Apoptosis and Related Proteins in Different Stages of Human Atherosclerotic Plaques

Mark M. Kockx, MD, PhD; Guido R.Y. De Meyer, PhD; Johannes Muhring, BSc; Willem Jacob, PhD; Hidde Bult, PhD; Arnold G. Herman, MD, PhD

Background—The transition of a fatty streak into an atherosclerotic plaque is characterized by the appearance of focal and diffuse regions of cell death. We have investigated the distribution of apoptotic cell death and apoptosis-related proteins in early and advanced atherosclerotic lesions.

Methods and Results—Human atherosclerotic plaques were studied by whole-mount carotid endarterectomy specimens (n=18). This approach allowed comparison of adaptive intimal thickenings, fatty streaks, and advanced atherosclerotic plaques of the same patient. The fatty streaks differed from adaptive intimal thickenings by the presence of BAX (P<0.01), a proapoptotic protein of the BCL-2 family. Both regions were composed mainly of smooth muscle cells (SMCs), and macrophage infiltration was low and not different. Apoptosis, as detected by DNA in situ end labeling (terminal deoxynucleotidyl transferase end labeling [TUNEL] and in situ nick translation) was not present in these regions. Apoptosis of SMCs and macrophages, however, was present in advanced atherosclerotic plaques that were present mainly in the carotid sinus. A dense infiltration of macrophages (5.8±3% surface area) was present in these advanced atherosclerotic plaques. Cytoplasmic remnants of apoptotic SMCs, enclosed by a cage of thickened basal lamina, were TUNEL negative and remained present in the plaques as matrix vesicles.

Conclusions—We conclude that SMCs within human fatty streaks express BAX, which increases the susceptibility of these cells to undergo apoptosis. The localization of these susceptible SMCs in the deep layer of the fatty streaks could be important in our understanding of the transition of fatty streaks into atherosclerotic plaques, which are characterized by regions of cell death. Matrix vesicles are BAX-immunoreactive cytoplasmic remnants of fragmented SMCs that can calcify and may be considered the graves of SMCs that have died in the plaques. (Circulation. 1998;97:2307-2315.)

Key Words: atherosclerosis ■ apoptosis ■ cholesterol ■ carotid arteries ■ muscle, smooth ■ cells

Recent work by our and other laboratories demonstrates the presence of apoptotic cell death in human and experimental atherosclerotic plaques. Most of these studies use DNA in situ end-labeling techniques (TUNEL or ISNT) to detect apoptotic cell death within the plaques. If specific labeling is avoided by optimization of the enzyme concentration and use of a calcium chelating agent, low levels of apoptotic cell death (never >2%) were found in the plaques. Human atherosclerotic plaques display a pronounced morphological variability in the different regions. Recently, it was demonstrated that apoptosis in atherosclerotic plaques is associated with macrophage infiltration, whereas lesions consisting only of SMCs present very little apoptosis, as demonstrated by the TUNEL technique. DNA fragmentation is a rather late stage of apoptotic cell death. Apoptosis occurs in at least two stages. After a signal, which may be either intrinsic or extrinsic to the cell, the cell enters a committed phase. This is terminated in cell autonomous fashion by a transition to a final execution phase. The latter, which includes DNA fragmentation, is brief and decisive.

Bennet et al have found that most smooth muscle derived from atherosclerotic plaques but not of the media die when brought in culture. This suggests that the SMCs of the atherosclerotic plaques but not the medial SMCs are committed to die.

In the present study, regional differences between the committed phases and the executive phases of apoptosis were studied in whole-mount human atherosclerotic plaques and the adjacent nonatherosclerotic intima. The executive phase of apoptosis was detected by DNA in situ end labeling. Changes in the expression of proteins of the BCL-2 family were used to detect the committed phase.

