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Membrane-Bound CC Chemokine Inhibitor 35K Provides Localized Inhibition of CC Chemokine Activity In Vitro and In Vivo

Christina A. Bursill,*† Jenna L. Cash, † Keith M. Channon,* and David R. Greaves2†

CC chemokines mediate mononuclear cell recruitment and activation in chronic inflammation. We have shown previously that gene transfer using recombinant adenoviruses, encoding a soluble CC chemokine-binding protein of vaccinia virus 35K, can dramatically reduce atherosclerosis and vein graft remodeling in apolipoprotein E knockout mice. In this study, we report the development of a membrane-bound form of 35K (m35K), tagged with GFP, which allows for localized, broad-spectrum CC chemokine blockade. In vitro experiments indicate that m35K-expressing cells no longer undergo CC chemokine-induced chemotaxis, and m35K-expressing cells can locally deplete the CC chemokines RANTES (CCL5) and MIP-1α (CCL3) from supernatant medium. This sequestration of CC chemokines can prevent chemotaxis of bystander cells to CC, but not CX3C chemokines. Intraperitoneal injection of mice with an adenovirus-encoding m35K leads to a significant (44%) decrease in leukocyte recruitment into the peritoneal cavity in a sterile peritonitis model. Intravenous adenovirus-encoding m35K delivery leads to m35K expression in hepatocytes, which confers significant protection against liver damage (75% reduction in liver enzymes) in a Con A-induced hepatitis model. In summary, we have generated a membrane-bound CC chemokine-binding protein (m35K) that provides localized broad-spectrum CC chemokine inhibition in vitro and in vivo. m35K may be a useful tool to study the role of CC chemokines in leukocyte trafficking and block the recruitment of monocytes in chronic inflammation. The Journal of Immunology, 2006, 177: 5567–5573.
but before the protease cleavage site. This fragment was then cloned into plasmid pShuttleCMV containing the 750-bp open reading frame of vaccinia viral 35K (Lister strain) and a hemagglutinin (HA) epitope tag ps35K (15) to create a final plasmid psm35K. This was used to generate recombinant Adm35K by transfection into 293 cells using the AdEasy system (20) (Fig. 1). Viruses were isolated by three rounds of plaque purification, amplified in 293 cells, and purified using CsCl gradient ultracentrifugation, as described previously (21).

Assessment of cell migration in vitro

The effect of m35K on CCR5-directed cell migration was assessed by two different approaches using an in vitro chemotaxis assay described previously (15). The 293 cells were transfected separately with plasmids encoding either: 1) CCR5, empty vector (pShuttle), and GFP; or 2) CCR5, m35K, and GFP. Transfected cells were harvested and allowed to migrate toward increasing concentrations of CC chemokine RANTES (Research Diagnostics). As a control, 293 cells were transfected separately with plasmids encoding either: 1) CX3CR1 (unrelated fractalkine receptor), empty vector, empty vector, and GFP; or 2) CX3CR1, m35K, and GFP. Transfected cells were harvested and allowed to migrate toward increasing concentrations of the CX3CR1 fractalkine (Research Diagnostics). To assess the effect of m35K on bystander CCR5 cell migration, 293 cells were transfected separately with plasmids encoding either: 1) CCR5 and GFP; or 2) m35K and GFP; or 3) empty vector and GFP (to make up total number of migrating cells). Transfected cells were harvested and placed on Transwell membranes. CCR5/GFP cells were allowed to migrate toward mouse plasma (10 µl) in the presence of increasing amounts of m35K/GFP cells (0–40 µl). As a control, the same experimental protocol was followed, but substituting CX3CR1 for CCR5 and allowing the cells to migrate toward fractalkine (20 ng/ml). Cell migration was measured as green cell pixel count from confocal images of cells that had migrated through the Transwell membranes (duplicates, three scans/membrane).

Animals and gene transfer

Adm35K, Ad35K, AdGFP, or PBS was administered to C57BL/6 mice via the tail vein (1 × 1011 viral particles (vp)). Five days postinfection, mouse plasma was collected using cardiac puncture from terminally anesthetized animals and isolated by centrifugation at 500 × g for 5 min. Livers were excised and stored at −20°C for 35K protein determination. A small piece of liver was also embedded in OCT (TissueTek) and then frozen at −80°C for sectioning. All animal procedures were conducted in accordance with the Animal (Scientific Procedures) Act 1986, and after appropriate Local Ethical Review.

