Monocyte Chemoattractant Protein-1 Induces a Novel Transcription Factor That Causes Cardiac Myocyte Apoptosis and Ventricular Dysfunction

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Abstract—Monocyte chemoattractant protein-1 (MCP-1; CCL2)–mediated inflammation plays a critical role in the development of ischemic heart disease (IHD). However, the gene expression changes caused by signal transduction, triggered by MCP-1 binding to its receptor CCR2, and their possible role in the development of IHD are not understood. We present evidence that MCP-1 binding to CCR2 induces a novel transcription factor (MCP-induced protein [MCPIP]) that causes cell death. Gene microarray analysis showed that when expressed in human embryonic kidney 293 cells, MCPIP induced apoptotic gene families before causing cell death. Mutagenesis studies showed that the structural features required for transcription factor–like activity were also required for causing cell death. Activation of caspase-3 was detected after MCPIP transfection and Z-VAD-fmk partially inhibited cell death. Cardiomyocyte-targeted expression of MCP-1 in mice caused death by heart failure at 6 months of age. MCPIP expression increased in parallel with the development of ventricular dysfunction. In situ hybridization showed the presence of MCPIP transcripts in the cardiomyocytes and immunohistochemistry showed that MCPIP was associated with the cardiomyocyte nuclei of apoptotic cardiomyocytes. CCR2 expression in cardiomyocytes increased with the development of IHD. MCPIP production induced by MCP-1 binding to CCR2 in the cardiomyocytes is probably involved in the development of IHD in this murine model. MCPIP transcript levels were much higher in the explanted human hearts with IHD than with nonischemic heart disease. These results provide a molecular insight into how chronic inflammation and exposure to MCP-1 contributes to heart failure and suggest that MCPIP could be a potential target for therapeutic intervention. (Circ Res. 2006;98:1177-1185.)

Key Words: MCP-1 ■ MCP-induced protein (MCPIP) ■ monocyte/macrophages ■ MCP-1–induced transcription factor ■ MCPIP-induced cell death

Inflammation is an important component of cardiovascular pathology associated with a number of types of heart diseases. However, the mechanism by which inflammation contributes to the development of cardiac dysfunction is poorly understood.1–4 The recruitment and activation of monocytes/macrophages through monocyte chemoattractant protein-1 (MCP-1; CCL2) are thought to be important events that contribute to the initiation and pathophysiology of cardiovascular diseases.5–7 MCP-1 is the main chemotactic factor for the migration of monocytes/macrophages and the pathogenesis of chronic inflammation.8,9 Eliminating MCP-1 function or blockade of MCP-1/CCR2 pathway has been shown to decrease neointimal hyperplasia after injury and atherogenesis in mice10–14 and attenuate postischemic myocardial remodeling and heart failure.15 In an attempt to mimic the inflammatory component implicated in the development of cardiovascular diseases, transgenic mice that express MCP-1 specifically in the heart were generated. Cardiac-targeted expression of MCP-1 results in monocyte/macrophage infiltration into the heart, and the mice experience a thrombotic occlusive arteriolar vasculopathy that results in ischemia, interstitial fibrosis, ventricular chamber dilation, and heart failure leading to death by ∼6 months of age.16,17

The binding of MCP-1 to CCR2, a trimeric G-protein–coupled receptor on the target cell membrane, initiates a series of signaling events that lead to chemotactic migration toward the source of MCP-1 production.18–22 Although the signaling triggered by MCP-1 binding to receptor has been studied extensively, very little is known about gene expression changes resulting from this signaling. We showed previously that IL-1β gene was upregulated by MCP-1 treatment of monocytes.23 We also used a genomic approach...
with gene arrays to identify MCP-1–induced genes in human monocytes. Analysis of changes in the expression of genes caused by MCP-1 treatment of human peripheral blood monocytes revealed transcriptional activation of a variety of genes encoding known proteins and several expressed sequence tags (ESTs) that were not homologous to any known proteins (our unpublished data, 2001). Here we report the identification of the most highly induced EST as a fragment of a cDNA encoding a heretofore unknown protein that we designate MCP-induced protein (MCPIP). We present evidence that this protein represents a novel transcription factor of which the expression causes cell death. We also present evidence that suggests that the expression of this transcription factor is probably linked to the development of ischemic heart disease in both a murine model and in human, providing novel insights into how chronic inflammation and exposure to elevated MCP-1 levels contributes to development of ischemic heart disease.