Methods

Human carotid endarterectomy specimens were obtained from patients (n=18) who had a carotid stenosis >70% as demonstrated by digital subtraction angiography and Duplex. The patients (9 women and 9 men) were normocholesterolemic and had a mean age of 72±5 years. The specimens were fixed in 4% formalin and opened along their longitudinal axes, and complete longitudinal sections of the paraffin-embedded specimens were mounted on APES (3-aminopropytriethoxysilane)

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Selected Abbreviations and Acronyms

<table>
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<tr>
<td>AEC</td>
<td>3-amino-9-ethyl carbazole</td>
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<td>ICE</td>
<td>caspase 1</td>
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<td>ISNT</td>
<td>in situ nick translation</td>
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<td>PAS</td>
<td>periodic acid–Schiff</td>
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<td>SMC</td>
<td>smooth muscle cell</td>
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<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase end labeling</td>
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Immunohistochemistry

The following primary monoclonal antibodies were used: α-SMC actin from Sigma Chemical Co; CD 68 (anti-macrophage), LCA (common leukocytic antigen CD-45), and BCL-2 from Dako, Glostrup, Denmark; BAX from Pharmingen, San Diego, Calif; and Ki67 from Immunotech, Marseille, France.

The following polyclonal antibodies were used: BAX, ICE, and BCL-2 from Santa Cruz and fibrinogen from Cappel. All antibodies were diluted in PBS. The monoclonal antibodies were detected by an indirect peroxidase antibody conjugate technique. The sections were incubated with a goat anti-mouse peroxidase antibody (Jackson) for 45 minutes. The polyclonal antibodies were detected by a PAP complex. For demonstration of the complex, AEC was used as a chromogen.

The specificity of the immunohistochemical reactions was checked by omitting the primary antibody and substituting an unrelated antibody at the same concentration. Both antibodies (monoclonal and polyclonal) against BAX and BCL-2 gave identical results.

DNA In Situ End Labeling

After deparaffinization and rehydration, tissue sections were incubated with 3% citric acid. This step removes all small calcium-containing vesicles that can be responsible for aspecific binding of the nucleotides. Both the TUNEL and ISNT techniques were used. For the TUNEL technique, ApopTagkit/Oncor (Gaithersburg) was used with minor modifications. For the ISNT technique, the sections were rinsed in a buffer (Tris-HCl 50 mmol/L, MgCl2 5mmol/L, BSA 0.0005%, pH 7.5) for 10 minutes, dried, and later incubated at 37°C for 1 hour with the same buffer containing 0.01 mmol/L dATP, dCTP, and dGTP (Sigma) and 0.01 mmol/L biotin-16 d-UTP (Boehringer Mannheim) with 20 U/mL of the Klenow fragment of DNA polymerase I (Boehringer Mannheim). Incorporated biotin-16 d-UTP was demonstrated by incubating the sections with a monoclonal antibody against biotin (Dako, Glostrup) at a dilution of 1/40 for 30 minutes. The antibody was visualized by a goat anti-mouse peroxidase (Jackson) at a dilution of 1/125 for 45 minutes.

In both the TUNEL and the ISNT techniques, the labeled antibody was visualized by AEC. Sections were lightly counterstained with hematoxylin and mounted in glycerin jelly. Negative controls included omission of terminal deoxynucleotidyl transferase or the Klenow fragment from the labeling mixture. Tonsils were used as a positive control.

To identify cell types undergoing apoptosis, double staining was performed by combining TUNEL and immunohistochemistry for CD-68 and α-SMC actin. Therefore, we used DAB instead of AEC as a chromogen. Moreover, we combined the TUNEL technique with a PAS stain.

Definitions

The following definitions are based on the definitions of the American Heart Association Medical/Scientific Statement.5

Adaptive Intimal Thickening

We have defined adaptive intimal thickening as the accumulation of SMCs between the endothelium and the media. The SMCs are oriented mainly in the longitudinal direction and are immunoreactive for α-SMC actin. The cytoplasm of the smooth muscle does not show lipid vacuoles. Macrophages, as detected by their expression of CD-68, are not present in this layer.

Fatty Streaks

This layer was defined as adaptive intimal thickening with lipid deposition. Lipid deposition was detected by the presence of small vacuoles in the cytoplasm of the SMCs. This was confirmed on adjacent sections stained with a Scharlach red fat stain. The percent of the total area that was immunoreactive for macrophages (CD-68) was <1%. Regions of pronounced cell loss were not present in this layer. It should be noted that the term “fatty streak” covers a spectrum of lesions from those consisting almost entirely of macrophages to those that are composed entirely of SMCs, with every gradation in between. The fatty streaks in our study were composed mainly of SMCs.