Biogel-induced peritonitis model

Biogel beads (0.5 ml of 2% w/v in PBS) were injected in combination with PBS, AdGFP, or Adm35K (1 × 1011 vp) into the peritoneal cavity of C57BL/6 mice (10 wk old; n = 10). Four days after treatment, mice were sacrificed and peritoneal exudates were collected into 10 ml of ice-cold PBS plus 2 mM EDTA. Cells were spun down at 1200 rpm for 10 min in a bench top centrifuge and resuspended in PBS before counting on a Coulter counter (Beckman Coulter) to assess the total number of cells in peritoneal exudates.

Con A-induced hepatitis

C57BL/6 mice (7–8 wk old) were injected with PBS, AdGFP, or Adm35K (1 × 1011 vp) via the tail vein. Three days after Ad gene transfer, mice were injected with Con A (13.5 mg/kg; Sigma-Aldrich) (22, 23). Eight hours following Con A administration, mice were sacrificed and blood was collected for measurement of plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (commercial kits: Infinity ALT and AST; Thermo Electron) and TNF-α levels (ELISA). Livers were flushed with 10 ml of PBS, and then frozen in OCT compound (TissueTek) for sectioning. Liver sections (5 µm) were stained with H&E according to standard protocols and analyzed by light microscopy.

FIGURE 1. Construction of Adm35K. Enhanced GFP (eGFP), the type II transmembrane domain of FasL (FasL TM), and 35K plus an HA tag were cloned to make plasmid Adm35K. ITR, inverted terminal repeats.

Western immunoblotting

Medium (15 µl) and cell lysates (20 µg) from cells infected with Ad35K or Adm35K, as well as plasma and livers from Adm35K-, Ad35K-, AdGFP-, or PBS-infected mice were separated on a 14% w/v stacking gel and transferred to polyvinylidene difluoride membrane (Millipore). The 35K protein was detected using rat anti-HA high-affinity mAb diluted 1/2000 (Roche), followed by an anti-rat secondary Ab conjugated to HRP diluted 1/1500. To extract m35K from mouse plasma, 180 µl of plasma was incubated with monoclonal anti-HA agarose-conjugated beads (Sigma-Aldrich) by methods described previously (15) before Western blotting.

To determine whether m35K expression is predominantly localized to the liver membranes, whole liver homogenates from AdGFP- and Adm35K-infected mouse livers were centrifuged at 100,000 × g for 1 h in an Ultracentrifuge (TL-100; Beckman Coulter). The supernatant (cytosolic fraction) was collected and the liver membrane pellet was resuspended in radioimmunoprecipitation assay buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% Nonidet P-40, 1 mM PMSE, 10 mM DTT, and protease inhibitor tablet) and then sonicated for 2 × 10-s bursts. Protein concentrations were determined, and 50 µg of protein from different liver fractions was used to detect m35K protein using Western blotting, as described above.

Local inhibition of CC chemokine activity in vivo

The 293 cells cotransfected with CCR5 and GFP were allowed to migrate toward plasma (10 µl) or liver homogenates (30 µl, 6 mg/ml) from mice infected with AdGFP, Ad35K, or Adm35K. For some experiments, chemotaxant ApoE KO mouse plasma (10 µl) was also included to stimulate migration (15–17).

CC chemokine scavenging

Chemotaxis buffer (DME, 25 mM HEPES, and 0.1% BSA) containing RANTES (25, 50 ng/ml) or MIP-1α (10, 20 ng/ml) was incubated for 1 h at 37°C with cells transfected with GFP (control plasmid) or m35K. This conditioned medium was collected, spun at 500 × g for 5 min, and then placed in the lower chamber of the chemotaxis assay. The 293 cells transfected with CCR5 plus GFP were allowed to migrate toward the conditioned medium, as described above.

Detection of CC chemokines and TNF-α in mouse plasma

Mouse RANTES was detected in mouse plasma (50 µl) using ELISA kits (Quantikine; R&D Systems), following manufacturer’s instructions. Anti-TNF-α capture and detection Abs were purchased from R&D Systems and used according to manufacturer’s suggested protocol.

