Glycated serum albumin induces chemokine gene expression in human retinal pigment epithelial cells

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Abstract: Chronic hyperglycemia is thought to be important in the development of diabetic neovascularization but the mechanisms involved remain poorly understood. Interleukin-8 (IL-8) is a leukocyte chemokine and activating agent with angiogenic properties that is present in diabetic vitreous and may play a role in diabetic vasculopathy. We studied IL-8 and monocyte chemotactic protein-1 (MCP-1) production by human retinal pigment epithelial (hRPE) cells exposed to glycated human serum albumin (GHSA). Enzyme-linked immunoassay GHSA (500 μg/mL)-treated hRPE cells secreted levels of IL-8 and MCP-1 detectable within 4 h and reached 26.0 ± 1.3 and 42.2 ± 0.4 ng/10^6 cells/mL after 24 h, respectively. Induction of IL-8 and MCP-1 by GHSA at concentrations ranging from 62.5 to 3,000 μg/mL exhibited dose-dependent kinetics. The GHSA-induced chemokine secretion by hRPE was almost completely inhibited by actinomycin D and cycloheximide, suggesting that de novo mRNA and protein synthesis are necessary for the GHSA-induced IL-8 and MCP-1 production. Northern blot analysis of GHSA-induced hRPE IL-8 and MCP-1 mRNA expression corresponded to the time- and dose-dependent increases measured by enzyme-linked immunosorbent assay. High concentrations of glucose (20 mM; 360 mg/dl) increased GHSA-induced hRPE IL-8 and MCP-1 secretion, whereas added insulin (0.5 ng/mL) inhibited IL-8 but not MCP-1 protein secretion and mRNA expression. GHSA also induced hRPE to secrete GRO-α, RANTES, and NAP-2 chemokines. GHSA induction of hRPE chemokines further suggests a role for the hRPE in leukocyte infiltration, vascular injury, and neovascularization. *J. Leukoc. Biol.* 60: 405–414; 1996.

Key Words: *interleukin-8 · monocyte chemotactic protein-1*

INTRODUCTION

Hyperglycemia is generally believed to be a major contributing factor leading to the development of diabetic retinopathy [1]. As blood glucose concentrations rise, excessive nonenzymatic glycation of a wide range of proteins including serum albumin occurs [2]. Glycation adducts may result in a series of pathophysiological consequences [for review see refs. 3–5]. Once advanced glycation end-products (AGEs) are formed, this process and its associated tissue damage become irreversible.

Protein glycation has been reported in crystalline lenses in the eyes of diabetic patients [6]. The link between hyperglycemia-associated excessive glycation and development of proliferative diabetic retinopathy (PDR) is suggested by several observations. For example, there is a close correlation between glycated hemoglobin levels and the severity of retinopathy [1]. A similar link between collagen browning and microangiopathy in skin biopsies from type 1 diabetes mellitus has also been reported [7]. Furthermore, lowering glycated hemoglobin by improving blood glucose control results in a beneficial impact on diabetic retinopathy [8]. Although these data support the role of glycated proteins in the pathogenesis of diabetic retinopathy, the mechanisms by which these proteins elicit and propagate ocular complications of diabetes remain unknown. Previous studies have predominately focused on AGEs and their pathophysiological impact, but little is known about the role of early adducts of glycated human serum albumin (GHSA).

Because retinal pigment epithelial (RPE) cells are strategically positioned at the blood-retinal barrier (BRB), RPE cells may participate in diabetic and other types of retinopathy. Indeed, RPE are known to produce a variety of cytokines, including IL-8 [9] and monocyte chemotactic protein-1 (MCP-1) [10] chemokines for leukocytes that have been implicated in the pathogenesis of these dis-

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Abbreviations: IL-8, interleukin-8; MCP-1, monocyte chemotactic protein-1; hRPE, human retinal pigment epithelial cell; GHSA, glycated human serum albumin; AGEs, advanced glycation end products; PDR, proliferative diabetic retinopathy; RPE, retinal pigment epithelial cells; BRB, blood-retinal barrier; hTNF-α, human tumor necrosis factor-α; HSA, human serum albumin; LPS, lipopolysaccharide; ELISA, enzyme-linked immunosorbent assay; EDTA, ethylenediaminetetraacetate; SDS, sodium dodecyl sulfate.

