Modulated Inflammation by Injection of High-Mobility Group Box 1 Recovers Post-Infarction Chronically Failing Heart

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Background—Inflammation plays an important role in the progress of adverse ventricular remodeling after myocardial infarction. High-mobility group box 1 (HMGB1) is a nuclear protein, which has recently been uncovered to also act as a modifier of inflammation when released. We hypothesized that HMGB1 injection could preferentially modulate local myocardial inflammation, attenuate ventricular remodeling, and subsequently improve cardiac performance of postinfarction chronic heart failure.

Methods and Results—Three weeks after left coronary artery ligation, HMGB1 (2.5 μg) or PBS was intramyocardially injected into rat hearts. At 28 days after injection, left ventricular ejection fraction was significantly improved after HMGB1 injection compared to PBS (39.3±1.4 versus 33.3±1.8%; P<0.01). Accumulation of CD45+ inflammatory cells, two thirds of which were OX62+ dendritic cells, in the peri-infarct area was significantly attenuated by HMGB1 injection. Dramatic changes in the expression of major proinflammatory cytokines were not detected by microarray or RT-PCR. Adverse ventricular remodeling including cardiomyocyte hypertrophy (cardiomyocyte cross-sectional area; 439±7 versus 458±6 μm²; P<0.05) and extracellular collagen deposition (collagen volume fraction; 11.9±0.4 versus 15.2±0.6%; P<0.01) was attenuated by HMGB1 injection. Analyses of signal transduction pathways revealed that HMGB1 injection activated ERK1/2, but not p38, Akt, and Smad3. Cardiac regeneration and neovascularization were not observed.

Conclusion—HMGB1 injection modulated the local inflammation in the postinfarction chronically failing myocardium, particularly via reducing the accumulation of dendritic cells. This modulated inflammation resulted in attenuated fibrosis and cardiomyocyte hypertrophy, which thereby improved global cardiac function. These data suggest that HMGB1 may be valuable for the chronic heart failure treatment. (Circulation. 2008;118[suppl 1]:S106–S114.)

Key Words: heart failure • ventricular remodeling • inflammation • myocardial infarction

Adverse ventricular remodeling is an underlying factor for the development of heart failure after myocardial infarction (MI). This cellular and molecular event that affects extracellular matrix as well as surviving cardiomyocytes is known to progressively continue, eventually leading to severe dilation and dysfunction of the heart. Previous reports have indicated that inflammatory and/or immunogenic processes play an important role in the pathogenesis of ventricular remodeling, giving rise to an expectation that modulation of such inflammation should attenuate remodeling and may thus recover post-MI failing heart. However, despite encouraging experimental data, clinical trials of anticytokine therapy targeting a single inflammatory cytokine showed only insufficient therapeutic efficacy in patients with post-MI chronic heart failure. For the future success of this type of therapy, therefore, refinement of the protocol including development of a more effective modifier of inflammation is needed.

High-mobility group box 1 (HMGB1) was initially identified as a nuclear protein which regulates the interaction and recruitment of various transcriptional factors to stabilize nucleosome. Recently, it has been revealed that exogenous HMGB1 (either passively released from necrotic cells, actively secreted from inflammatory cells or externally injected) acts as a cytokine to modulate inflammation through its high affinity receptors on the surface of target cells, including the receptor for advanced glycation end products and toll-like receptors 2 and 4. Similar to other
damage-associated molecular pattern (DAMP) molecules such as heat shock proteins and adenosine. HMGB1 is reported to exhibit variable actions to inflammation, including both initiating/amplifying and attenuating the response. Such differential effects appear to be dependent on the condition or type of cells/organs as well as the duration/degree of extracellular HMGB1 upregulation. The reported function of HMGB1 to repair damaged tissues for maintaining the organ homeostasis is likely to be dependent on such highly-balanced, double-edged effects.

HMGB1 is also reported to have angiogenic and vasogenenic abilities. In addition, it has recently been demonstrated that HMGB1 has the potential to enhance myocardial regeneration by activating c-kit cardiac resident stem cells in an acute MI model. These findings encouraged us to investigate the hypothesis that administration of HMGB1 could preferentially modulate local myocardial inflammation, attenuate adverse ventricular remodeling, enhance myocardial/vascular regeneration, and subsequently improve cardiac performance in post-MI chronic heart failure.