Results

Detection of recombinant m35K protein in cell medium and cell lysates

Cells infected with Adm35K expressed a protein that was localized to cell membranes when viewed under the confocal microscope (Fig. 2A). Using Western blotting with an anti-HA Ab, a protein of the expected size was observed (65 kDa, 35K plus GFP), which was present in cell lysates, but not in cell medium (Fig. 2B). As a control, cells were also infected with Ad35K. These cells expressed a protein of molecular mass 35 kDa that was detected using an anti-HA Ab (Fig. 2B). As shown previously (15), this protein was present in the cell medium, but was not present at detectable levels in the cell lysates, indicating that the majority of 35K is made by the cell and immediately secreted into the medium. Together, these experiments show that m35K encodes a membrane-bound GFP-35K fusion protein.

m35K inhibits cell migration in vitro

To determine the effect of m35K on CCR5-directed cell migration in vitro, cells were transfected with CCR5 and either m35K or empty vector, and then allowed to migrate toward increasing concentrations of RANTES. In chemotaxis assays, the migration of cells expressing both CCR5 and m35K toward RANTES was significantly attenuated compared with cells expressing CCR5 only.
In contrast, the migration of cells expressing the unrelated fractalkine receptor (CX3CR1) and m35K toward fractalkine was not different from cells expressing CX3CR1 only, indicating that the inhibitory effects of m35K on cell migration are specific to the CC chemokine class (Fig. 3B).

To determine whether m35K could sequester CC chemokine activity and inhibit cell migration, cells were transfected with CCR5 plus GFP or m35K plus GFP. Using the chemotaxis assay, the inclusion of increasing volumes of cells expressing m35K plus GFP to CCR5 plus GFP cells significantly inhibited CCR5-directed bystander cell migration ($p < 0.05$) toward chemoattractant mouse plasma (Fig. 4A). In contrast, the inclusion of increasing volumes of cells expressing m35K plus GFP to CX3CR1 plus GFP cells did not affect the migration of CX3CR1-directed cell migration toward fractalkine (Fig. 4B).

**In vivo gene transfer of m35K**

To investigate the effects of recombinant m35K expression in vivo, Adm35K, AdGFP, or Ad35K ($1 \times 10^{11}$ vp) was administered to mice as a single injection via the tail vein. Five days post-Ad infection, the efficiency of i.v. AdGFP and Adm35K gene transfer was demonstrated by the presence of numerous fluorescent green cells in liver sections, viewed by confocal microscopy (Fig. 5A). The GFP fluorescence in Adm35K liver sections appeared to be localized to cell membranes of hepatocytes; this was in contrast to the cytosolic GFP expression seen in AdGFP liver sections.

Using Western blotting, a 65-kDa m35K protein was clearly visible in the liver homogenates (30–90 ng) of mice infected with
Adm35K (Fig. 5B, lanes 6–8), whereas no m35K was detected in the livers of control mice infected with AdGFP (Fig. 5B, lane 3) or Ad35K (Fig. 5B, lane 4). No m35K protein was detected in the plasma (180 μl) of mice infected with Ad35K after HA agarse bead pull-down, indicating that m35K protein expression is localized to the liver (Fig. 5B, lane 2). To determine whether the expression of m35K is predominantly in the cell membranes, membrane and cytosolic fractions from whole liver homogenates were prepared. m35K protein was detected using Western blotting. A 65-kDa band corresponding to m35K was clearly visible in the whole homogenate and the membrane fraction of m35K livers (Fig. 5C, lanes 3 and 5). A very light band was detected in the cytosolic fraction (Fig. 5C, lane 7). No bands were visible in the liver preparations of GFP liver. This provides further evidence that m35K expression is stably localized to the cell membranes of hepatocytes.

Ad-mediated m35K provides localized inhibition of CC chemokine activity in vivo

To determine whether m35K CC chemokine inhibition is localized to the liver, CCR5-directed migration was assessed toward chemotactractant mouse plasma spiked with liver homogenate from mice infected with AdGFP, Ad35K, or Adm35K. The addition of liver homogenates from Adm35K mice reduced CCR5-directed cell migration by 80% (p < 0.05). AdGFP and Ad35K liver homogenates did not affect CCR5-directed cell migration (Fig. 6A).