Materials and Methods

Tissue Samples
Human heart tissues were obtained from the Ohio State University hospital. All animal and human materials used were in accordance with the approval of institutional review boards and animal use committees.

Purification of Human Monocytes
Human monocytes were isolated from buffy coat preparations (American Red Cross) using Ficoll-Plaque PLUS (Amersham Pharmacia Biotech AB), an indirect magnetic labeling system and a monocyte isolation kit. Flow cytometry using double staining with antibodies CD14-FITC and CD45-PE showed >90% purity.

Cloning of Human MCPIP From Human Monocytes After Treatment With MCP-1
Monocytes were treated with 7 nmol/L MCP-1, and total RNA was isolated with Trizol reagent (GIBCO); cDNA was prepared using Moloney Murine Leukemia Virus Reverse Transcriptase (GIBCO). The human MCPIP (hMCPIP) cDNA was prepared by polymerase chain reaction (PCR) from total cDNA as template and the following primers: 5′-CGCATATGAGTGGCCCCTGTGGAG-3′ (sense) and 5′-CGGGATCCTTACTCACTGGGGTGCTGG-3′ (antisense). The expected size PCR product was recovered, ligated to the vector pCR2.1 (Invitrogen), and the reaction mixture was used to transform TOPO10 competent cells. Colonies were screened, and inserts were sequenced.

Expression of Human MCPIP in Escherichia coli and Preparation of Antibodies
hMCPIP open reading frame (ORF) from pCR2.1/hMCPIP obtained with BamHI and NdeI, was expressed in pET16b in E. coli BL21. Rabbit polyclonal antibody was prepared against the recombinant hMCPIP.

In Situ Hybridization
A 406-bp cDNA fragment from murine MCPIP (mMCPIP) ORF (from 403 to 809 bp) and 352-bp fragment from CCR2 ORF (from 722 to 1073 bp) were generated by PCR with specific primers, cloned into dual-promoter vector pCRII, and the ligation mixture was used to transform competent cells of TOPO10. The recombinant plasmids, linearized with KpnI, was used as template for in vitro transcription with RNA polymerase and digoxigenin (DIG)-labeled uridine-triphosphate using a DIG RNA Labeling Kit (Roche). Frozen optimum cutting temperature compound–embedded sections were hybridized with DIG-labeled RNA probes (antisense or sense) and processed using standard procedures with anti-DIG antibodies conjugated–alkaline phosphatase.

Construction of Mutant MCPIP
Standard PCR methods were used to generate the mutants. The nuclear localization signal (NLS) sequence RKKP was mutated to GGGP, the two conserved amino acids KC within zinc finger motif was changed to GG. The following primers were used: for NLS-primer 1 (antisense): 5′-AAGTGATGAGACCTCCACCCAGG-AAGTTGCAGCT-3′, for zinc finger motif mutation, primer II (sense): 5′-CTATGGAATCATGAGGTTCCATCTCCACCCAGG-3′, for cloning the whole ORF of hMCPIP, primer III (sense): 5′-CGGAATTCAATGAGTGGCCCTGTGGAG-3′, and
primer IV (antisense): 5’-CGGGATCCGCTCACTGGGGT-
GCTGGGA-3’; EcoRI and BamHI sites are used in the primer III or IV. PCR products were ligated to the N terminus of enhanced green fluorescence protein within the vector pEGFP-N1 by using EcoRI and BamHI and used to transform TOPO10 competent cells. Dele-
tion of praline-rich regions was created using QuikChange Site-
directed Mutagenesis Kit from Stratagene. The 298–378 deletion was created using 5’-AGCGCCAGACCTCAGAAGAGGAAAAG-
GAGG-3’ and 5’-CCTCCTTTTCCTCTTCTGAGGTCTGGCGCT-3’ as
mutagenic oligonucleotides. The oligonucleotides used for the 458–536 deletion were 5’-GGGGTTCGAGGAGGAGGCGGGCAGG-
GCTGGCAGG-3’ and 5’-GCCCCACGGGCCTGC-CAGCC-
CCGCCCTCCTCAGAACCCC-3’, and for the deletion of the both regions, deleted proline2 region construct was used as a template and the same primers for the deletion of the first praline-rich region were used. All substitution and deletion mutants were confirmed by sequencing.

