AGEs mediated expression and secretion of TNF alpha in rat retinal microglia

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Abstract

Diabetic retinopathy induces an inflammatory response in the retina characterized by an increase in inflammatory cytokines and the activation of microglia. The degree of microglia activation may influence the extent of retina injury following retinal metabolic stress. We have previously shown that DR rats have elevated levels of advanced glycation end products (AGEs) in their blood. We have also suggested that AGEs might be involved in microglial activation and production of tumor necrosis factor α (TNFα). In this study, we attempted to confirm that AGEs induce the release of TNFα from rat retinal microglia using an in vitro microglia culture system, and concurrently to explore the mediating mechanisms. AGEs increased the protein secretion and mRNA expression of TNFα in cultured rat retinal microglia. These effects of AGEs were primarily mediated by reactive oxygen species (ROS). Furthermore, the inhibitors for mitogen-activated protein kinases (MAPK; p38, JNK and ERK 1/2) and nuclear factor-kB (NF-kB) could significantly decrease AGEs-induced TNFα release. AGEs-activated microglia showed an increase of NF-kB p65 nuclear translocation. These observations indicated that pathophysiological levels of AGEs may alter rat retinal microglia function by up-regulating TNFα expression and release via enhanced formation of intracellular ROS. AGEs-induced ROS subsequently activates MAPK (p38, JNK and ERK1/2) and NF-kB.

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1. Introduction

Diabetic retinopathy (DR) is one of the leading causes of blindness in working-age adults. The mechanisms of vascular and neuronal pathology in this disease are not yet fully understood. Among several pathogenic mechanisms that may contribute to DR are the formation and accumulation of advanced glycation end products (AGEs) (Lu et al., 1998; Stitt et al., 2005). AGEs are known to accumulate in the diabetic retina where they may impair retinal function in vitro and in vivo (Stitt, 2003). The action of AGEs may form on amino groups of proteins, lipids, and DNA though a number of complex pathways, including nonenzymatic glycation by glucose and reaction with metabolic intermediates and reactive dicarbonyl intermediates (Singh et al., 2001). These reactions not only modify the structure and function of proteins, but also cause the formation of intramolecular and intermolecular cross-links (Wautier and Guillausseau, 2001).

The retina is composed of many classes of cells and each may be affected by diabetes. The number and activity of quiescent microglia in retina were changed in DR (Krayd et al., 2005; Rungger-Brandle et al., 2000; Zeng et al., 2000). Perivascular microglia were closely apposed to retinal vessels. This vessel association would favor the initial microglia activation by cytokines released by the blood vessel cells and neurons.
In long-standing diabetes, however, microglia are often found in the inner retina and in association with blood vessels (Zeng et al., 2000). An increasingly damaged blood–retinal barrier might trigger this behavior. It remains unclear why diabetes would incite microglia activation in the retina; but research on retinal microglia activation may provide substantial insights to the DR pathogenesis (Gardner et al., 2002).

Activated microglia release TNF α as shown in in-vivo and in-vitro studies (Krady et al., 2005; Nakamura et al., 1999). TNF α is an important pro-inflammatory factor in retinal and central nervous system (CNS) neurodegenerative diseases, such as DR, Parkinson’s disease (Sriram et al., 2002; Zeng et al., 2000). TNF α has the potential to induce apoptosis, fibroblast proliferation, NF-κB activation and cell adhesion molecule activation (Hirsch et al., 2003). There is convincing evidence to suggest that neuropathy is a feature of DR as ganglion cell death was observed in the diabetic retina (Barber, 2003).

Wong et al. (2001) have shown that AGES induce microglial activation. In this study, we explore the mediating mechanisms of microglial activation induced by AGES. Our results indicate that AGES not only promote TNF α mRNA expression but also induce the release of TNF α from rat retinal microglia. Therefore, AGES may exaggerate the pathological process of DR by inducing TNF α production from microglia, thereby triggering the infiltration of leukocytes to the site of vascular injury and causing vascular inflammation.