Methods

Animal Care
All studies were performed with the approval of the institutional ethics committee and the UK Home Office. The investigation conforms to the Principles of Laboratory Animal Care (National Society for Medical Research) and the Guide for the Care and Use of Laboratory Animals (National Institutes of Health). All procedures and evaluations, including assessment of cardiac parameters, were carried out in a blinded manner.

Generation of Post-MI Chronic Heart Failure and HMGB1 Injection Into the Heart
Sprague-Dawley female rats (150 to 200 g; Harlan) underwent left thoracotomy under 1.5% isoflurane inhalation and mechanical ventilation using a volume-controlled ventilator (Harvard Apparatus). The left coronary artery (LCA) was ligated with 6 to 0 polypropylene (BairMedic) at the level of the bottom edge of the left atrial appendage. At 21 days after LCA ligation, under thoracotomy, HMGB1 protein (total 2.5 μg in 100 μL PBS, R&D) or the same volume of PBS as controls were injected into 2 sites of the heart. 

Assessment of Cardiac Function
Cardiac function and dimensions were assessed at 1 day before and 28 days after injection with echocardiography using Sequoia 512 and 15-MHz probe (Siemens Medical) under 1.5% isoflurane inhalation via a nose cone (n=7 and 8 in the control-treated and HMGB1-treated groups, respectively). Left ventricular diastolic and systolic areas were obtained from parasternal short-axis views with 2-dimensional tracing. Left ventricular ejection fraction was calculated from these obtained data.

Sample Preparation for Histological Studies
At set time points, the hearts were collected, perfused with 4% paraformaldehyde, and fixed in 4% paraformaldehyde on ice for 30 minutes. After this, the hearts were washed with PBS and incubated with PBS containing 30% sucrose (wt/vol) at 4°C overnight. The fixed hearts were then cut transversely into 3 pieces, each of which was embedded in OCT compound (BDH), frozen in liquid nitrogen-cooled isopentane and stored at −80°C. Cryosections were cut from these samples for histological studies below.

Assessment of Infarct Size, Fibrosis, Neovascularization, and Cardiomyocyte Hypertrophy
For investigating infarct size and collagen deposition, cryosections from 28-day samples (n=4 in each group) were stained with picrosirius red F3B (BDH) and observed with an all-in-one microscope system (Keyence, BZ8000). At least 1 cryosection from each of 3 pieces of heart sample was examined. Infarct size was measured as a percentage of scar length to total LV circumference. Fibrosis in the peri-infarct and remote areas was semiquantitatively assessed by morphometric determination of collagen volume fraction using ImageJ software. To assess neovascularization, cryosections from 28-day samples were stained with anti-von Willebrand factor antibody (1:200 dilution, Abcam) and then with a fluorescent conjugated secondary antibodies (Molecular Probes). The number of capillary vessels in 10 to 16 different fields of the peri-infarct area per heart was counted (n=4 in each group).

Sampling for Cardiomyocyte Fraction
Cardiomyocytes in the peri-infarct and remote area per heart were analyzed with a Keyence BZ8000 fluorescence microscope and the positive cell number was counted.

Analysis of Proliferating Cells in the Heart and Cardiomyocyte Fraction
To investigate myocardial regeneration, both 3-day (n=5 in each group) and 28-day (n=4 in each group) samples were double-stained with antiphosphorylated histone H3 (phhistone H3; 1:100 dilution, Upstate) and either anti-α-sarcormeric actin (1:200 dilution, Daco) or anti-Nkx2.5 (1:100 dilution, Santa Cruz), anti-CD34 (1:100 dilution, Santa Cruz), anti-CD45 (1:400 dilution, BD Pharmingen), anti-PECAM1 (1:100 dilution, Santa Cruz), or anti-VEGFR2 (1:100 dilution, Abcam) antibodies. Appropriate fluorophore-conjugated secondary antibodies (Molecular Probes) were applied and nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma). Images were obtained and analyzed with a fluorescence microscope (Keyence, BZ8000). Five to 10 different fields in each of the peri-infarct and remote area per heart were analyzed to count the number of positive cells. For investigating cardiomyocyte fraction, cryosections of 28-day samples (n=4 in each group) were stained with anti-α-sarcormeric actin (1:200 dilution, Daco) antibody and then with a fluorescent conjugated secondary antibody (Molecular Probes). The fraction of cardiomyocytes in the infarct area was semiquantitatively assessed using ImageJ software.