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These regions were defined by the presence of foci of pronounced cell loss. These regions were defined as necrotic cores. The cores could be detected by the presence of fibrin(ogen). The region between the endothelium and the necrotic core was defined as the fibrous cap. Unstable atherosclerotic plaques were defined by the presence of numerous foam cells of macrophage origin within the fibrous caps.

Quantification

The images were analyzed with a color image analysis system (PC Image Color, Foster Findlay Associates). Each whole-mount carotid endarterectomy specimen, consisting of a complete cast of the atherosclerotic carotid bifurcation, was projected, and the contours were drawn on paper. This allows mapping of the atherosclerotic plaques present in each specimen (Figure 1). Subsequently, 15

Figure 1. Example of a whole-mount carotid endarterectomy specimen used in this study. The specimen, consisting of a complete cast of the atherosclerotic carotid bifurcation, was projected, and the contours were drawn on paper (white lines). The specimens contained the inner wall of the distal common carotid artery (communis), proximal part of the external carotid artery (externa), and carotid sinus. The specimen also contained adjacent inner media that was cleaved during the surgical procedure in the communis region and at the flow divider (blue region). In each section, advanced atherosclerotic plaques (red regions) alternate with regions of fibromuscular intimal thickening (green region) and fatty streaks (orange region).
regions (5 different regions in triplicate), each 65 x 100 μm, from each carotid endarterectomy specimen were quantitatively analyzed. These 5 different regions were located in the media (n = 3), adaptive intimal thickening (n = 3), fatty streak (n = 3), fibrous caps (n = 3), and necrotic cores (n = 3). For each region, the percent immunoreactive areas for α-SMC actin, CD-68, LCA, and BAX were measured. The latter variables were expressed as percent of the total area.

Transmission Electron Microscopy

The fragments for transmission electron microscopy were fixed for 2 hours in 1% (vol/vol) glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.4). They were postfixed for 30 minutes in 1% (vol/vol) osmium tetroxide in 0.1 mol/L sodium cacodylate buffer (pH 7.4). After dehydration in an ethanol gradient, they were embedded in LX-112 (Ladd Research Industries). Selection of the zones most representative for the lesions was made from 2-μm sections oriented in a transverse plane (perpendicular to the bloodstream) and stained with toluidine blue. Sections (50 nm thick) were cut on an Ultratome Nova (Reichert-Jung). They were stained for 30 minutes at 40°C with uranyl acetate and for 15 minutes at 20°C with lead citrate in an Ultrostainer 2168 (LKB). The sections were examined in a Jeol-1200 EX transmission electron microscope at 80 kV. Photographs were made with electron microscopy film 4489 Estar Thick Base (Kodak).

Statistical Analysis

Data are expressed as mean±SEM. The five different regions were compared for their BAX immunoreactivity by use of one-way ANOVA followed by the Tamhane’s T2 test. The SPSS package for Windows (SPSS Inc) was applied for these purposes. A 1% level of significance was selected.

Results

Human Atherosclerotic Plaques (n = 18)

The carotid endarterectomy specimens were cut longitudinally, and whole-mount sections were made of all cases. The specimens were complete casts of carotid bifurcations and consisted of intima and atherosclerotic plaques of the communis part, the proximal part of the external carotid artery, and the carotid sinus. This approach allows a comparative study of the different stages of atherosclerotic plaques present in each specimen. A trichrome-Masson stain showed that the cell density in these different regions was variable. In general, advanced atherosclerotic plaques showed a severe loss of SMCs, most extreme in the necrotic core. This indicates that cell death must have occurred during plaque progression. In this study, we compared the cell composition of each region, expression of apoptosis-related proteins, and cell replication and apoptotic cell death.

Adaptive Intimal Thickening

This stage was often present in the communis and the distal part of the interna (distally from the carotid sinus), regions that also contained adjacent fragments of the inner media.

