VEGF-C mediates cyclic pressure-induced endothelial cell proliferation

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1Department of Biomedical Engineering, Rensselaer Polytechnic Institute, Troy, New York 12180; 2Department of Molecular Biology and Department of Cardiovascular Research, Genentech, Inc., South San Francisco, California 94080; and 3Department of Biomedical Engineering, University of Virginia, Charlottesville, Virginia 22908

Submitted 3 June 2002; accepted in final form 30 September 2002

Shin, Hainsworth Y., Michael L. Smith, Karen J. Toy, P. Mickey Williams, Rena Bizios, and Mary E. Gerritson. VEGF-C mediates cyclic pressure-induced endothelial cell proliferation. Physiol Genomics 11: 245–251, 2002. First published October 8, 2002; 10.1152/physiolgenomics.00068.2002.—Mechanical forces modulate endothelial cell functions through several mechanisms including regulation of gene transcription. In the present study, gene transcription by human umbilical vein endothelial cells (HUVEC) either maintained under control pressure (that is, standard cell culture conditions equivalent to 0.15 mmHg sustained hydrostatic pressure) or exposed to 60/20 mmHg sinusoidal pressures at 1 Hz were compared using Affymetrix GeneChip microarrays to identify cellular/molecular mechanisms associated with endothelial cell responses to cyclic pressure. Cyclic pressure selectively affected transcription of 14 genes that included a set of mechanosensitive proteins involved in hemostasis (tissue plasminogen activator), cell adhesion (integrin-α2), and cell signaling (Rho B, cytosolic phospholipase A2), as well as a unique subset of cyclic pressure-sensitive genes such as vascular endothelial growth factor (VEGF)-C and transforming growth factor (TGF)-β2. The present study also provided first evidence that VEGF-C, the most highly induced gene under 60/20 mmHg, mediated HUVEC proliferation in response to this cyclic pressure. Cyclic pressure is, therefore, a mechanical force that modulates endothelial cell functions (such as proliferation) by activating a specific transcriptional program.

mechanical forces; expression profiling; microarray

THE IMPORTANT ROLE of the vascular endothelium in regulating blood vessel function (i.e., hemostasis, vaso-motor tone, barrier properties, metabolism of various blood-borne substances, and immune/inflammatory responses) is controlled and/or affected by a diverse array of biological (e.g., biomolecules, nutrient supply, pH, oxygenation) and physical (e.g., mechanical, thermodynamic, electrical) stimuli that modulate the endothelial cell phenotype through transcriptionally independent and dependent mechanisms (6). Among these stimuli, mechanical forces resulting from the hemodynamics of the circulation have been correlated with changes in vascular wall structure/function associated with normal physiology as well as with pathological conditions such as hypertension and atherosclerosis (13). These pathologies involve endothelial dysfunction leading to intimal hyperplasia, vascular wall tissue hypertrophy, and altered regulation of underlying vascular smooth muscle cell function (18). A direct relationship between mechanical forces and endothelial-related cardiovascular pathologies, however, remains to be determined.

In the vasculature, endothelial cells are exposed to three types of mechanical forces: 1) fluid shear stress, due to blood flow; 2) wall stretch, due to compliance of the blood vessel wall tissue; and 3) fluid pressure, due to containment of blood within the lumen of the vasculature. In vitro cell culture models have provided evidence that pressures affect endothelial cell functions such as proliferation as well as synthesis and release of proteins and growth factors. Sustained hydrostatic pressures in the range of 1–11 mmHg (comparable to pressures on vascular wall tissues of the microcirculation) for up to 7 days elicited increased proliferation of bovine pulmonary artery endothelial cells (BPAEC) through the release of basic fibroblast growth factor (bFGF) from cytoplasmic pools (1). Furthermore, exposure of human umbilical vein endothelial cells (HUVEC) to 3 mmHg sustained hydrostatic pressure for up to 4 days elicited increased cell proliferation mediated, at least in part, from upregulated expression of integrin-αv (22). In the case of pulsatile pressures, exposure of bovine aortic endothelial cells to pulsatile ambient pressure of 160/110 mmHg for time periods ranging from 1–5 days resulted in reduced cell proliferation associated with the release of an unidentified soluble growth inhibitor (28).