2. Materials and methods

2.1. Cell culture

Microglia were isolated from retinas of newborn Sprague–Dawley (SD) rats by methods described previously (Roque and Caldwell, 1993; Wang et al., 2005). The ARVO Statement for the Use of Animals in Ophthalmic and Vision Research was followed in all aspects. Briefly, retinas were dissected from newborn (within 72 h) SD rats with blood vessels removed. Tissues were collected, and digested with 0.125% trypsin for 20 min before mechanical dissociation. The trypsin was inactivated with DMEM/F12 (1:1) (Invitrogen, CA) plus 10% fetal bovine serum (FBS) (Hyclone, UT). The cells were collected 20 min before mechanical dissociation. The trypsin was inactivated with DMEM/F12 (1:1) (Invitrogen, CA) plus 10% fetal bovine serum (FBS) (Hyclone, UT). The cells were collected by centrifugation, re-suspended in culture medium and plated to T75 cell culture dishes at a density of 1 × 10⁶ cells/mm². After 2 weeks, microglia were harvested in culture media with serum from the flasks by shaking the flasks at 100 rpm for 1 h, the cell suspension was centrifuged and the detached cells were re-plated in DMEM/F12 (1:1) plus 10% FBS at designated densities for various experiments described below.

2.2. Immunocytochemistry and flow cytometry (FCM)

The morphology of microglia in culture was carefully examined by phase contrast and fluorescent microscopy. The purity of the microglial cultures was evaluated by immunocytochemical staining and flow cytometric (FCM) analysis for CD11b (Lambertsen et al., 2007; Milligan et al., 1991) and CD45 (Herber et al., 2006), the microglial markers. The proliferation of microglia after AGES treatment was measured by immunohistochemistry Ki67 and PCNA.

2.3. Microglial activation: AGES effect on CD11b and CD45 expression

Purified retinal microglia were seeded into 6-well tissue culture plates and incubated overnight. One day after microglia seeding, serum-free medium containing 50 μg/ml AGES (Catalog A8301; Sigma, MO) was added to each culture well for 24 h, and labeled for CD11b and visualized with 3,3′-diaminobenzidine (DAB). CD11b and CD45 expression was analyzed by FCM.

2.4. Measurement of TNF α protein release

Purified retinal microglia were treated with AGES of different concentrations for periods ranging from 24 h to 72 h. Microglia were treated with medium containing 0 (control), 10, 50, 100, 500, 1000 μg/ml AGES for 24 h and cell viability was determined by [3H]thymidine conversion. AGES at 10–1000 μg/ml did not induce obvious cell death in cultured microglia during the 24 h incubation (data not shown). The supernatant of microglia culture was harvested and transferred to polypropylene tubes and stored at −80°C for not more than 1 week before the measurement of TNF α. TNF α in cultured microglia supernatants were determined by ELISA kit (R&D Systems, MN).

For reversibility of AGES on microglial activation, purified retinal microglia were treated with AGES (100 and 500 μg/ml) for 24 h and the supernatant of microglia culture was harvested. Then AGE-containing medium was removed and replaced with AGE-free medium for 24 h and the supernatant of microglia culture was harvested. For specificity of AGES on microglial activation, we also measured the effects of insulin (10 nM) on TNF α production from purified microglia.

2.5. Semi-quantitative RT-PCR

RT-PCR was performed as previously described (Bhat et al., 1998). Briefly, RT was performed using ThermoScript™ RT Reagents (Invitrogen, CA). The sequence of primers for rat TNF α used in this study was (CAC GCT CTT CTG TCT ACT CAC GCT CTT CTG TCT ACT; GGA CTC CGT GAT GTC TAA GT). The sequence of primers for rat β-actin was (GCC ACT GCC GCA TCC TCT T; ATC GTA CTC CGT CTT GCT GA). The fluorescent images were digitally captured for analysis of intensity with Quantity One 1-D Analysis Software (Bio-Rad, CA). Levels of TNF α mRNA were normalized relative to that of β-actin in the same sample.

2.6. Measurement of total antioxidative capacity (T-AOC) release

The anti-oxidative capacity in the defensive system of an organism is closely related to general health (Chularojmontri et al., 2005; Lin et al., 2005). Conditioned medium was
collected from purified microglia cultures treated with 0, 10, 100, 500 μg/ml AGEs for 30 min. T-AOC release from cultured microglia was determined by T-AOC detection kits (Nanjing Jiancheng Chemical Co, Nanjing, China).