Plasma from AdGFP, Ad35K, or Adm35K mice was also included in the chemotaxis assay. As shown previously (15–17), plasma from Ad35K mice induced significantly lower levels of cell migration than AdGFP plasma (p < 0.05). Adm35K plasma, however, induced an intermediate level of cell migration that is significantly greater than Ad35K plasma, but lower than AdGFP plasma (p < 0.05; Fig. 6B).

Ad-mediated m35K reduces CC chemokine RANTES in mouse plasma

To determine whether m35K influenced CC chemokine levels in mouse plasma, we used a specific ELISA to quantify the concentration of RANTES in plasma from mice that had been injected with PBS, AdGFP, Ad35K, or Adm35K. RANTES concentrations were significantly higher (p < 0.05) in Ad-infected mouse plasma (AdGFP, Ad35K, Adm35K) than nonviral PBS control plasma. Interestingly, plasma from mice infected with Ad35K had significantly higher levels of RANTES protein than AdGFP control mice (p < 0.05; Fig. 6C), despite significantly lower CC chemokine bioactivity as measured in the chemotaxis assay (Fig. 6A). Plasma from Adm35K mice, however, had significantly lower concentrations of RANTES (p < 0.05) than both Ad35K and AdGFP (Fig. 6C).
Scavenging of CC chemokine activity by m35K

To determine whether m35K could scavenge CC chemokines, medium containing increasing amounts of RANTES and MIP-1α was incubated with 293 cells expressing m35K or GFP. This conditioned medium was then included in the chemotaxis assay. Medium that had been exposed to m35K-expressing cells induced significantly lower levels of CCR5-directed cell migration (p < 0.05) than medium exposed to GFP-expressing cells (Fig. 7). This reduction in CC chemokine activity following incubation with m35K-expressing cells occurred for both RANTES and MIP-1α at all treatment concentrations tested.

Ad-mediated m35K reduces leukocyte recruitment in peritonitis and hepatitis models

To determine whether 35K could inhibit chemokine-induced recruitment of monocytes and macrophages in vivo, Biogel beads were injected i.p. into mice in combination with PBS, Ad35K, or AdGFP. Frustrated phagocytosis of Biogel beads by resident peritoneal macrophages leads to leukocyte recruitment into the peritoneal cavity, which consists predominantly of macrophages 4 days postinjection (19). Four days after treatment, there was a significant increase in total cell counts in peritoneal exudates (43%) between mice injected with AdGFP plus Biogel compared with PBS plus Biogel control mice (Fig. 8A). This is consistent with previous reports that Ad infection increases inflammation, mediated in part by induction of CC chemokines (24, 25). Interestingly, there was a significant 44% reduction in total cell counts in the Adm35K plus Biogel compared with the AdGFP plus Biogel mice, indicating that m35K gene transfer to the peritoneal cavity was able to reduce CC chemokine-directed inflammatory cell recruitment.

CC chemokines have been implicated in the inflammatory effects of Con A-induced hepatic injury (23, 26–28). We therefore investigated the effect of liver-localized, broad-spectrum CC chemokine inhibition via Ad-mediated delivery of Adm35K, on the Con A hepatitis model. Systemic administration of Con A resulted in a large elevation in plasma ALT, AST, and TNF-α after a 7-h incubation period compared with control mice injected with PBS only (Fig. 8B). There were no significant differences in liver enzymes or TNF-α between Con A-only mice and those injected with AdGFP plus Con A. Interestingly, mice administered Adm35K plus Con A had significantly reduced liver damage, as plasma ALT and AST were each reduced by 75% (p < 0.05) compared with control GFP mice. Using histology on liver sections from these mice, m35K mouse livers had less hepatodegenerative changes and inflammatory infiltrate compared with AdGFP control mouse livers (Fig. 8C). In addition to this, Adm35K plus Con A mice had significantly lower levels (76%; p < 0.05) of TNF-α compared with control GFP mice (Fig. 8B). This indicates that localized CC chemokine blockade, established using Ad-mediated m35K gene delivery to hepatocytes, confers significant protection against liver injury in the Con A model of hepatitis.