The adaptive intimal thickening was composed primarily of longitudinally oriented SMCs that strongly expressed α-SMC actin (10.5±1.1% of the total area; Figure 1). The SMCs showed a morphology similar to the medial SMCs. Macrophages, as detected by their immunoreactivity for CD-68, were very rare and occupied <0.1% of the total area. Scattered rare lymphocytes, detected by their immunoreactivity for LCA, could be found and covered <1% of the total area. Fibrinogen and von Willebrand factor depositions were absent. In general, most of the SMCs within the media and the adaptive intimal thickenings expressed α-SMC actin but not BAX (Figures 2A and 3). BCL-2 and ICE could not be
detected. Cell replication (as demonstrated by Ki-67) and apoptotic cell death (as demonstrated by DNA in situ end labeling) could not be detected.

Transmission electron microscopy of this region showed SMCs with intact cytoplasm. The cytoplasm contained microfilaments and subplasmalemmal vesicles. A thin basal lamina surrounded each smooth muscle. The extracellular matrix contained cross-banded collagen fibers. Cytoplasmic remnants were located in the superficial layer. The smooth muscle that surrounded the necrotic cores, numerous foam cells of macrophage origin, and few residual SMCs (<0.2% of the total area) were located around the necrotic cores and fibrous caps of atherosclerotic plaques. These regions showed a high expression of BAX and nuclei that were labeled by the TUNEL technique. This indicates apoptotic cell death in those regions (see also Figure 4). Data are expressed as mean±SEM. *P<0.01 versus media and intima.

Advanced Atherosclerotic Plaques

Most of the advanced atherosclerotic plaques were located at the outer wall of the carotid sinus, opposite the bifurcation flow divider. The plaques showed a shoulder, a fibrous cap, and a necrotic core. The core was largely acellular, indicating that cell death must have occurred. Surrounding the necrotic cores, numerous foam cells of macrophage origin, as demonstrated by their immunoreactivity for CD-68, were present (5.8±3.2% of the total area). Lymphocytes occupied <0.2% of the total area. Multinucleated giant cell transformation was frequent in this region. However, α-SMC immunoreactivity was nearly absent in these regions (<0.2% of the total area). This is a consequence of smooth muscle cell loss and the fact that the remaining SMCs lose their α-SMC actin content.

In the fibrous caps, most of the foam cells were of macrophage origin and showed granular material within their cytoplasm. These cells expressed both BAX (Figure 2C) and ICE. BCL-2 was not detectable. The foam cells surrounding the necrotic cores and in the fibrous caps showed nuclei that were labeled with Ki-67 and DNA in situ end labeling. This indicates that both cell replication and apoptotic cell death occurred in these regions. Around the necrotic core, BAX-immunoreactive material and cell remnants could be detected (Figures 2D and 3). Moreover, with the DNA in situ end labeling, nuclear fragments could be detected within and around the necrotic cores, including the fibrous cap. This could indicate an ongoing process of cell death in this region. Immunohistochemical staining of adjacent sections and/or double staining with TUNEL and α-SMC actin or CD-68 identified both apoptotic SMCs and macrophages. Double staining, however, often failed to establish the identity of apoptotic nuclei and nuclear remnants. Therefore, we combined the TUNEL technique with a PAS stain. Foam cells of macrophage origin showed nuclei that were labeled (Figure 4A). SMCs (Figure 4B, bottom) but not the macrophages (Figure 4A, bottom) were surrounded by a cage of PAS-positive basal lamina. The SMCs in the fibrous cap especially were surrounded by prominent cages of PAS-positive material. Using this technique, we could detect TUNEL-labeled...
nuclei and nuclear fragments that were enclosed by a cage of PAS-positive material, indicating smooth muscle apoptosis. (Figure 4B, top and bottom). This indicates that the SMCs die within their cage of thickened basal laminae. Moreover, we could detect cages of PAS-positive material that contained small vesicles (1 μm) that were not labeled by the TUNEL technique (Figure 4C, top and bottom) and associated labeled vesicles (Figure 5). Cages of PAS-positive material that were empty were also present.