2.7. Measurement of intracellular reactive oxygen species (ROS) generation

AGEs (Sigma, MO) were added to cultures together with a ROS-prober dye 2,7-dichlorofluorescin diacetate (DCFH-DA, 5 μM/l) (Molina-Jimenez et al., 2004). DCF fluorescence was monitored with a laser confocal scanning microscope (Leica, Germany).

2.8. Inhibitors of mitogen-activated protein kinases (MAPK) and nuclear factor-kB (NF-kB) treatment

Each culture well was fed with medium containing 500 μg/ml AGEs (Sigma, MO) or with other pharmacological reagents for 24 h. Microglia were pre-treated with the inhibitors for extracellular signal-regulated kinase (ERK) 1/2 MAPK (U0126, 2.6 and 26 μM), p38 MAPK (SB203580, 5.3 and 53 μM), c-Jun NH2-terminal kinase MAPK (curcumin, 5 and 50 μM), and NF-kB (PDTC, 1 and 10 nM) for 60 min, respectively. Media of treated microglia cultures were harvested. TNFα proteins were determined by ELISA kit (R&D Systems, Minneapolis, MN).

2.9. Localization of NF-kB immunofluorescence

For immunofluorescence localization of NF-kB by laser confocal scanning microscopy, fixed cells were treated with a rabbit polyclonal antibody against the p65 subunit of NF-kB, followed by FITC-conjugated goat anti-rabbit IgG antibody. The nuclei were stained with PI. NF-kB is known to regulate various proinflammatory cytokines and mediators such as TNFα, IL-1β, and IL-6, and cellular adhesion molecules (Carter et al., 1998). We localized NF-kB with the immunostaining method described previously (Hsuan et al., 1999). To acquire dual-color images, the double-stained microglia were observed with a confocal laser scanning microscope (Model, TCS NT; Leica, Germany). The samples labeled with both FITC and PI were excited at 488 nm, and the fluorescence emissions were captured at 510–550 nm (530 nm in the centre) and 590–620 nm (605 nm in the centre) band pass with spectral grating, respectively. Images were recorded via digital photography with the accompanying software.

2.10. Statistics

All experiments were performed at least three times. Graphical representation of data was from combined results, rather than representative data from one single experiment. The graphical data were presented as the arithmetic mean percentages of control ± standard errors of the mean (SEM). Experimental data were analyzed with one-way analysis of variance (ANOVA) for significant differences. p < 0.05 were considered significant.

3. Results

3.1. Effect of AGEs on microglial morphology

In control cultures (Fig. 1A), most microglia had characteristic thin thread-like processes (arrow). In microglia (Fig. 1B) treated with 500 μg/ml AGEs for 24 h, the cell body of microglia appeared to be more oval shaped, phase bright (arrow). The microglia under AGEs treatment acquired a time-dependent change in morphology. These changes were obvious in microglia with 72 h of treatment (Fig. 1C). Many cell bodies of AGEs-treated microglia appeared larger and bore long blunt ruffles with thin thread-like projections (arrow). However, the proliferation markers of PCNA and Ki67 were negative in purified microglia after AGEs treatment.

3.2. Purification of rat retina microglia

The culture of retinal microglia appeared to be homogeneous with many dark cells under the phase contrast microscope (Fig. 8A). Immunocytochemical staining showed that

Fig. 1. Effects of 500 μg/ml AGEs on retinal microglial morphology. (A) Microphotograph of retinal microglia without AGEs treatment. (B) Microglia from (A) treated with 500 μg/ml AGEs for 24 h. (C) Microglia from (A) treated with 500 μg/ml AGEs for 72 h. Figures are representative of three separate experiments. Bar =50 μm.
the majority of cells in the cultures were positively labeled for CD11b (Fig. 8B). The labeling was localized in the cytoplasm (Fig. 8C). We further double-immunostained these microglia with two of their markers, CD45 and CD11b (Fig. 8D, E, F). Both markers were labeled in the cell membranes. Flow cytometry analysis of these microglia demonstrated that 95% of them stained positively for CD11b (Fig. 8G), and 84% stained positively for CD45 (Fig. 5H). This indicated that the culture had high purity of microglia. GFAP staining is negative in a purified microglial culture system.