Cell Culture, Transfection, and Measurement of Cell Death and Viability
Human embryonic kidney (HEK)293 (4 × 10⁵) cells, grown in DMEM supplemented with 10% FBS, 1% of penicillin and strepto-
mycin, were transiently transfected with pEGFP/MCPIP or its mutants or control pEGFP-N1 using LipofectAMINE 2000 per the

manufacturer protocol (Invitrogen). After 30 hours, cells were stained with propodium iodide and examined with a confocal microscope (MRC-600 Series Laser Scanning Confocal Imaging System; Bio-Rad). Cell viability and death were measured by trypan blue and TUNEL assays using standard procedures.36

In Vivo Labeling of Active Caspase-3 and Inhibition of Cell Death by Caspase Inhibitor
HEK293 cells (10⁶) were treated with 10 μmol/L biotin-Val-Ala-Asp-(OMe)-fluoromethyl ketone (MP Biomedicals) for 1 hour at 37°C in 5% CO₂, followed by lysis in 20% glycerol, 0.1% Triton X-100, 8% 0.5 mol/L EDTA, 1% 1 mol/L dithiothreitol. Biotinylated proteins, captured with streptavidin-conjugated beads (Calbiochem) overnight at 4°C, were subjected to SDS-PAGE on a 12% polyacry-
lamide gel. Immunoblot was done with caspase-3 antibody (1:2000; Cell Signaling) and horseradish peroxidase–anti-rabbit secondary antibody (1:5000) and visualized with Super Signal West Pico Chemiluminescent Substrate from Pierce. Effect of 100 μmol/L Z-VAD-fmk on cell death was assessed by trypan blue staining.

In Vitro Assay for Transcription Factor Activity
We constructed a fusion protein of GAL4DNA binding with MCPIP and its mutants as test plasmid. The reporter plasmid had five GAL4
binding sites linked to firefly luciferase gene, and in the positive control, activating transcription factor 4 was used to activate the luciferase gene.\textsuperscript{37} Experiments were repeated at least three times.

**Real-Time PCR and RT-PCR**

Total RNA was isolated with the RNAeasy kit (Invitrogen) from human and murine hearts, and first-strand cDNA was synthesized using 1 μg total RNA (DNase-treated) using iScript cDNA synthesis kit (Bio-Rad); β-actin served as internal control. Primers designed for real-time PCR and RT-PCR using Primer software were: β-actin: human: F-5'-GGACTTTGACAGAAGATG-3', R-5'-AGGAGGGCTGGAAGAG-3'; mouse: F-5'-AGAAACACGCT-TCAGCCCAAA-3', R-5'-GAGCTCAGGACTGCTGAA-3'. MCPIP, human: F-5'-AACGAGAAGAAGAAGATCCTGG-3', R-5'-ATTGAGGAAGGATCATGAGCAG-3'; mouse: F-5'-CTGGTAGACGAGCAGTTGAGAATTA-3', R-5'-GTACTCTCTC- GATGGGTAGGTGG-3'. MCP-1, mouse: F-5'-CAGGTCCCTGTCACTGCTGAA-3', R-5'-CTGGGAAGGATCATGAGCAG-3'.

**Localization of MCPIP and Apoptosis in the Heart**

Paraffin-embedded sections were blocked in 3% hydrogen peroxide incubated with rabbit polyclonal anti-human MCPIP antibody or isotype control overnight at 4°C, incubated with horseradish peroxidase–conjugated goat anti-rabbit antibody (Santa Cruz Biotechnology), visualized with diaminobenzidine, and sections were counterstained with hematoxylin. For dual labeling, sections were first stained by TUNEL for identifying apoptotic nuclei with streptavidin-fluorescent (R&D Systems) and then by immunohistochemistry for determination of MCPIP expression with goat anti-rabbit IgG conjugated with Alexa 594 (Invitrogen), visualized under Nikon fluorescence microscopy.

**Apoptosis Microarray Analysis**

The nonradioactive human apoptosis oligomicroarrays (Super Array; Biosciences Corp.) were used. The HEK293 cells were transfected (LipofectAMINE) with MCPIP–GFP or GFP alone as control for 6 hours in serum-free medium and then incubated in complete medium for 16 hours before the isolation of RNA. The expression profiling was done according to manufacturer instructions.