In an earlier study (24), we used the same cyclic pressure system as in the present study and demonstrated increased HUVEC proliferation in response to 60/20 mmHg at 1 Hz for 24 h; this effect was equipotent to a 20 ng/ml dose of bFGF. On the other hand, cells exposed 140/100 mmHg cyclic pressure for 24 h exhibited reduced...
proliferation rates (24). Although the governing mechanical parameter (maximum pressure vs. minimum pressure vs. mean pressure) of the pressure stimulus was not established, the results of Shin et al. (24) provided evidence that endothelial cell proliferation is dependent upon the level of the applied cyclic pressure, a differential response that may involve mechanosensitive cell/molecular mechanisms, such as expression of a bioactive protein mediator(s).

In the present study, we investigated the role of gene transcription by HUVEC exposed to 60/20 mmHg cyclic pressure at 1 Hz for 24 h to identify cellular/molecular mechanisms involved in the proliferative response of HUVEC under cyclic pressure.

MATERIALS AND METHODS

Cyclic pressure experiments. HUVEC (Clonetics, Wakersville, MD) were seeded at a density of 15,500 cells/cm² onto rigid, tissue culture, polystyrene substrates (BD Biosciences; Franklin Lakes, NJ) and cultured overnight in endothelial growth media (Clonetics) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT) and 0.4% bovine brain extract (BBE; Clonetics). Prior to experiments, the culture media was replaced with Media 199 (GIBCO BRL, Carlsbad, CA) containing 1% FBS and 0.04% BBE, and the cells were either maintained under control (0.15 mmHg) sustained hydrostatic pressure conditions (due to the volume of media used to culture the cells) or exposed to 60/20 mmHg cyclic pressure at 1 Hz in a sterile, humidified, 5% CO₂/21% O₂/74% N₂ atmosphere at 37°C for various time periods ranging from 1 to 24 h according to established protocols (24). During the cyclic pressure experiments, the gas phase over the supernatant was pressurized, but the fluid layer over the cells was stationary; the cells were, therefore, exposed to a normal force equal to the applied cyclic pressure.

**RNA extraction.** Total RNA was extracted from HUVEC monolayers using Tri Reagent-LS (Molecular Research Center, Cincinnati, OH) following the manufacturer’s instructions, resuspended in diethyl pyrocarbonate-treated water, and purified of contaminating DNA using RNeasy Mini kit (Qiagen, Valencia, CA) following the manufacturer’s protocol. The total amount of RNA in each HUVEC sample was determined spectrophotometrically (λ = 260 nm). All such samples were stored at −20°C until gene expression analyses.

**Affymetrix GeneChip microarray analysis.** Preparation of cRNA, hybridization, and scanning of microarrays were performed following manufacturer’s (Affymetrix, Santa Clara, CA) protocols. Fluorescently stained microarrays were visualized with a GeneArray scanner (Hewlett-Packard, Loveland, CO), and expression data were analyzed using GeneSpring software. Gene transcripts expressed by HUVEC either maintained under control (0.15 mmHg) sustained pressure or exposed to 60/20 mmHg cyclic pressure at 1 Hz for 24 h were compared using Affymetrix GeneChip HuGene FL arrays containing total RNA of HUVEC extracted from each of two separate cyclic pressure and control experiments. This analysis was used to generate a profile of differentially expressed genes; only those genes that exhibited a greater than threefold change (either increased or decreased) in microarray intensity levels from four out of four pairwise (100% agreement) comparisons (control vs. cyclic pressure) were considered different.

**Quantitative RT-PCR (TaqMan) analysis.** Altered expression of a select number of genes, as predicted by Affymetrix analysis, was independently confirmed and quantified using total RNA collected from three to four separate cyclic pressure experiments and TaqMan (Applied Biosystems, Foster City, CA) analysis according to established procedures (11) with gene-specific oligonucleotide primers and probes either previously synthesized (5, 11) or, in the case of TGF-β2 and syntenin, custom-designed according to standard procedures (11). The oligonucleotide sequences for the forward primer, reverse primer, and the TaqMan probe used in the present study to detect TGF-β2, were gctgagetectttctgatc, cgagttgtgcagtagtagaca, and tggtagcgtgcgtcagct, respectively. For syntenin, the following sequences for the forward primer, reverse primer, and TaqMan probe were used: tggtaggtgtgagtttggg, tgcggctattagcteca, and aagactctagatcatggtaaaacgtgcaggg, respectively. Measurements for gene-specific mRNA expression values were standardized to corresponding values for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for each RNA sample and expressed as a gene-specific mRNA expression unit. Mean gene-specific mRNA expression units for HUVEC exposed to cyclic pressures were normalized to those of HUVEC maintained under control (0.15 mmHg) sustained pressure conditions.