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eases. Nonetheless, mechanisms by which leukocytes are attracted into ocular tissues affected by diabetes are poorly understood. The association of PDR with excessive protein glycation [11] may reflect a mechanistic linkage between glycated proteins and induction of RPE-derived leukocyte chemokines IL-8 and MCP-1. IL-8 is a chemotaxin and activator of neutrophils and eosinophils [12, 13]. Increased levels of IL-8 have been measured in the vitreous of patients with PDR and may in part be responsible for increased vascular permeability and angiogenesis [14]. MCP-1, a member of the CC chemokine family, is a chemoattractant for monocytes and lymphocytes [15, 16] causing monocyte/macrophage infiltration in tissues. We have also detected increased MCP-1 levels in the vitreous from patients with PDR [14]. In this study, we examined GHSA-induced chemokine secretion by human RPE (hRPE) with emphasis on the gene expression and secretion of IL-8 and MCP-1.

MATERIALS AND METHODS

Materials

Human interleukin-1β (hIL-1β), human tumor necrosis factor-α (hTNF-α), and monoclonal antibody against hIL-1β (anti-hIL-1β) were purchased from R & D Systems, Minneapolis, MN. Monoclonal antibody against hTNF-α (anti-hTNF-α) was purchased from Genzyme, Cambridge, MA. Human serum albumin (HSA), globulin-free HSA, globulin- and fatty acid-free HSA, polymyxin B, lipopolysaccharide (LPS), porcine insulin, and other chemicals were purchased from Sigma Chemical Co., St. Louis, MO. Specific human IL-8, TNF-α, MCP-1, GRO-α, NAP-2, IL-1β, IP-10, ENA-78, MIP-1α, MIP-1β were produced in rabbits as previously described by immunization of rabbits with corresponding cytokines with complete Freund's adjuvant [17]. No cross-reaction with any other cytokines known so far was observed for any of them. GHSA, which contains 1–5 moles of fructosamine per mole albumin, was purchased from Sigma and made as described by Baynes et al. [18]. The GHSA preparation did not contain measurable AGEs as determined in our laboratory by fluorescence assays (from 360 to 600 nm) upon excitation at 370 nm [19] or 350 nm [20].

Cell isolation and culture

The hRPE cells were isolated from donor eyes as previously described by Elner [10] and cultured in Dulbecco's modified essential medium (DMEM) containing 15% fetal bovine serum. The cultured hRPE cells formed monolayers showing typical polygonal morphology and pigmentation of scattered cells. Unless otherwise specified, the culture media were collected after 24-h stimulation of near confluent hRPE monolayers with GHSA. The media were immediately frozen at -70°C until enzyme-linked immunosorbent assays (ELISA) were performed. All the samples were assayed in triplicate. All experiments were carried out at least three times using cell lines isolated from at least three different donors.

ELISA for hRPE-secreted cytokines

The cytokine levels in the hRPE supernatants were determined by a modification of a double-ligand ELISA method as previously described [21]. Briefly, the 96-well microtiter plates were coated with rabbit anti-cytokine antibody for 16 h at 4°C. Nonspecific binding was blocked with 2% bovine serum albumin. Diluted hRPE supernatants (50 μL) were added and incubated for 1 h. The plates were then subjected to sequential incubations with biotinylated rabbit anti-cytokine (1:1000) for 45 min and streptavidin-peroxidase conjugate for 30 min. Chromogen substrate (OPD) was added and the plates were incubated to the desired extinction when the reaction was terminated with 3 M H_2SO_4. Absorbance for each well at 490 nm was read in an ELISA reader and calibrated using half-log dilution standards for corresponding cytokine concentrations ranging from 1 pg to 100 ng/well. This ELISA method consistently detected cytokine concentrations greater than 10–50 pg/mL in a linear fashion.

IL-1β and TNF-α neutralization

Neutralization concentrations of hIL-1β and hTNF-α were determined by use of the manufacturer's ND_{50}. Five times the ND_{50} was used in order to inhibit ~100% of the biological activity of each cytokine. The final concentrations for anti-hIL-1β and anti-hTNF-α were 1 and 3 μg/mL, respectively. Antibodies were added 1 h before challenging the hRPE cells with various stimuli. Our studies demonstrated that these antibodies blocked chemokine secretion by hRPE cells exposed to 2 ng/mL of hIL-1β or 20 ng/mL of hTNF-α when the above concentrations of anti-hIL-1β or anti-hTNF-α were used.