3.3. Activation of microglia: effect of AGES on CD11b and CD45 expression

In control cultures (Fig. 8B), most microglia were stained light brown and many microglia did not exhibit many processes. After 500 μg/ml AGES treatment (Fig. 8C), most microglia showed activation as indicated by ramified morphology with darker staining in the figure.

Cell surface expression of CD11b and CD45 was determined by two-color FCM analysis. Area R1 in Fig. 8I shows that in a control culture 47% of the cells counted were positively co-labeled with CD11b and CD45. Fig. 8J shows the two-color FCM analysis of microglia in cultures treated with AGES for 24 h. Area R1 in Fig. 8J shows that 70% of the cells counted were positively co-labeled with CD11b and CD45. These data indicate that AGES activate microglia.

3.4. Effect of AGES on TNF α release

Culture media were collected at 24, 48 and 72 h after AGES treatment. The levels of TNF α in control culture media without AGES treatment did not change throughout the experimental period. Treatment with 0, 10, 100, and 500 μg/ml AGES induced a dose-dependent release of TNF α at 24, 48 and 72 h (Fig. 2). AGES at 10 μg/ml could induce a significant elevation in TNF α levels in culture media. As shown in Fig. 2, the levels of TNF α in microglia cultures treated with various concentrations of AGES (10—500 μg/ml) for the 72 h period were always significantly high compared with levels in the control. Cultures treated with 500 μg/ml AGES acquired the highest levels of TNF α in the medium. This level remained high throughout the 72 h experimental period. It seemed that there was apparently cell loss after 72 h (Fig. 1C) of 500 μg/ml AGES treatment. Cells were counted at each time point. There was an 18% cell loss after 24 h of 500 μg/ml AGES and a 52% cell loss after 72 h of 500 μg/ml AGES. Apparently, quantification of cell counts is more appropriate. Fig. 2 shows the TNF α protein level with normalization.

3.5. Effect of AGES on TNF α mRNA expression

We treated microglia with 500 μg/ml AGES for 8, 12 and 24 h and measured the TNF α mRNA in culture. After normalization against β-actin mRNA, RT-PCR, assays revealed that the mRNA level of TNF α was significantly increased after AGES treatment (Fig. 3).

3.6. Measurement of total antioxidative capacity (T-AOC)

When treated with 10 μg/ml AGES, the microglia T-AOC level was not significantly different from that of the controls (Fig. 4). When treated with 100 and 500 μg/ml AGES, the T-AOC level was significantly decreased compared to the level in the untreated controls. The detrimental effects of AGES on T-AOC levels were dose-dependent. The lowest level of T-AOC was at 500 μg/ml AGES treatment (Fig. 4).

3.7. Effect of AGES on intracellular ROS levels

To examine whether AGES might induce ROS production in cultured retinal microglia, we measured the intracellular ROS level using the redox-sensitive fluorescent dye DCFH-DA.

![Fig. 2. The release of TNF α from retinal microglia in culture with (10, 100, 500 μg/ml) or without AGES treatment. Culture supernatants were collected at various time points and assayed for TNF α with an ELISA kit. Results were the means ± SEM of three individual experiments. *p < 0.05 compared with the controls.](image-url)

![Fig. 3. Comparison of the transcriptional levels of TNF α in microglia cultures with or without 500 μg/ml AGES treatment. β-actin was used as internal reference. (A) Representative gel of RT-PCR for TNF α and β-actin in retinas for indicated time periods of three independent experiments. (B) Densitometric analysis of TNF α mRNA. Intensity of corresponding mRNA expression for β-actin was considered 100%. Data were expressed as mean ± SEM. *p < 0.05 compared with the controls.](image-url)
Under 500 μg/ml AGEs, the fluorescent intensity increased sharply in the first 30 s of treatment and continued to increase up to 90 s (Fig. 5). The increment began to level off from 90 s and then the level gradually decreased over a 50 min experimental period. We have tested other compounds, such as glucose and albumin, as controls on intracellular ROS formation in microglia. We did not detect any effect of these other compounds on ROS formation (data not shown).