**Statistical Analysis**

The experimental data were analyzed by using SPSS statistical software (SPSS Inc.) under Windows XP. All values are presented as mean ± SEM. Real-time PCR data were expressed as fold upregulation compared with sex- and age-matched wild-type controls. Results were compared between groups by ANOVA analysis followed by t tests. Differences were considered significant at a P value of <0.05.

**Results**

**Human MCPIP Gene and Protein**

Treatment of human peripheral blood monocytes with MCP-1 resulted in the transcriptional activation of a variety of genes, including those that encode a variety of cytokines and chemokines, extracellular matrix degrading enzymes, cell adhesion proteins, and a set of ESTs (our unpublished data, 2001). The most highly induced EST, representing unidentified genes, was matched with a human cDNA clone with GeneBank accession number AW206332, which maps to a gene for a novel protein (accession number AY920403). Protein motif analysis showed that MCPIP contains two praline-rich potential activation domains (Figure 1A), one between residues 100 and 126 with 37% proline residues and the other at 458 to 536 with 28% proline residues. It also contains a putative monopartite NLS sequence (RKKP) and a single zinc finger motif. Thus, MCPIP has features characteristic of a transcription factor.

Mouse genome data search revealed a gene highly homologous to human mcpp gene. RT-PCR of mRNA isolated from a 6-month-old MCP mouse heart gave the mMCPIP cDNA that showed a 596-aa ORF (accession number AC205257) that would encode a protein containing 599 amino acids with a calculated mass of 65.8 kDa (accession number AY920403). Protein motif analysis showed that MCPIP contains two praline-rich potential activation domains (Figure 1A), one between residues 100 and 126 with 37% proline residues and the other at 458 to 536 with 28% proline residues. It also contains a putative monopartite NLS sequence (RKKP) and a single zinc finger motif. Thus, MCPIP has features characteristic of a transcription factor.

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AY920404). Sequence of this cDNA showed 80% identity at the nucleotide level and 82% identity at the amino acid level to that of human MCPIP. The mMCPIP expressed in HEK293 cells strongly cross-reacted with rabbit anti-hMCPIP antibodies (data not shown).

**Induction of MCPIP by Treatment of Human Monocytes With MCP-1**

To verify the data from gene arrays, we examined the production of MCPIP transcripts in human monocytes after treatment with 7 nmol/L MCP-1 by RNA blot analysis with the cloned cDNA for hMCPIP as a probe. The results showed that the expected 1.8-kb transcript was found only in MCP-1–treated human monocytes (Figure 1B). Anti-CCR2 blocked MCP-1–induced synthesis of MCPIP (Figure 1C), showing that this induction involved MCP-1 binding to its receptor CCR2.

**Localization of MCPIP**

Because the structural features suggested MCPIP to be a transcription factor, we tested whether it is localized in the nucleus. MCPIP–GFP expressed in HEK293 cells was found to be localized in the nucleus, whereas in the control, GFP was found to be distributed throughout the cell (Figure 2A). Propidium iodide that stained the nucleus (red) was colocalized with GFP, resulting in the yellow color on merging of the two images.

**Cell Death Caused by Expression of MCPIP**

In situ TUNEL assay was performed on HEK293 cells after transfection with MCPIP–GFP or GFP alone (control). Transfection with either plasmid resulted in the appearance of robust and equal GFP fluorescence within 16 hours after transfection. During the next five days, blebbing of plasma membrane, nuclear condensation, and disintegration became clear. Trypan blue staining showed that MCPIP expression caused cell death. Expression of MCPIP caused apoptosis detectable by TUNEL positivity (Figure 2B). Apoptosis was also demonstrated by poly (ADP-ribose) polymerase (PARP) cleavage in HEK293 cells transfected with MCPIP–GFP (Figure 2C). MCPIP caused cell death also in cardiomyoblast cell line H9C2. Three days after infection with adenovirus expressing MCPIP–GFP, 26% cells were trypan blue positive, whereas GFP control and uninfected control showed 15₁/₁₀₀₀ and 12₁/₁₀₀₀ trypan blue staining cells, respectively.