**HUVEC proliferation in response to cyclic pressure.** To test whether VEGF-C signaling pathways mediate cyclic pressure-induced HUVEC proliferation, fusion proteins (R & D Systems, Minneapolis, MN) of human IgG with the extracellular, ligand-binding domains of either flt-1 (flt-1/IgG), kdr (kdr/IgG), or flt-4 (flt-4/IgG) were used. These soluble, protein chimeras competitively bound available VEGF ligands and, therefore, blocked respective VEGF activity by interfering with ligand–receptor interactions. HUVEC were exposed to 60/20 mmHg cyclic pressure at 1 Hz in the presence and absence of 100 ng/ml concentrations of either flt-1/IgG, kdr/IgG, or flt-4/IgG for 24 h. In parallel experiments, cells were maintained under control (0.15 mmHg) sustained pressure conditions in the presence of 20 ng/ml VEGF-A with and without 100 ng/ml of either flt-1/IgG, kdr/IgG, or flt-4/IgG.

In other experiments, HUVEC (in fibronectin-coated wells of 48-well cell culture plates) were exposed to 60/20 mmHg cyclic pressure at 1 Hz in the presence and absence of 10 µg/ml mouse monoclonal anti-human VEGF-C (kindly provided by Anan Chantharapai of Genentech, South San Francisco, CA) for 24 h. In parallel experiments, cells were maintained under control (0.15 mmHg) sustained pressure conditions in the presence of 100 ng/ml VEGF-D (R & D Systems) with and without 10 µg/ml monoclonal anti-human VEGF-C.

At the end of experiments, cells were fixed with FixDenat (Roche Applied Science, Indianapolis, IN) and stained in situ with 4 µM 4’,6-diamidino-2-phenylindole (DAPI, Sigma, St. Louis, MO). DAPI-stained cell nuclei from three to five random fields per well were imaged using a fluorescence microscope (Eclipse TE300; Nikon, Melville, NY) with ultraviolet optics and counted using Openlab software (Improvision, Lexington, MA). Mean nuclei counts of DAPI-stained cell nuclei from three to five random fields per well were imaged using a fluorescence microscope (Eclipse TE300; Nikon, Melville, NY) with ultraviolet optics and counted using Openlab software (Improvision, Lexington, MA). Mean nuclei counts of DAPI-stained HUVEC exposed to cyclic pressure were normalized to those of similarly stained controls. These experiments were repeated at least three separate times.

**RESULTS**

Cyclic pressure alters endothelial expression of genespecific transcripts. Exposure of HUVEC to cyclic pressure affected 14 genes (Fig. 1) whose expression levels
were either increased or decreased by at least a factor of 3. Specifically, compared with cells maintained under control (0.15 mmHg) sustained pressure conditions, HUVEC exposed to 60/20 mmHg cyclic pressure exhibited increased expression of VEGF-C, tissue plasminogen activator (tPA), interleukin-8 (IL-8), cytosolic phospholipase A2 (cPLA2), carbonic anhydrase-related protein (CARP), TGF-β2, unknown protein KIAA0101, syntenin, and integrin-α2. Among these nine genes, VEGF-C and tPA were the most highly induced mRNA transcripts (Fig. 1). Furthermore, under 60/20 mmHg cyclic pressure, HUVEC exhibited decreased expression of the following five transcripts: chemokine (C-X-C motif) receptor-4 (CXCR-4), transglutaminase-2 (TGM-2), LIM-domain binding protein-AB1 (LIM-AB1), Rho B, and Claudin-5 (Fig. 1). TaqMan analysis independently confirmed and provided a more precise quantification of the changes in transcriptional levels of seven of these genes including VEGF-C, tPA, IL-8, TGF-β2, integrin-α2, syntenin, and CXCR-4 (Table 1). There was agreement for all seven of these genes tested with respect to the direction of change predicted by either Affymetrix GeneChip probe array (Fig. 1) or TaqMan (Table 1) analyses. The magnitude of the change in expression levels predicted by the two analytical methods were somewhat different; this result was not surprising, considering differences in the methods of analysis and normalization, as well as in the resolution of the techniques used.