Microarray Analysis
At 3 days after injection, the hearts were excised and the whole LV walls were frozen and homogenized in TRIzol (Invitrogen) to extract total RNA. RNA was purified using an RNEasy kit after the digestion of the genome by DNaseI (Qiagen). Double-stranded cDNA was synthesized from 5 μg of total RNA, and biotinylated target cRNA was synthesized by Affymetrix IVT labeling kit. After fragmentation, 15 μg of cRNA was hybridized with the mouse Genome 230 2.0 Array (Affymetrix). GeneChips were then scanned in a GeneChip Scanner 3000 (Affymetrix). The obtained data were analyzed with GeneSpring GX 7.3.1 software (Agilent Technologies). Two independent experiments were performed.

Quantitative RT-PCR
cDNA was obtained by reverse transcription from 1 μg of total RNA obtained as above (n=5 in each group). Primers, reagents, and
TaqMan probes for IL-1β and IL-6 were purchased from Applied Biosystems. Standard curves for expression of each gene were generated by serial dilution of known quantities of the respective cDNA gene template. Relative quantification of the signals was performed by normalizing with the GAPDH signal. Samples were prepared in triplicate and run on Prism 7900 (ABI).

**Western Blotting**

Frozen whole LV specimens were dispersed mechanically (n=4 in each group) in lysis buffer. After measuring protein concentration, 50 to 100 μg protein was subjected to SDS-PAGE and transferred to PVDF membrane. Immunodetection was performed first against phosphorylated Akt, ERK1/2, p38, and Smad3 and then against respective total level of each protein (antibodies from Cell Signaling Technology). The signals were developed with horseradish peroxidase conjugated specific secondary antibodies and the ECL system (Amersham). Developed films were scanned and quantitative analysis was performed using ImageJ software. The proportion of phosphorylated to total protein was normalized to the corresponding control-treated group.

**Statistical Analysis**

All values are expressed as mean±SEM. The differences in the data between 2 groups (capillary density, infarct size, cross-sectional area, histone H3 + cell number, cardiomyocyte fraction, ERK1/2 expression) were determined with the nonparametric Mann-Whitney U test. Comparison among multiple groups (IL-1β and IL-6 expression) was performed by 1-way ANOVA followed by Fisher protected least significant difference test. A repeated-measures ANOVA was used to compare the echocardiography data, CD45 + cell number, OX62 + cell number, and collagen fraction. P<0.05 was considered to be significant.

**Statement of Responsibility**

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

**Mortality**

After LCA ligation and before HMGB1/PBS injection, 12.5% (5/40) rats died presumably as a result of acute heart failure. All deaths occurred within 48 hours after LCA ligation. At 21 days after LCA ligation, the surviving 35 rats were randomized and received either HMGB1 or PBS injection into the peri-infarct zone. After either injection, there was no mortality.

**Functional Improvement by HMGB1 Injection**

Cardiac function was evaluated at 28 days after HMGB1 or PBS injection with echocardiography. The left ventricular ejection fraction was significantly larger in the HMGB1-treated group compared to the control-treated group (Figure 1A and 1B). Left ventricular systolic areas tended to be larger in the control-treated group compared to the HMGB1-treated group (Figure 1B). There was no significant difference in heart rate between the groups (Figure 1B).

**Modulated Inflammation by HMGB1 Injection**

The myocardial inflammatory response was evaluated by counting the number of inflammatory cells accumulated in the heart and by measuring mRNA expression levels for inflammatory cytokines. At 3 days after injection, the number of CD45 + panleukocytes in the peri-infarct area was significantly decreased in the HMGB1-treated group compared to the control-treated group (169.0±8.8 versus 232.4±8.9/mm², P<0.01; Figure 2A through 2C), although no significant change was observed in the remote area (161.5±9.4 versus 149.4±6.0/mm²). Approximately 70% of CD45 + cells in either group displayed a typical morphology of dendritic cells (DCs), which were also seen in OX62 + cells in our study. Consistent to this, the number of OX62 + DCs was similarly reduced in the HMGB1-treated group compared to the control-treated group in the peri-infarct area (118.3±6.4 versus 170.7±7.1/mm², P<0.01; Figure 2D through 2F), but not in the remote area (80.6±3.7 versus 93.9±5.2/mm²; Figure 2F).

