instead of a squamous monolayer as observed in control embryos.

Decrease in Lumen Diameter and Fibrillin-2 Expression at HH40

By HH40, the NT embryos showed a decrease in lumen diameter of both left and right brachiocephalic arteries (Figure 4a) compared with control embryos (Figure 4d). The strongest reduction was directly downstream of the bifurcation of the ascending aorta and both brachiocephalic arteries.
At this site, the brachiocephalic arteries were almost completely obliterated (Figure 4a). The lumen diameter of the ascending aorta was also reduced. The left and right ductus arteriosus in the NT embryos showed lumen diameters comparable to those of control embryos (not shown).

Fibrillin-2 expression is strong and regular in the intima-like layer, the media, and the adventitia of the brachiocephalic arteries of control embryos (Figures 4e and 4f), whereas fibrillin-2 expression in the media of the brachiocephalic arteries of NT embryos was only low (Figures 4b and 4c).

No distinct differences between the elastin fibers in the control and NT embryos could be noted. Fibronectin and endothelin-1 mRNA expression were low in both control and NT embryos at HH40. The lumen shape, fibrillin-2 expression, and vessel morphology of the dorsal aorta was not affected by homocysteine treatment.

**Increased Vessel Wall Thickness and Number of Actin-Positive Cell Layers in the Brachiocephalic Arteries of NT Embryos**

We measured the vessel wall thickness at HH35 and HH40 of the dorsal aorta and brachiocephalic arteries at positions indicated in Figure 1 and compared the values of NT embryos with control embryos using a Mann-Whitney test. At HH35, the values were not significantly different.

At HH40, the vessel wall thickness of the dorsal aorta in control and NT embryos was not significantly different either. In contrast, the vessel wall thickness of the brachiocephalic arteries in the NT embryos was significantly (67%, \( P < 0.001 \)) more than in the control embryos (Figure 5). The vessel wall thickness being 262 \( \mu \text{m} \) (IQR 63 \( \mu \text{m} \)) and 157 \( \mu \text{m} \) (IQR 53 \( \mu \text{m} \)), respectively. The values for intima-like layer thickness were 73 \( \mu \text{m} \) (IQR 22 \( \mu \text{m} \)) and 49 \( \mu \text{m} \) (IQR 11 \( \mu \text{m} \)), representing a significant 49% increase (\( P < 0.001 \)) of intima thickness in NT embryos. The media layer thickness was significantly (70%, \( P < 0.001 \)) increased (184 \( \mu \text{m} \), IQR 49 \( \mu \text{m} \)) compared with control embryos (108 \( \mu \text{m} \), IQR 32 \( \mu \text{m} \)). As an additional measurement for media thickness, we counted the actin positive layers. The number of actin layers was significantly (22%, \( P < 0.001 \)) increased (14 layers, IQR 3 layers) compared with control embryos (11.5 layers, IQR 1.75).

The pharyngeal arch arteries of HH35 and HH40 control and NT embryos were analyzed for the frequency of cell death. The number of apoptotic cells was very low and

![Figure 3](http://circres.ahajournals.org/)

**Figure 3.** Left ductus arteriosus (LVI) in homocysteine-circulatory system–injected (CS) embryos stained for fibrillin-2 (fib-2). a, No endothelial cell detachment was observed in CS embryos treated with 30 \( \mu \text{mol/L} \). Area between arrows shows cells with a cuboidal instead of squamous shape. b, Higher magnification of a, cuboidal endothelial cells between arrows. c, 300 \( \mu \text{mol/L} \) CS resulted in a slightly irregular-shaped endothelial layer, an irregular array of nuclei is visible. Bar in a=150 \( \mu \text{m} \); bars in b and c=75 \( \mu \text{m} \).

![Figure 4](http://circres.ahajournals.org/)

**Figure 4.** a through c, Brachiocephalic artery of NT embryo (HH40). a, Section stained for actin using the HHF35 antibody, showing narrow, slit-like lumen (arrows) surrounded by the intima-like layer (I) and actin lamella in the media (M). b, Fibrillin-2 (Fib-2) is expressed in the intima-like layer and adventitia, however only very low in the media. c, Higher magnification of b, showing low fibrillin-2 expression in the media. d through f, Brachiocephalic artery of control embryo (HH40). d, Open lumen surrounded by the intima-like layer and actin lamella in the media. e, Fibrillin-2 is expressed intensely and uniformly in the vessel wall. f, Fibrillin-2 fibers are noted between the smooth muscle cell layers of the media. Bars in a and d=150 \( \mu \text{m} \); bars in b and e=300 \( \mu \text{m} \); bars in c and f=75 \( \mu \text{m} \).
Discussion

Our study demonstrates that homocysteine affects the cardiac neural crest cell–derived pharyngeal arch arteries in the developing chick embryo. Endothelium detachment, decreased fibrillin-2 and fibronectin expression, and thickening of the vessel wall were observed in the neural crest–derived pharyngeal arch arteries, but not in the mesoderm-derived dorsal aorta, coronary arteries, subclavian arteries, or pulmonary arteries. Endothelium detachment in relation to homocysteine has not been described in embryos before. The embryos displayed a large variation in the extent of endothelium detachment, this might be explained by the biological variance between embryos. Because endothelial cell detachment was never observed in any of the control embryos, it is of special interest that the NT embryos displayed endothelial detachment. The majority of the endothelial cell detachment (68%) was observed on the inner curvature of the pharyngeal arch arteries. The difference between the inner and outer curvature might suggest a relation between severity of endothelial cell detachment and hemodynamics. Studies in adult baboons did show endothelium desquamation, but only after months of hyperhomocysteinemia. Oxidative stress and disruption of calcium-dependent cell adhesions were proposed as mechanisms through which homocysteine damages the endothelium. However, the results from our studies reveal that the embryonic endothelium detachment cannot be explained by a general effect of homocysteine thiolactone only, because in that case we would expect all vessels to show endothelial detachment and a similar morphology of the pharyngeal arch arteries in the NT embryos and the CS embryos.