To test whether caspase-3 activation occurs in the MCPIP-expressing cell, an activation-specific labeling of the caspases was done using biotin-Z-VAD-fmk that would covalently attach biotin only to enzymatically active caspase. Recovery of the biotin-labeled protein with avidin and immunological detection showed 17-kDa and 12-kDa species expected from activation of caspase-3 (Figure 2D). That the MCPIP-induced cell death probably involved caspase activation was shown by the finding that pan-caspase inhibitor Z-VAD-fmk partially inhibited MCPIP-induced cell death (Figure 2E).

**Transcription Factor–Like Activity of MCPIP**

The ability of MCPIP to transactivate transcription was tested in an in vitro system. Cotransfection of HEK293 cells with GAL4–MCPIP and the pGal4-Luc reporter demonstrated that MCPIP activated transcription of the luciferase reporter gene showing 865-fold/mg protein after transfection for 24 hours, whereas the positive control containing activating transcription factor-4 (ATF4) showed 1263-fold. This result demonstrated that MCPIP could act as a positive regulator of transcription.

To determine whether cell death caused by MCPIP is related to its transcription factor–like activity, we compared
the effects of mutations in the putative domains thought to be important for transactivation on the transactivation and the cell death–inducing activities of the MCPIP (Figure 3A and 3B). Mutation of the Zn finger domain caused a drastic decrease in both transactivation and death-inducing ability. Mutation of either praline-rich domain or both caused drastic reduction in cell death–inducing activity. Mutation of the putative NLS showed very little effect on transcription factor activity and cell death–inducing activity. Confocal microscopy showed that this mutation did not affect nuclear localization (data not shown). Thus, the structural features that are essential for the transcription factor–like activity are also essential for cell death–inducing activity, indicating that MCPIP transcriptionally activates genes of which expression is involved in cell death.

Induction of Apoptotic Genes by MCPIP Expression in HEK293 Cells

To determine whether expression of MCPIP causes detectable upregulation of genes known to be involved in cell death, microarray analysis was done with RNA isolated soon after MCPIP expression was clearly indicated by the fluorescence of the fused GFP (16 hours) but before cell death was detectable. This gene expression profile showed that MCPIP caused induction of several genes the products of which are known to be involved in cell death (Table 1).

Association of MCPIP Expression With Ischemic Heart Disease

Because MCP-1 and cell death has been associated with development heart disease6,7,24 and MCPIP induces death in cell cultures, we examined whether MCPIP expression is associated with the heart disease in the transgenic mouse model for heart failure, in which cardiac inflammation is induced by cardiomyocyte-targeted expression of MCP-1.16,17 Real-time PCR analysis of the MCPIP transcript levels showed that the transgenic animals expressed much higher levels of MCPIP when compared with age and sex-matched wild-type controls. As the MCP mice aged, levels of MCP-1 and MCPIP transcripts increased in the heart, and fractional shortening decreased (Figure 4A through 4C).

In situ hybridization showed that MCPIP transcripts were in cardiomyocytes (Figure 5A). MCPIP immunostaining further demonstrated the accumulation of this protein in myocyte of MCP mice, and MCPIP staining was associated with the nuclei of cardiomyocytes (Figure 5B). In MCP mice, MCPIP levels were easily detected immunologically when the animals reached 2 months of age. When clinical symptoms of heart failure were very obvious at 6 months of age (fractional shortening <20%), the nuclei of many cardiomyocytes were degraded and appeared like vacuoles, and in such cases, MCPIP staining was associated with the periphery of such vacuoles; vacuolation is a characteristic ultrastructural feature of heart failure in the MCP mice as is found also in the human failing myocardium.24 In the hearts of wild-type mice, MCPIP staining was hardly detectable at any age (Figure 5B). In 4-month-old MCP mouse hearts, most cells strongly expressing MCPIP were also found to be TUNEL positive (Figure 5C).

Induction of CCR2 in Cardiomyocytes

Real-time PCR showed elevation of CCR2 transcript levels in MCP mouse hearts at 2 months of age, before the development of clinical manifestation of the heart disease and CCR2 levels increased further until 4 months of age (Figure 6A). In situ hybridization clearly showed that CCR2 transcript was present within the cardiomyocytes of MCP mice (Figure 6B).