The expression level of inflammatory cytokines, such as IL-1β, IL-6, TNFa, and IL-10, were not high enough to be detected by microarray analysis (Table). Quantitative RT-PCR demonstrated an increased expression of IL-1β and IL-6 in both the control-treated and HMGB1-treated groups compared to normal intact hearts. No significant change in mRNA expression of either inflammatory cytokines, however, was observed between the injection groups (Figure 3A and 3B).

**Changes in Fibrosis, Neovascular Formation, Infarct Size and Cardiomyocyte Hypertrophy**

At 28 days after injection, picrosirius red staining detected severe collagen deposition between cardiomyocytes in the peri-infarct area of the control-treated group (Figure 4A) and this appeared to be reduced in the HMGB1-treated group (Figure 4B). Quantification demonstrated that the...
collagen volume fraction was significantly reduced in the HMGB1-treated group compared to the control-treated group in the peri-infarct area (11.9±0.4 versus 15.2±0.6%, P<0.01; Figure 4C), but not in the remote area (9.1±0.3 versus 9.9±0.3%). Microarray analysis using 3-day samples detected an altered expression of fibrosis-related genes including reduction of TIMP1, a major fibrogenic factor, in the HMGB1-treated group, although not statistically significant (Table).

Capillary density was assessed to investigate the effect of HMGB1 on neovascular formation using immunolabeling for von Willebrand factor. There was no significant difference in capillary density between the control-treated (901.8±28.6/mm²) and HMGB1-treated groups (919.6±26.5/mm²; Figure 4D through 4F).

Cardiomyocyte cross-sectional area was measured to assess the extent of post-MI cardiomyocyte hypertrophy. As a result, cardiomyocyte hypertrophy was significantly attenuated in the HMGB1-treated group compared to the control-treated group (439±7 versus 458±6 μm², P<0.05; Figure 4G). There was no difference in infarct size between the control and HMGB1-treated groups (34.7±1.4 versus 36.7±1.3%, Figure 4H).

**Myocardial Regeneration**

The myocardial regenerative response was evaluated by detecting/characterizing the proliferating cells within the myocardium by immunofluorescence for histone H3, a cell-cycle (M phase) marker, and by measuring the cardiomyocyte fraction. At 3 days after injection, the number of histone H3+ cells in the peri-infarct area was significantly increased in the HMGB1-treated group (7.9±1.1/mm²) compared to the control-treated group (4.7±0.4/mm², P<0.05; Figure 5A through 5C). However, none of these cells were positive for α-sarcomeric actin (Figure 5A and 5B) or Nkx2.5 at 3 days or 28 days after injection (data not shown). In addition, cardiomyocyte fraction in the infarct area at 28 days after injection assessed by immunolabeling for α-sarcomeric actin was not significantly changed between the control and HMGB1-treated group (9.7±0.8 versus 8.7±1.7%; Figure 5D thorough 5F). Coimmunostaining for histone H3 and either CD34, CD45, PECAM1, or VEGFR2 at 3 days after injection revealed that about one half of histone H3+ cells were CD45+ hematopoietic cells in both groups (Figure 5G through 5J). The majority of histone H3+ cells were negative for Nkx2.5, CD34, PECAM1, and VEGFR2 after either HMGB1 or PBS injection (data not shown). It was confirmed that all antibodies used in this study reacted to positive controls in rat.

**Signal Transduction Pathways**

Major signal transduction pathways which are reportedly involved in ventricular remodeling were analyzed. Phosphorylated ERK1/2 level was increased 1.2±0.1-fold in the HMGB1-treated group compared to the control-treated group (P<0.05, Figure 6A and 6B). On the other hand, Akt, p38, and Smad3 were not affected by HMGB1 injection (data not shown).