**VEGF-C mediates cyclic pressure-induced proliferation.** Proliferation of HUVEC exposed to 60/20 mmHg cyclic pressure at 1 Hz for 24 h was not affected by

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**Table 1. Effects of cyclic pressure on gene expression by HUVEC: TaqMan confirmation of results from Affymetrix analysis**

<table>
<thead>
<tr>
<th>NIH Accession Number</th>
<th>Gene Name Abbreviation</th>
<th>Normalized TaqMan Expression Units, % of controls</th>
</tr>
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<tbody>
<tr>
<td>U43142</td>
<td>vascular endothelial growth factor-C (VEGF-C)</td>
<td>336.5 ± 55.9*</td>
</tr>
<tr>
<td>K03021</td>
<td>tissue plasminogen activator (tPA)</td>
<td>193.3 ± 28.3*</td>
</tr>
<tr>
<td>M28130</td>
<td>interleukin-8 (IL-8)</td>
<td>163.6 ± 5.6*</td>
</tr>
<tr>
<td>M19154</td>
<td>transforming growth factor-β2 (TGF-β2)</td>
<td>135.2 ± 11.0*</td>
</tr>
<tr>
<td>M28249</td>
<td>integrin-α2</td>
<td>162.1 ± 16.7*</td>
</tr>
<tr>
<td>U83463</td>
<td>syntenin</td>
<td>118.4 ± 4.1*</td>
</tr>
<tr>
<td>L06797</td>
<td>chemokine (C-X-C motif) receptor-4 (CXCR-4)</td>
<td>54.3 ± 5.6*</td>
</tr>
</tbody>
</table>

Values are means ± SE. HUVEC, human umbilical vein endothelial cells. *P < 0.05 (compared to parallel controls using paired Student’s t-test).
coincubation with flt-1/IgG, but was partially blocked by kdr/IgG, and completely abolished in the presence of excess flt-4/IgG (Fig. 2A). In contrast, increased cell density of HUVEC in response to 20 ng/ml VEGF-A was completely abolished by coincubation (for the duration of these experiments) with either flt-1/IgG or kdr/IgG, but not with flt-4/IgG (Fig. 2B). Moreover, the pressure-induced proliferative response of HUVEC was blocked by 10 μg/ml mouse monoclonal antibody to VEGF-C (Fig. 3; CP+α-VEGF-C). This antibody, however, did not inhibit increased HUVEC proliferation in response to 100 ng/ml exogenous VEGF-D (Fig. 3). These findings are consistent with the hypothesis that cyclic pressure-induced HUVEC proliferation was mediated by VEGF-C.

DISCUSSION

The present in vitro study used a cell culture model and investigated the transcriptional profile for HUVEC in response to a well-defined sinusoidal pressure regime of 60/20 mmHg at 1 Hz for 24 h. To ensure that HUVEC were exposed to pressure alone without contributions from other mechanical forces pertinent to the hemodynamic milieu (e.g., substrate stretch, fluid shear stress), the cyclic pressure experiments were conducted with cells cultured on rigid, tissue culture, polystyrene substrates and under no-flow conditions. Most importantly, the present in vitro study used cyclic pressure levels that simulated pertinent physiological/pathological conditions and stimulated, but neither “compressed” nor injured/killed, the cells.

Blood pressures in the circulation vary (depending on the location within the vasculature) and are cyclic (due to the pumping of the heart) with mean values in the range of either 20–80 mmHg or 20–35 mmHg in the arterioles and capillaries, respectively (4, 23). Moreover, pulsatile pressures in the range of 60/20 mmHg occur at the level of the human pulmonary artery during primary pulmonary hypertension (14) as well as pulmonary hypertension secondary to connective tissue disease (20); these pathological conditions are associated with pulmonary vessel remodeling (10), intimal hyperplasia (21), as well as dysregulated endothelial growth (3) and gene expression (27) associated with formation of plexiform lesions (resulting from either neoplastic or angiogenic endothelial growth) on the vascular wall.