3.8. Signaling mechanisms involved in AGEs-induced release of TNF α

To identify the mechanism of AGEs-induced TNF α release, microglia were pre-treated with the inhibitors for extracellular...
signal-regulated kinase (ERK) 1/2 MAPK (U0126, 2.6 and 26 μM), p38 MAPK (SB203580, 5.3 and 53 μM), c-Jun NH2-terminal kinase MAPK (curcumin, 5 and 50 μM), and NF-kB (PDTC, 1 and 10 nM). Then microglia were subjected to 500 μg/ml AGEs for 24 h after the incubation of the above agents for 60 min. MAPK (ERK1/2, p38, JNK) and NF-kB inhibitors significantly inhibited AGEs-induced TNF α release in retinal microglia (Fig. 6). The highest inhibition on TNF α release was acquired with 26 μM U0126. p38 MAPK (SB203580, 5.3 and 53 μM), c-Jun NH2-terminal kinase MAPK (curcumin, 5 and 50 μM), and NF-kB (PDTC, 1 and 10 nM) partially inhibited TNF α release by AGEs treatment.

3.9. Effect of AGEs on NF-kB p65 nuclear translocation

NF-kB translocation from the cytosol to the nucleus was used as an index of microglia activation. A change in color of the nucleus from red to yellow (due to co-localization of green FITC fluorescence and red PI fluorescence) was indicative of NF-kB translocation (Table 1). In the unstimulated group, NF-kB p65 protein was mainly located in the cytoplasm (Fig. 7A1, A3); there was little staining for p65 in the nuclei, indicating lack of NF-kB translocation to the nucleus (Fig. 7A3). In microglia that were activated by AGEs an increase in NF-kB translocation was observed (Fig. 7B, C). There are more yellowish nuclei visible in Fig. 7B3 than in Fig. 7C3. We counted more than 120 microglia and found that there was more NF-kB p65 being translocated to the nucleus from the cytoplasm in 500 μg/ml AGEs treatment than in 100 μg/ml AGEs (Table 1).

4. Discussion

Krady et al. (2005) have shown that retinal microglia become activated with changes in morphology and function. The signal pathways by which microglia become activated are not defined and need to be explored. Our previous work indicated that AGEs were elevated in the serum of diabetic rats and might be involved in the activation of retinal microglia. The results of our present study demonstrated that AGEs activation of microglia not only increased the level of TNF α in the microglia, but also induced a release of TNF α from microglia (Figs. 2 and 3). This action involved ROS, MAPK, and NF-kB signaling pathways.

The use of primary culture of retina microglia offers the advantage of studying the response of microglia to varied treatments in vitro. In our laboratory, primary culture of retinal microglia achieved over 95% purity, as confirmed by immuno-cytchemistry and FCM determination with CD11b staining. Possible contaminating cells in the cultures, such as astrocytes, were not significantly present and did not contribute to the results we obtained.

AGEs are known to increase in the diabetic retina where they may have important effects on the disease progress (Wautier and Guillausseau, 2001). Blood–retinal barrier breakdown begins to occur in diabetes at mildly elevated blood glucose concentrations and, therefore, an increase in vascular permeability can be expected to occur rather early in diabetes (Xu et al., 2001). Leakage from the vascular bed leads to increased glucose and AGEs levels within the neuronal parenchyma. The AGEs receptor is expressed on a wide range of cells including microglia (Dukic-Stefanovic et al., 2003). Leakage of AGEs--serum factors may directly promote retinal microglial activation. Microglial activation can produce inflammatory agents and further exacerbate DR. Identification of the molecular basis underlying AGEs-induced microglia activation in DR might help explain the accelerated vascular inflammation and breakdown of the blood–retinal barrier observed in diabetes.