Transmission electron microscopy of plaque tissue confirmed that few intact smooth muscles remained present. The remaining SMCs were strangely shaped, with thin elongated cytoplasmic extensions and prominent cages of hypertrophic, multilamellated basal laminae. Moreover, small membrane-bound vesicles of varying size were shed from the SMCs, and often SMCs had died by disintegration into myriad vesicles (Figure 6). These vesicles were enclosed by prominent cages of basal lamina. These ultrastructural findings fit with the TUNEL + PAS stain (Figure 4B and 4C, top and bottom). The basal laminae around the SMCs were irregularly thickened and multilaminated, and fragments of thickened basal laminae unassociated with cells could be found. Macrophages, not enclosed by a basal lamina, completely filled with lipid vacuoles and myeloid bodies could also be demonstrated.

**Discussion**

The present study compares the presence of apoptotic cell death and apoptosis-related proteins in different stages of atherosclerotic plaque formation.

Complete specimens of human atherosclerotic plaques and adjacent nonatherosclerotic intimal thickenings were obtained during a carotid endarterectomy. The specimens were examined in toto with longitudinal whole-mount...
sections. The same tissue section of a carotid endarterectomy specimen contained regions of adaptive intimal thickening, adjacent media, fatty streaks, and advanced atherosclerotic plaques (for definitions, see the AHA Medical/Scientific Statement). With this approach, we could compare the different regions with respect to cell composition, apoptotic cell death, apoptosis-related proteins, and cell replication.

Most advanced atherosclerotic plaques were located at the outer wall of the carotid sinus, opposite the bifurcation flow divider, confirming the studies of Glagov et al. One difference between regions with minimal atherosclerotic plaque formation and advanced plaques was the pronounced cell death in the latter. The focal regions of extensive cell death are known as the necrotic cores. Discrete necrotic core formation is reported to be present in early atherosclerosis. The necrotic cores do not contain SMCs and interstitial collagen fibers. This implies that the plaques can destabilize by expansion of these regions.

Figure 6. Transmission electron microscopy of an advanced atherosclerotic plaque. Two smooth muscle cells are demonstrated that are completely disintegrated into myriad vesicles (granulovesicular degeneration). The prominent basal laminae (bl) around these clusters of vesicles led us to conclude that the vesicles are of SMC and not of macrophage origin. This electron microscopy picture is the explanation for the vesicles of Figure 4B and 4C (bottom) and Figure 5. Col indicates cross-banded collagen. Scale bar=2 μm.
could be found both proximal (in the communis part) and
distal (in the interna) to the advanced atherosclerotic
plaques present in the carotid sinus. The fatty streaks are
considered early atherosclerotic lesions.5 It is important to
note that the term “fatty streaks” covers a spectrum of
lesions from those consisting almost entirely of macro-
phages to those composed mostly of SMCs. The fatty
streaks in our study were composed mainly of lipid-laden
SMCs. The lesions were similar to those described in the
aorta of young adults by Katsuda et al.19 Interestingly, the
lipid-laden SMCs that were the predominant cells in the
fatty streaks of our study and in the study of Katsuda et al
were found primarily in the deeper layer of the lesions.
Foam cells of macrophage origin were scarce and, if
present, located in the superficial layer.

In the adaptive intimal thickening and fatty streaks, cell
replication and apoptotic cell death were absent. In the
advanced atherosclerotic plaques, apoptotic cell death and
cell replication were present in regions with macrophage
infiltration. The overall number of TUNEL-positive nuclei in
the plaques was low if an optimal enzyme concentration and
pretreatment with a calcium chelating agent were used.21
Foci of TUNEL-positive nuclei and nuclear fragments could be
found around the necrotic cores and fibrous cap.