**Discussion**

We have demonstrated that injection of HMGB1 improved global function of post-MI chronically failing hearts in association with attenuated accumulation of inflammatory cells, particularly DCs. In the HMGB1-treated heart, cardiomyocyte hypertrophy and pathological extracellular matrix accumulation, both of which are important factors of post-MI ventricular remodeling, were significantly attenuated. On the other hand, reduction of infarct size, enhancement of neovascular formation and myocardial regeneration were not observed. Therefore, attenuated ventricular remodeling is considered to be the major factor responsible for the improved global cardiac function after HMGB1 injection.
This study demonstrated that the accumulation of inflammatory cells (CD45-H11001/panleukocytes) in the peri-infarct area was significantly reduced by HMGB1 injection at the chronic phase of heart failure. This area is considered to be a major stage for the progress of ventricular remodeling. Considering the evidence that OX62-H11001/DCs also express CD45, it was estimated that 70% of accumulated CD45-H11001/panleukocytes were DCs in both the HMGB1 and control groups. Consistent with this, approximately two thirds of accumulated CD45-H11001/panleukocytes presented a typical morphology of DCs, similarly to OX62-H11001/DCs in our study. DCs are potent antigen presenting cells and can also affect the immune response by modulating the expression profile of inflammatory cytokines. Persistent accumulation of DCs in the ischemically damaged area has been observed not only in the heart but also in other organs such as brain and liver. Although the detailed role of DCs in ischemic heart failure is uncertain, the reverse correlation between the level of the DC accumulation and ventricular remodeling in our study may suggest that DCs play a role in the progress of post-MI chronic heart failure. The effect of HMGB1 on activation and recruitment of DCs is still controversial or may be double-edged. Some reports have demonstrated the proinflammatory properties of extracellular HMGB1 on DCs, whereas others have reported that exogenous HMGB1 attenuates the proinflammatory function of DCs by inhibiting the secretion of proinflammatory cytokines. Here we provided additional information that exogenous HMGB1, at the dosage used in this study, reduced the accumulation of DCs in the post-MI chronically failing myocardium in rat.

This experiment was repeated twice and data is expressed as an averaged change. ND indicates not determined because its low signal (the expression value lower than background).

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Continued upregulation of inflammatory cytokines associated with post-MI inflammatory response is an integral component in myocardial remodeling as well as repair. Contrary to our prediction, HMGB1 injection did not dramatically alter the expression of major inflammatory cytokines.
including IL-1β or IL-6 in the present study. Nevertheless, this treatment provided a significant increase of left ventricular ejection fraction at 28 days after injection. We speculate that accumulated subtle changes in the cytokine-profile as a whole, rather than a substantial change in the expression of a single cytokine, might be beneficial to introduce therapeutic effects in chronically failing hearts undergoing complex processes of adverse remodeling. As responders to such mildly modified inflammation and also as responsible factors for the functional recovery of the failing heart by HMGB1 injection, our results highlighted cardiomyocyte hypertrophy and fibrosis. Both these are key factors to determine ventricular remodeling and dysfunction of the post-MI heart, and are therefore major targets of the treatment for post-MI chronic heart failure.

In our study, microarray data showed HMGB1 injection appeared to induce down-regulation of TIMP1, which is a key fibrogenic factor, suggesting a possible role of TIMP1 on HMGB1-mediated reduction of fibrosis in post-MI chronic heart failure.

To identify the molecular mechanism underlying HMGB1-induced beneficial effects, we also analyzed major signal transduction pathways which are involved in ventricular remodeling, particularly hypertrophy and fibrosis. As a result, it was shown that HMGB1 injection significantly activated ERK1/2, whereas Akt, p38, and Smad3 were not affected. To date, the direct role of ERK1/2 pathway on cardiomyocyte hypertrophy or fibrosis in post-MI chronic heart failure remains unknown. However, it has been reported that ERK1/2 is an important survival factor for cardiomyocytes, suggesting that ERK1/2 activation by HMGB1 could prevent cardiomyocyte loss and/or could preserve cardiomyocyte function in the failing, remodeling heart. As a secondary consequence to this, post-MI progression of fibrosis in the peri-infarct zone might be attenuated. Although some angiogenic factors including fibroblast growth factors are known to be upstream molecules of ERK1/2, the capillary density in our model of post-MI chronic heart failure was not increased by HMGB1 treatment.