Discussion

In this study, we report the successful construction of a membrane-bound version of the vaccinia virus CC chemokine-binding protein m35K. This construct contains an intracellular GFP domain to allow for easy detection using confocal microscopy. Both in vitro and in vivo studies have demonstrated that infection with a recombinant adenovirus encoding this form of the 35K protein (Adm35K) results in high level expression of m35K protein that is localized to cell membranes and readily detectable using confocal microscopy (GFP) and Western blotting. Importantly, m35K expression is able to strikingly inhibit CC chemokine-directed cell
m35K PROVIDES LOCALIZED CC CHEMOKINE INHIBITION

Migration and CC chemokine activity in vitro and in vivo. We have demonstrated previously the importance of the CC chemokine class in atherosclerosis using gene transfer of native soluble 35K (16, 17). Broad-spectrum CC chemokine inhibition by m35K may be a useful tool to study the effect of localized CC chemokine blockade.

The addition of a transmembrane domain allows the location of 35K protein expression to be controlled. This could potentially allow for more useful clinical applications of broad-spectrum CC chemokine blockade. Previous studies have found that soluble 35K vaccinia viral protein binds to and inactivates nearly all of the CC chemokine class, providing a broad-spectrum blockade of CC chemokine activity (12, 14, 29, 30). In addition to this, Ad35K to ApoE KO mice significantly reduced native atherosclerosis, macrophage recruitment, and CC chemokine activity (16). In the vein graft model, Ad35K dramatically reduced accelerated atherosclerosis (17). These studies, using Ad35K, provide a striking example that CC chemokine inhibition can reduce inflammatory diseases, such as atherosclerosis, that are characterized by monocyte recruitment and macrophage differentiation.

This study set out to investigate whether m35K could also inhibit CC chemokine activity. The single pass type II transmembrane domain was cloned from FasL, including an extra 25 aa past the transmembrane domain, but before the protease cleavage site that normally generates soluble FasL. The m35K protein therefore arranges itself such that the N terminus (GFP) is on the inside of the cell and the C terminus (35K plus HA tag) is on the outside of the cell (31). Potentially, the extra 25 aa after the transmembrane domain may help to extend 35K out from the cell membrane, making it more accessible to circulating chemokines. By virtue of being anchored to a membrane, there are different mechanisms by which 35K may act to reduce leukocyte chemotaxis: 1) by direct, cell-autonomous inhibition of chemotaxis, or 2) inhibition of bystander cell chemotaxis. Using an in vitro bioassay that assesses cell migration, we found that coexpression of m35K with CCR5 significantly attenuated CCR5-directed cell migration toward RANTES (Fig. 3). The exact mechanism by which localized m35K cell expression inhibits cell migration is difficult to pinpoint in these experiments. It is possible, however, that m35K can bind to and reduce the effective chemokine concentration in the local microenvironment, which therefore destroys the chemoattractant chemokine gradient that is required to trigger cell migration (32). Without this gradient, the cells have no directional stimulus and will not migrate. Separate studies also demonstrated that the addition of m35K-expressing cells to CCR5-expressing cells inhibited their normal CCR5-directed cell migration toward RANTES (Fig. 4). This suggests that m35K-expressing cells are able to sequester CC chemokines, thereby reducing the surrounding CC chemokine activity and thus reducing bystander cell migration. Such a mode of action may find therapeutic application especially if m35K expression was targeted to specific cell types. For example, if m35K was expressed on circulating monocytes/macrophages, it may inhibit their ability to be recruited to the endothelium. The observed bystander effect would also lead to reduced chemotaxis of surrounding cells. m35K-directed expression on the endothelium may have similar effects.

This study also demonstrated that m35K expressed on a cell surface can sequester the CC chemokines RANTES and MIP-1α from supernatant medium. The exact mechanism of CC chemokine binding to the 35K protein has not yet been elucidated, although based on structural evidence from in vitro studies, it is thought to compete for binding with their cognate G protein-coupled receptors by interacting with specific residues conserved among many of the CC chemokines (29, 33). The ability of m35K, anchored to a cell membrane, to sequester CC chemokine activity could potentially be a valuable tool to prolong vein graft patency and transplant rejection time, as inflammatory cell infiltrate, triggered by surgery and mediated by CC chemokines, is the primary cause of graft and transplant failure (17, 34).