Elevated Levels of MCPIP in Ischemic Human Hearts

Because MCPIP expression is associated with ischemic heart failure in the murine model, we considered the possibility that MCPIP expression may be a feature of human ischemic heart disease. Therefore, we measured the MCPIP transcript levels in human heart tissue from explanted hearts by real-time PCR. In these patients of comparable age, seven were classified as ischemic and the other six nonischemic. Patients were classified as having ischemic cardiomyopathy based on a clinical history of documented coronary artery disease, myocardial infarction, or evidence of ischemia by exercise or pharmacological stress testing before transplantation. Nonischemic patients showed diastolic-dilated cardiomyopathy and normal coronary angiogram with no specific reason for cardiac dysfunction. The clinical data on the patients indi-
cated that the hearts of both groups were compromised to a comparable degree (Table 2). Remarkably, the ischemic hearts showed much higher levels of MCPIP than the nonischemic hearts (Table 2).

**Discussion**

It is becoming widely recognized that MCP-1 is involved in the development of cardiovascular diseases.\(^6\)\(^7\) The signaling events initiated by MCP-1 binding to CCR2 probably leads to transcriptional activation of genes for which products could lead to pathophysiological changes. However, little is known about them. Gene array analysis revealed that a set of genes are upregulated (unpublished data, 2001). These included a number of heretofore unidentified genes. Among the highly induced nonannotated human genes, the most highly induced one, that we designate MCPIP, has structural motifs that are characteristic of transcription factors. We demonstrate with an in vitro assay that MCPIP can activate transcription of a reporter gene.

Expression of MCPIP–GFP in HEK293 cells showed nuclear localization of the protein and caused cell death. Mutagenesis of the various motifs in MCPIP that are characteristic of transcription factors affected the in vitro transcriptional activity of MCPIP and the death-inducing activity in HEK293 cells in a similar manner. This finding strongly suggests that MCPIP mediates transcriptional activity of genes involved in cell death. Microarray analysis of the transcripts present in HEK293 cells soon after MCPIP expression indicated that MCPIP expression caused upregulation of transcript known to be involved in cell death. These include components of caspase activation, cytochrome C release, production of reactive oxygen, unique subsets of BCL2 family of genes, and tumor necrosis factor receptor family members.\(^25\)\(^\text{–}\)\(^28\) It is probable that MCPIP-induced proteins are involved in one or more of the multiple forms of cell death that occur in ischemic heart disease.\(^29\) Although the components involved in the cell death caused by MCPIP remain to be elucidated, we present evidence that caspases are involved. Using an activation-specific avidin labeling method, we demonstrate caspase-3 activation and show inhibition of cell death by a pan–caspase inhibition. PARP cleavage also demonstrated apoptosis.
The present findings on the MCP model appear to be reflected in human disease. In human myocardium, chemokines and their receptors were reported to be upregulated, especially CCR2 and CXCR4.32 Real-time PCR analysis of transcripts in human heart tissue obtained during heart transplant surgery of individuals of comparable age showed that hearts with ischemic disease contained much higher levels of MCPIP transcripts than in the hearts with nonischemic disease, although both groups were at end stages of the disease with comparable degrees of functional compromise.

Many reports implicate involvement of MCP-1 in the development of ischemic heart disease,5–7 although in some circumstances, MCP-1 shows a protective effect against ischemia/reperfusion injury.33,34 We found that chronic expression of MCP-1 in cardiomyocytes leads to ischemic heart disease that manifests the clinical, pathological, ultrastructural, and molecular features found in human ischemic heart disease.18,19 How chronic inflammation and prolonged exposure of the myocardium to elevated levels of MCP-1 cause heart failure remains unclear. The present findings that MCP-1 treatment of human monocytes causes induction of a novel transcription factor, MCPIP, that is also induced in the heart of the murine MCP model of ischemic heart disease and found at much higher levels in human hearts with ischemic disease when compared with nonischemic heart disease, all strongly suggest that the newly discovered transcription factor causes cell death and thus plays an important role in the development of ischemic heart disease. This finding provides a novel insight into the molecular mechanism by which chronic inflammation and exposure to MCP-1 contributes to development of heart failure. If confirmed in further studies, this transcription factor could be a potential target for therapeutic intervention.

Acknowledgments

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References


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