In this study there appears to be several potential limitations. First, we used PBS injection as a control following many recent reports, although injection of denatured HMGB1 might be the more suitable control. The lack of fully established denaturation methods prevented us from being able to carry out the experiment. In addition, it is highly likely that PBS injection resulted in a similar, if not identical, outcome to that of the “more
appropriate control injection, in terms of cardiac function, ventricular remodeling, gene/protein expression and signal transduction. Second, gene expression is known to be different between the infarct, border, and remote areas, but we did not separate each of these areas for protein/gene analysis. After 2-stage surgery (coronary artery ligation and HMGB1 injection) under repeated thoracotomy, it is extremely difficult to clearly identify the border of each area in most samples due to adhesion. We did not take a risk of cross-contamination between areas by uncertain separation, and instead concentrated to provide more reliable information on the inclusive changes in gene expression in the whole LV. These limitations on the experimental design would be challenged in the next stage study.

Recently, Limana et al have shown that HMGB1 injection into the mouse heart with acute MI amplified cardiomyogenic regeneration, leading to the improvement of cardiac performance. They demonstrated that infarcted ventricular myocardium was dramatically regenerated with newly formed small cardiomyocytes which were derived from cardiac resident stem cells. In our study, to the contrary, the α-sarcomeric actin staining demonstrated that HMGB1 injection did not increase the mass of cardiomyocytes in the infarct area. In addition, there was no phistone H3+/Nkx2.5− or phistone H3+/α-sarcomeric actin+ cells at any time points studied in either group. Although our results do not exclude their attractive idea of the augmented cardiac regeneration by HMGB1, we concluded that cardiac regeneration was unlikely to be involved in the improved cardiac function in our rat model of post-MI chronic heart failure, despite the use of the equivalent amount of HMGB1 for injection. Given that a typical mammalian cell contains $1 \times 10^6$ HMGB1 molecules and that the molecular weight of HMGB1 is 25, it is estimated that release of 2.5 $\mu$g of HMGB1 requires $1 \times 10^7$ necrotic cells. Different from the case of acute MI, it is unlikely that such a substantial amount of cell death occurs at once in post-MI chronic heart failure, suggesting that the amount of injected HMGB1 was larger than the typical amount of intrinsically released HMGB1 in chronic ischemic heart failure. The reasons for such a discrepancy in myocardial regeneration by HMGB1 are uncertain, but this may be due to the different species used for the experiment and/or different natures of the host myocardium (acute MI versus chronic heart failure). It has been reported that human cardiac stem cells isolated from post-MI chronic failing hearts showed the advanced cellular senescence and the reduced growth response compared to those from acute MI hearts. Such an impaired regenerative ability of the chronically failing heart may also explain the discrepancy observed here.

We found that HMGB1 injection increased the number of phistone H3+ proliferating cells in the myocardium. A half of these phistone H3+ cells were CD45− hematopoietic cells, and the majority of phistone H3+ cells were negative for Nkx2.5, CD34, PECAM1 and VEGFR2 after

Figure 5. Myocardial regeneration. A–C, Immunolabeling demonstrated that phistone H3+ proliferating cells (green color) were increased in the peri-infarct area of the HMGB1-treated group (A) compared to the control-treated group (B) at 3 days after injection. None of these phistone H3+ cells was positive for α-sarcomeric actin (red color). Blue color indicates nucleus (DAPI). Scale bar=200 μm. Quantification showed a significant increase of the number of positive cells in the HMGB1-treated group compared to control-treated (CON) group (C). Data are expressed as means±SEM, *P<0.05 versus CON, n=5 in each group. D–F, At 28 days after injection, viable cardiomyocytes were observed in the infarct areas of the control-treated (D) and HMGB1-treated groups (E) by immunolabeling for α-sarcomeric actin (red color). Scale bar=300 μm. Cardiomyocyte fraction was not different between the groups (F). Data are expressed as means±SEM n=4 in each group. G–I, Immunolabeling demonstrated that CD45 (G; red color) was expressed in about one half of the phistone H3+ proliferating cells (H; green color). Cells were counterstained with DAPI (I; blue color) and the images are merged in J. Scale bar=50 μm.
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Disclosures

None.

References


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