DR induces an inflammatory response in the retina characterized by an increase in inflammatory cytokines and the activation of microglia. The degree of microglial activation may influence the extent of retinal injury following an inflammatory stimulus. Cytokines released by activated microglia regulate the influx of inflammatory cells to the damaged area. Thus, a therapeutic strategy to reduce cytokine expression in microglia would be neuroprotective (Krady et al., 2005; Wang et al., 2005). Our hypothesis, regarding the involvement of microglia activation in DR, is that intracellular hyperglycemia

<table>
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<tr>
<th>Group</th>
<th>AGEs concentration (μg/ml)</th>
<th>NF-kB p65 (FN/FC)</th>
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<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>1.042 ± 0.34</td>
</tr>
<tr>
<td>AGEs 100</td>
<td>1.477 ± 0.51*</td>
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</tr>
<tr>
<td>500</td>
<td>2.024 ± 0.36*</td>
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Retina microglia were treated with AGEs of different concentrations for 24 h. NF-kB translocation from the cytosol to the nucleus was used as an index of activation. FN stands for green fluorescence in the nucleus, FC stands for green fluorescence in the cytosol. Results were means ± SEM of three individual experiments. *p < 0.05 compared with the AGEs-treated cultures. (n > 120 cells each for (A) and (B), (C)).

![Fig. 6. Effects of MAPK or NF-kB inhibitors on AGEs-induced secretion of TNF α in cultured retinal microglia. Cells were pretreated with the inhibitors of extracellular signal-regulated kinase (ERK) 1/2 MAPK (U0126, 2.6–26 μM), p38 MAPK (SB203580, 5.3–53 μM), c-Jun NH2-terminal kinase MAPK (curcumin, 5–50 μM), NF-kB (PDTC, 1–10 nM) for 60 min, respectively. Then cells were stimulated by 500 μg/ml AGEs for 24 h. Data are expressed as mean ± SEM. *p < 0.05 compared with AGEs treated alone. Data are representative of at least three independent experiments.](image-url)
causes abnormalities in blood flow and increases vascular permeability in the early course of diabetes. Leakage of serum factors might promote microglial activation (Nakamura, 2002). Microglia start to proliferate, assume an activated morphologic form, and produce various cytokines, such as TNF-α leading to neuronal injury (Jeng et al., 2005).

In this work we tested the function of ROS as a mediator in AGEs-induced microglia activation. We found that antioxidative capacity was extremely decreased in cultured microglia after 30 min with AGEs treatment. Consistently, AGEs abruptly increased intracellular ROS within 30 s. This observation agrees with previous reports that the ROS is increased in AGEs-induced secretion of cytokines from endothelial cells (Yamagishi and Takeuchi, 2004). Brownlee (Krady et al., 2005) reported that the overproduction of superoxide by the mitochondrial electron-transport chain might reflect a hyperglycemia-induced process to leading various diabetic complications.

Previous studies focused on the influence of AGEs on the vasculature (Chakravarthy et al., 1998). Our results suggest that AGEs might act directly on microglia to initiate and promote the vascular changes of DR. AGEs might up regulate TNF-α expressions via microglia and macrophages in the retina, resulting in an influx of inflammatory cells into the damaged retina.

In conclusion, this study demonstrated that AGEs significantly enhance TNF-α production and release from retinal microglia. The intracellular oxidative products and subsequent activation of multiple signaling molecules, including MAPKs...
Fig. 8. The culture of retinal microglia appeared to be homogeneous under the phase contrast microscope (A). Immunocytochemical staining showed that majority of the cells in cultures was positively labeled for CD11b (B). We further double-immunostained these microglia with two of their markers, CD45 and CD11b (D–F). Both markers were labeled in the cell membranes. Flow cytometry analysis of these microglia demonstrated that 95% of them stained positively for CD11b-PE (G), and 84% stained positively for CD45-FITC (H). This indicated that the culture had high purity of microglia. In control cultures (A), most microglia were stained light brown and many microglia did not exhibit many processes. After treatment with 500 mg/ml AGEs (B), most of these microglia showed activation as indicated by ramified morphology with darker staining in the figure (C). Cell surface expression of CD11b and CD45 were determined by two-color FCM analysis. Area R1 in Fig. 5I shows that, in a control culture, 47% of the cells counted were positively co-labeled with CD11b and CD45; (J) shows the two-color FCM analysis of microglia in cultures treated with AGEs for 24 h. Area R1 in (J) shows that 70% of the cells counted were positively co-labeled with CD11b and CD45. These data indicate that AGEs might activate microglia.
and NF-kB, mediated the effects of AGEs in retinal microglia. AGEs may act directly on microglia to initiate and promote the advancement of DR.

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