Protein synthesis inhibition of hRPE cells

Nearly confluent monolayers of hRPE cells were pre-incubated with 1 μg/mL actinomycin D or 10 μg/mL cycloheximide for 1 h. GHSA was added to the culture and co-incubated with actinomycin D or cycloheximide for 24 h. Subsequently, IL-8 and MCP-1 were determined by ELISA.

RNA extraction and Northern blot analysis

Total cellular RNA was isolated from hRPE cells and Northern blot analysis was carried out as described previously [9]. Briefly, the hRPE cells were washed with phosphate-buffered saline and solubilized in a solution consisting of 25 mM Tris (pH 8), 4.2 M guanidine isothiocyanate, 9.5% Sarkosyl, and 0.1 M 2-mercaptoethanol. After homogenization, the resulting suspension was added to an equal volume of 100 mM Tris (pH 8), containing 10 mM ethylenediaminetetraacetate (EDTA) and 1% sodium dodecyl sulfate (SDS). Total RNA was extracted with chloroform-phenol and chloroform-isoamyl alcohol, precipitated in ethanol, and dissolved in 10 mM Tris (pH 8) buffer containing 0.1 mM EDTA and 0.1% Sarkosyl. RNA was separated by electrophoresis on a 1% formaldehyde agarose gel, blotted onto nitrocellulose membranes, and immobilized. The membranes were prehybridized with buffer consisting of 1× Denhardt's, 0.5% SDS, 100 μg/mL tRNA, 0.05% sodium pyrophosphate, 50 μg/mL polyadenylic acid in 6× saline sodium citrate solution at 56°C, then hybridized at 68°C with ^{32}P-labeled probes complementary to the nucleotides 262 to 291 and 256 to 285 for IL-8 [22] and MCP-1 [23], respectively, as previously described [9, 10]. Equivalent amounts of total RNA (11 μg) loaded per gel lane were verified by monitoring 18S and 28S RNA.

Data analysis

The mean cytokine concentration ± SE was determined for each assay condition. Various assay conditions were compared using Student's *t*-test and probability values less than 0.05 were considered to be statistically significant.

RESULTS

GHSA-induced IL-8 and MCP-1 secretion

Unstimulated hRPE cells or hRPE cells treated with nonglycated HSA, globulin-free HSA, or globulin- and

TABLE 1. Glycated HSA-Induced Expression of IL-8 and MCP-1 in hRPE cells[a]

Condition	IL-8 (ng/10^6 cells/mL)	MCP-1 (ng/10^6 cells/mL)
	Experiment A	
Control	0	4.2 ± 0.4
GHSA	26.0±1.3	42.2 ± 0.4
HSA	0	2.2 ± 0.2
GFHSA	0	5.1 ± 0.6
GFFAFHSA	0	3.8 ± 1.0
	Experiment B	
Control	0	1.0 ± 0.0
+ Anti-hIL-1β	0	1.3 ± 0.2
+ Anti-hTNF-α	0	1.3 ± 0.2
IL-1β		
0.02 ng/mL	18.1 ± 5.1	23.9 ± 2.4
2.0 ng/mL	241.1 ± 62.5	74.7 ± 8.0
Anti-hIL-1β + IL-1β (2.0 ng/mL)	1.1 ± 0.2	2.9 ± 0.6
TNF-α		
0.2 ng/mL	0.3 ± 0.1	13.6 ± 0.9
20 ng/mL	28.7 ± 2.8	42.7 ± 1.6
Anti-hTNF-α + TNF-α (20 ng/ml)	0.3 ± 0.0	1.7 ± 0.4
GHSA	26.0 ± 1.5	40.3 ± 2.1
+ Anti-hIL-1β	23.8 ± 2.3	47.0 ± 2.2
+ Anti-hTNF-α	20.2 ± 2.4	42.1 ± 1.2
	Experiment C	
Control	0	1.6 ± 0.02
PMX B	0	2.60 ± 0.6
LPS		
100 ng/mL	11.47 ± 0.3	24.97 ± 1.02
100 ng/mL + PMX B	0.57 ± 0.01	3.12 ± 0.04
	Experiment D	
Control	0	3.4 ± 0.2
PMX B	0.8 ± 0.01	5.7 ± 0.3
GHSA	25.9 ± 4.6	43.1 ± 4.2
GHSA + PMX B	25.7 ± 3.7	41.9 ± 2.1