Because these labeled nuclei were focally present, an
estimation of percentages of TUNEL-positive nuclei de-
pends completely on the selected regions and the number
of nuclei used in the denominator. Therefore, we have
chosen to separate only regions with and without TUNEL-
positive nuclei. The TUNEL-positive nuclei and nuclear
fragments belong to the macrophages and SMCs, as
demonstrated with TUNEL + CD-68 and TUNEL + a-SMC
actin. This confirms the findings of other groups.1,2,6 A
significant fraction of the labeled nuclei and nuclear
fragment could not be stained by CD-68 or a-SMC
actin, which could reflect a loss of specific markers during
apoptosis. A feature of smooth muscles in atherosclerotic
lesions from those consisting almost entirely of macro-
phages to those composed mostly of SMCs. The fatty
streaks in our study were composed mainly of lipid-laden
SMCs. The lesions were similar to those described in the
aorta of young adults by Katsuda et al.19 Interestingly, the
lipid-laden SMCs that were the predominant cells in the
fatty streaks of our study and in the study of Katsuda et al
were found primarily in the deeper layer of the lesions.
Foam cells of macrophage origin were scarce and, if
present, located in the superficial layer.

The topographical relationship between macrophage
infiltration and TUNEL positivity suggests that the inflam-
mascular cells by multiple prolife or prodeath factors.42
example to illustrate that apoptosis is regulated in mam-

Another consideration is that the SMCs of the ather-
sclerotic plaques could be programmed to die and that one
of the above-mentioned macrophage-derived factors is
capable of completing the programmed SMC death. This is
suggested by the finding that SMCs derived from the
atherosclerotic plaque but not from the media die when
brought in culture.14 Interestingly, the fatty streaks in our
study differed from adaptive intimal thickenings by the
presence of BAX, a proapoptotic protein of the BCL-2
family. It was demonstrated that BCL-2 heterodimerizes in
vivo with a conserved homologue, BAX, that accelerates
programmed cell death.38–40 Recent data state that BAX
expression can activate a common pathway of apoptosis
both caspase-dependent and -independent.41 However, apo-
ptotic cell death, as detected by DNA in situ end labeling,
is not present in either of these regions. This is a good
example to illustrate that apoptosis is regulated in mam-
malian cells by multiple prolife or prodeath factors.42
However, the expression of BAX in the foam cells of SMC
origin in the fatty streaks indicates that these cells become
susceptible for apoptosis. Interestingly, in both our study
and the study of Katsuda et al,19 the lipid-laden SMCs in
the fatty streaks were located mainly in the deep layer of
the lesions. It is known that the regions of cell death in the
full-blown atherosclerotic plaques are present primarily in
the deep layer of the plaques.

The SMCs that showed BAX expression contained lipid
vacuoles and often were transformed in foam cells. This
indicates a possible relationship between the accumulation
of lipid in the cytoplasm of SMCs and a change in the
BAX/BCL-2 ratio. It was demonstrated that apoptosis can
be induced by lipid peroxides, particularly oysterols, in
cultured vascular SMCs through caspase 3 (CPP-32 pro-
tease) activation and BCL-2 protein downregulation.43 In a
recent study, caspase 3 was detected in human atheroscle-
rotic plaques, and colocalization of caspase 3 expression
and TUNEL positivity was found.44
The increased BAX protein expression within the advanced atherosclerotic plaques was located in the regions around the necrotic core and the fibrous cap. The increased BAX expression was the consequence of an increased cytoplasmic immunoreactivity within remaining SMCs and the strong immunoreactivity of BAX protein expression within the cytoplasmic remnants of apoptotic SMCs called the matrix vesicles.11 The vesicles were enclosed by cages of thickened PAS-positive basa lamina, which was confirmed by transmission electron microscopy. This provides further argument that these matrix vesicles are cytoplasmic remnants of SMC death. The cytoplasmic remnants of SMC and matrix vesicles did not show ICE expression.

We conclude that SMCs within the fatty streaks increase their expression of BAX and become susceptible for apoptosis. The localization of these susceptible SMCs in the deep layer of the fatty streak could be important to our understanding of the transition of a fatty streak into an atherosclerotic plaque, which is characterized by the appearance of focal and diffuse regions of cell death mainly in the deep layer of the plaques. Cytoplasmic remnants of apoptotic SMCs, which are TUNEL negative, remain present in the plaques as matrix vesicles. These vesicles are excessive in regions of the plaque with pronounced cell loss, possibly by a lack of scavenging. The vesicles that can calcify can be considered the graves of SMCs that have died in the plaques.

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References


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