The present study provided the first evidence that exposure of HUVEC to pulsatile pressures (i.e., 60/20 mmHg) affected the transcription of various genes involved in important physiological processes such as inflammation (IL-8, CXCR-4), hemostasis (tPA), barrier function (claudin-5), angiogenesis (VEGF-C), cell differentiation (TGF-B2), cell adhesion (integrin-α2), and cell signaling (syntenin, Rh-B, actin-binding LIM-AB1 protein, cPLA2). It is important to note that some of these cyclic pressure-induced genes encode proteins whose expression or cellular distribution in endothelial cells is regulated by other mechanical forces (specifically, fluid shear stress and cyclic stretch)

Fig. 2. Kdr/IgG and flt-4/IgG, but not flt-1/IgG, block cyclic pressure-induced increases in HUVEC density. A: compared with cells maintained under control (0.15 mmHg) sustained pressure conditions (CTL), HUVEC exhibited 30% increased cell density after exposure to 60/20 mmHg cyclic pressure (CP) at 1 Hz in the absence or presence of 100 ng/ml flt-1 IgG for 24 h. This increased HUVEC density, however, was partially blocked by exposing HUVEC to cyclic pressure in culture media containing 100 ng/ml kdr/IgG and completely blocked with 100 ng/ml flt-4/IgG fusion protein. B: HUVEC maintained under control (0.15 mmHg) sustained pressure conditions in the presence of 20 ng/ml VEGF-A for 24 h exhibited increased cell densities relative to controls. When cells in media containing VEGF-A were cultured in the presence of either flt-1/IgG or kdr/IgG, but not flt-4/IgG, HUVEC densities were similar to those of controls. Values are means ± SE; n = at least 3 experiments. *P < 0.05 compared with CTL; #P < 0.05 compared with CP; and ‡P < 0.05 compared with VEGF-A (Student’s t-test, paired values).
in the focal adhesion assembly/reorganization and cytoskeletal actin-stress-fiber formation associated with responses of endothelial cells to shear stress (7), mechanical strain (29), and sustained hydrostatic pressure (22). Cyclic pressure, therefore, induces transcription of some genes that are similar to those reported when endothelial cells were exposed to other mechanical forces (i.e., fluid shear stress and cyclic stretch) pertinent to the vascular milieu.

At the same time, however, the present study provided the first evidence of unique responses of HUVEC to cyclic pressure. In fact, cyclic pressure affected transcription of a number of HUVEC genes that had not been previously associated with mechanical forces. Among these, transcription of VEGF-C, TGF-β2, KIAA0101 (a protein of unknown function), CARP, and syntentin (a cytosolic, syndecan-4-binding protein) was upregulated; in contrast, genes encoding CXCR-4 (a G-protein-coupled receptor for stromal cell-derived factor-1), TGM-2 (a catalyst for polyamine cross-linking), actin-binding LIM-domain protein (or KIAA0059), and claudin-5 (a tight junction transmembrane protein) were downregulated in HUVEC under cyclic pressure. These transcripts, therefore, represent a novel and select subset of cyclic pressure-responsive, endothelial-related genes.

Most importantly, the gene transcriptional profile observed in the present study identified a candidate growth factor, namely VEGF-C, which could mediate the increased proliferation of HUVEC observed under 60/20 mmHg cyclic pressure (24). VEGF-C (also known as VEGF-related protein or VRP) is produced by endothelial cells (30) and is a potent endothelial mitogen that has been implicated in angiogenesis/vasculogenesis during embryonic development of the venous and lymphatic circulation as well as in maintenance of differentiated lymphatic endothelium (16).