We propose that endothelium detachment results from an abnormal interaction between the differentiated cardiac neural crest cells in the smooth muscle cell layers and the endothelium, resulting in an abnormal distribution of extracellular matrix proteins. During normal development, extracellular matrix proteins, like fibronectin and fibrillin-2, are synthesized by smooth muscle cells and their immediate precursors, and these proteins play a role in the adherence of the endothelium to the smooth muscle cell layers. In the NT embryos, both fibrillin-2 and fibronectin expression in the pharyngeal arch arteries were decreased. Our findings on a relation between endothelium detachment and fibronectin are supported by in vitro studies that mildly increased homocysteine concentrations do not result in endothelial detachment when the cells are cultured on fibronectin-coated plastic, but do result in endothelium detachment when cells are cultured on noncoated plastic. Furthermore, Bellamy and McDowell speculated that the similarities between hyperhomocysteinemic patients and patients with Marfan syndrome (mutation in fibrillin gene) might suggest that homocysteine may interfere with fibrillin distribution. Our findings support a relation between homocysteine and fibrillin-2 distribution.

In vitro studies have demonstrated that homocysteine decreases endothelin-1 production in endothelial cells. However, the endothelin-1 mRNA expression in the detached endothelium of NT embryos at HH35 was not downregulated. Furthermore, endothelin-1 mRNA expression was low in both control and NT embryos at HH40, indicating that the decreased lumen diameter was not due to a change in the expression of vasoconstrictor gene endothelin-1. The reduction in lumen diameter was observed in the brachiocephalic arteries and the ascending aorta, but not in the also neural crest–derived ductus arteriosus segments. This could be related to the phenotype of these pharyngeal arch arteries. The left and right ductus arteriosi are the only pharyngeal arch arteries in chick embryos to develop a muscular phenotype concomitant with a low number of elastic fibers, compared with an elastic phenotype with numerous elastic lamellae. The reduction in lumen diameter might, therefore, be related to a changed functionality of the elastic lamellae. A relation between homocysteine and elastic fibers is supported by studies in human vessels where the most intense effects of homocysteine were observed on the aorta and carotids, which were explained by the high number of elastic laminae in these vessels. However, in our study we did not observe any changes in the structures of the elastic fibers.

Our studies are the first to show endothelium detachment as a result of elevated homocysteine concentrations during embryonic development. Only fetal studies in human describe endothelium detachment as a physiological process in closure of the ductus arteriosus. During this process, endothelial cells separate and invaginate creating a wide subendothelial region, followed by intimal thickening and contraction of the smooth muscle cell layer.

In the NT embryos at HH40, only small areas of endothelial detachment were observed, whereas no abnormal embryo lethality was observed between HH35 and HH40, suggesting...
that the severe endothelium detachment phenotype evolved into another phenotype. By HH40, the brachiocephalic vessel wall thickness was increased by 67%. Only very low numbers of apoptotic cells were observed in the pharyngeal arch arteries of control and NT embryos at HH35 and HH40, suggesting that the increase in vessel wall thickness in not due to a change in apoptotic rates. In the brachiocephalic arteries both the thickness of the intima and the media were increased; this might suggest that endothelium detachment stimulated proliferation of the cells in both the intima and the media layer. Furthermore, the deposition of elastin is probably slightly increased, whereas the ratio media thickness/number of actin layers is 13.1 in NT embryos and 9.4 in control embryos.

Several clinical studies have reported a relation between hyperhomocysteinemia and increased carotid arterial wall thickness in adults. Increased carotid wall thickness has been shown to be a predictor for the occurrence of plaques and early atherosclerosis. In addition, the theory of atherogenesis as a response to injury has described endothelial cell injury as the initiating event, which might be followed by an inflammation response, leading to decreased membrane fluidity affecting the attachment of foam cells to the endothelium, eventually resulting in the manifestation of atherosclerosis. Excessive amounts of homocysteine have been established as a major independent risk factor for arteriosclerosis in adults. Our findings are the first available data that homocysteine can cause endothelium detachment and vessel wall thickening in embryos. The observation that homocysteine results in embryonic endothelial injury might suggest that these embryos will be more susceptible to develop vessel pathology later in life. Maternal hypercholesterolemia has been described to, at least in part, increase the susceptibility to atherosclerosis as observed in their offspring later in life. During recent years, more emphasis has been placed on pathophysiological events occurring during fetal development as factors affecting development of atherosclerosis throughout childhood and adolescence.

Hyperhomocysteinemia with an estimated prevalence of 1:70 in the general population is not a rare phenomenon, this means large numbers of pregnant women will expose their yet unborn offspring to hyperhomocysteinemic concentrations. Further studies are necessary to address the impact of high maternal homocysteine concentrations on embryonic vessel morphology.

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References


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