m35K and native soluble 35K gene delivery had different effects on CC chemokine levels and bioactivity. The addition of liver homogenates from Adm35K significantly reduced CCR5-directed cell migration toward chemotactant mouse plasma (Fig. 6A). In contrast to this, Ad35K liver homogenates had no effect. This suggests that m35K is present in liver homogenates and is able to sequester the CC chemokines, hence reducing chemotaxis. Plasma from Adm35K mice also had reduced CC chemokine activity, as it induced significantly less CCR5-directed cell migration than AdGFP control mouse plasma (Fig. 6B). This reduction in cell migration toward m35K mouse plasma was not as striking as that seen toward Ad35K plasma and may be due to the localization of m35K to just the liver membranes compared with the systemic expression of soluble 35K. As demonstrated previously (15), mouse RANTES concentration was significantly elevated in Ad35K mouse plasma despite its lower chemotactic activity (Fig. 6C). Previous studies have also found that the RANTES ELISA measures both free and bound RANTES (15). Taken together, these results indicate that Ad-mediated 35K is binding CC chemokines and sequestering them into the circulation. In contrast to this, m35K significantly reduced plasma RANTES concentration (Fig. 6C). It therefore appears that m35K, expressed on the liver membranes, is binding circulating CC chemokines from the plasma, where they remain bound, effectively lowering plasma CC chemokine concentrations. These results demonstrate the different effects of m35K on CC chemokine levels and bioactivity due to its localization on liver membranes compared with systemically expressed soluble 35K. In summary, m35K is able to provide a localized inhibition of CC chemokine activity while anchored to liver membranes. It is also able to reduce systemic CC chemokine activity, but is not as effective as soluble 35K protein.

Further proof that the m35K can produce localized CC chemokine inhibition in vivo was found in this study using two different animal models of inflammation. Ad-mediated delivery of Adm35K i.p. resulted in reduced inflammatory cells in peritoneal exudates, using the Biogel-induced peritonitis model. This is consistent with previous results in our laboratory that have shown soluble 35K protein was able to reduce cells counts in peritoneal exudates using this model (15). In our second inflammatory model, we investigated the effect on m35K on Con A-induced hepatitis. Intravenous delivery of Adm35K was able to significantly reduce liver damage and inflammation, as ALT, AST, and TNF-α levels were significantly lower compared with control AdGFP mice. Studies have found that CC chemokines are involved in Con A-induced hepatitis; for example, MIP-1α has been found to be proinflammatory (23, 26), whereas other studies have found MCP-1 and CCR5 are hepatoprotective (27, 28). m35K provides a broad-spectrum inhibition of the CC chemokine class (14), so multiple CC chemokine pathways would be inhibited. In this model, liver-localized broad-spectrum CC chemokine inhibition appears to result in an overall protection to Con A-induced hepatitis. In addition, Con A is a good model to demonstrate localized inhibition of the CC chemokine class by m35K, as the inflammatory pathology is predominantly present in the liver (35). The expression of m35K is also predominantly in the liver, when using i.v. Ad delivery (18). m35K is therefore present at the site of interest, potentially maximizing its effect on the CC chemokine-mediated pathology.

The chemokine receptor D6 is a promiscuous transmembrane domain receptor that recognizes most CC chemokines (36–38) and
therefore has similar properties to m35K. Interestingly, D6 is unable to signal after ligand engagement, and instead internalizes and then degrades the ligand (36). It is thought to play a role in resolving inflammation after the initial inflammatory response by scavenging CC chemokines (38). Recent studies found that D6 KO mice had increased inflammatory chemokines (36) and excess residual chemokines (38) after an inflammatory insult. The ability of D6 to reduce and resolve CC chemokines clearly highlights a role for m35K in inflammatory disease pathologies.

In conclusion, membrane-bound CC chemokine inhibitor m35K may provide a novel approach to achieve broad-spectrum blockade of CC chemokine activity. We have demonstrated that m35K can directly inhibit cell migration and reduce CC chemokine activity both in vitro and in vivo. The use of Ad-mediated delivery of m35K via Adm35K may provide a promising strategy to reduce the severity of local inflammatory responses and to investigate mechanisms underlying chronic inflammatory diseases.

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Disclosures

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