Like other members (specifically, VEGF-A, VEGF-B, and VEGF-D) of the VEGF family of growth factors, VEGF-C stimulates cell functions (e.g., proliferation, migration) through selective binding and phosphorylation of tyrosine kinase growth factor receptors (VEGFRs; specifically, flt-1, kdr, and/or flt-4) followed by activation of downstream signaling molecules (31). VEGF-C (15) and VEGF-D (2) exhibit binding affinity for flt-4, lower binding affinity for kdr (also known as flt-2), but no detectable affinity for flt-1. In contrast, VEGF-A binds both flt-1 and kdr, but not flt-4 (15), whereas VEGF-B binds only flt-1 (16). Using this receptor selectivity to evaluate the potential role of VEGF-C as a cyclic pressure-induced mitogen, we found that HUVEC proliferation in response to 60/20 mmHg was mediated by a factor whose activity was inhibited by soluble receptor chimeras for kdr and flt-4, but not for flt-1 (Fig. 2A); furthermore, this factor could also be neutralized by a monoclonal antibody directed against VEGF-C (Fig. 3). These observations substantiate our conclusion that VEGF-C mediated cyclic pressure-induced HUVEC proliferation. Although the results of the present study provided evidence that endothelial cells increase transcription of VEGF-C in
response to cyclic pressure, we cannot rule out the possibility that additional sources of this growth factor (e.g., VEGF-C stored either within cells or sequestered in the extracellular matrix) contributed to the proliferative responses of HUVEC to 60/20 mmHg.

It should be noted that VEGF-C was the only member of the VEGF-related family to be induced by cyclic pressure. Different VEGF ligands have been associated with other mechanical forces; for example, VEGF-A transcripts were elevated in bovine endothelial cells exposed to 9% cyclic stretch of the substrate for 9 h (25). Although the DNA microarrays used in the present study contained oligonucleotide probes for VEGF-A, neither rigorous Affymetrix (Fig. 1) nor TaqMan (data not shown) analyses revealed changes in the transcription of this gene by HUVEC exposed to cyclic pressure; this result substantiated the conclusion that HUVEC proliferation under cyclic pressure was independent of VEGF-A expression and, for this reason, different from that observed under cyclic stretching (25).

It is of interest to note that the mechanism(s) of enhanced endothelial proliferation under either sustained hydrostatic or cyclic pressures involves known angiogenesis-associated molecules such as bFGF (1) and integrin-αV (22), as well as VEGF-C, IL-8, and CXCR-4 (present study). Since the sustained hydrostatic (1–11 mmHg) and cyclic (60/20 mmHg) pressures used in these studies are values characteristic of those present in the microcirculatory milieu (Sweeney TE, personal communication), it is possible that the results of these in vitro studies elucidate a biochemical-and/or molecular-level link between pressure and angiogenic phenomena associated with capillary formation in vivo. In addition, since the magnitude (maximum pressure of 60 mmHg and minimum pressure of 20 mmHg), pulse pressure (40 mmHg), and periodicity (1 Hz) of the cyclic pressure stimulus used in the present study are conditions also pertinent to the hemodynamic milieu of the large arteries, the gene transcription profile of endothelial cells under pulsatile pressures provides molecular-level information that may elucidate aspects of vascular conditions such as the neoplastic endothelial hyperplasia associated with plasmapheresis formation during pulmonary hypertension (27).

In summary, we report for the first time that, in addition to a set of previously reported mechanosensitive genes, exposure of HUVEC to cyclic pressure affects transcription of a novel subset of pressure-responsive genes. The molecular fingerprint revealed by microarray analysis provides critical new information regarding potential pathways linking cyclic pressure and endothelial cell function(s) pertinent to cardiovascular physiology and pathology. In addition, we provide the first evidence that the mechanotransduction of cyclic pressure into a functional endothelial response, i.e., proliferation, is mediated by VEGF-C.

This research was conducted at Genentech, Inc. We thank the following colleagues for assistance with various aspects of the project: Anan Chantharapai, Department of Immunology, for the mouse monoclonal VEGF-C antibody; Hans-Peter Gerber, Department of Molecular Oncology, for the VEGF-C TaqMan primers and probe set; Connie Zlot, Department of Cardiovascular Research, for assistance with bioinformatics analyses; Aldona Kallock, Department of Cardiovascular Research, for administrative assistance; and the Oligonucleotide Synthesis team, for generating TGF-β2 and synergist TaqMan primers and probe sets. We also acknowledge Jiro Nagatomi, Department of Biomedical Engineering at Rensselaer Polytechnic Institute, for technical assistance with the design and construction of the cyclic pressure system.

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REFERENCES


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