[a]Induction of IL-8 and MCP-1 was determined by ELISA after challenge with listed stimuli for 24 h as described in Materials and Methods. The levels of IL-1β and TNF-α were not detectable by the ELISA. The concentrations of GHSA, anti-hIL-1β, anti-hTNF-α, and polymyxin B were 500, 1, 3, and 10 μg/mL, respectively. Abbreviations: Anti-hIL-1β, monoclonal antibodies to human IL-1β; anti-hTNF-α, monoclonal antibodies to human TNF-α; GHSA, glycated human serum albumin; GFHSA, globulin-free human serum albumin; GFFAFHSA, globulin- and fatty acid-free human serum albumin; PMX B, polymyxin B.

fatty acid-free HSA did not produce detectable levels of IL-8 or significantly induce MCP-1 secretion over constitutive levels (**Table 1**, experiment A). HRPE exposure to GHSA (500 μg/mL) for 24 h resulted in secretion of significant levels of antigenic IL-8 (26.0 ± 1.3 ng/10^6 cells /mL, $P < 0.001$) and MCP-1 (42.2 ± 0.4 ng/10^6 cells/mL, $P < 0.001$). Because our previous studies showed that IL-1β, TNF-α, and LPS [9, 10] induce hRPE IL-8 and MCP-1, we studied whether IL-8 and MCP-1 induction by GHSA was due to production of hRPE IL-1β and TNF-α. Consistent with previous reports, IL-1β, TNF-α, and LPS all stimulated hRPE cells to secrete IL-8 and MCP-1 (Table 1, experiments B and C). The levels of IL-8 and MCP-1 induced by these stimuli were equivalent to or higher than those induced by 500 μg/mL of GHSA. The secretion induced by IL-1β, TNF-α, or LPS alone was abolished when neutralizing anti-hIL-1β (1 μg/mL) and anti-hTNF-α (3 μg/mL) antibodies or 10 μg/mL of polymyxin B, an LPS inhibitor [24], were used, respectively. The presence of anti-hIL-1β, anti-hTNF-α, and polymyxin B alone did not alter the basal levels of MCP-1 secretion. Under the same conditions, the GHSA-induced IL-8 and MCP-1 secretion

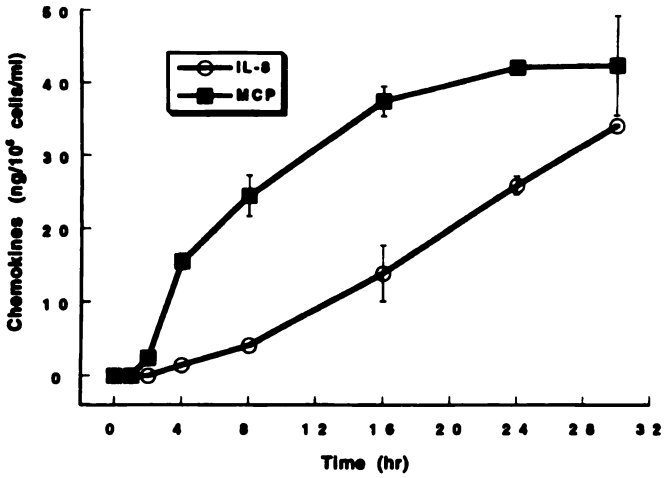

Fig. 1. Time-dependent IL-8 and MCP-1 secretion by GHSA-stimulated human RPE cells. The cells were incubated in medium containing 500 μg/mL of GHSA. Media were collected at the times indicated. Chemokine concentrations were determined by ELISA.

was not reduced by these antibodies or polymyxin B (Table 1, experiments B, C, and D). Furthermore, the levels of IL-1β or TNF-α in GHSA (500 μg/mL) -treated hRPE cells were not detectable by sensitive ELISA (>50 pg/mL), further suggesting that IL-1β and TNF-α did not induce any of the GHSA-induced IL-8 and MCP-1.

Time- and dose-dependent induction of hRPE IL-8 and MCP-1 secretion by GHSA

After challenge by 500 μg/mL of GHSA, antigenic IL-8 was detected by 4 h and MCP-1 by 2 h after GHSA exposure. MCP-1 levels peaked at 24 h, whereas antigenic IL-8 levels continued to show increase during incubations up to 30 h (**Fig. 1**).

Dose-dependent production of IL-8 and MCP-1 was determined by ELISA after 24-h incubation with GHSA concentrations ranging from 62.5 to 3,000 μg/mL. Regression analysis (**Fig. 2**) of GHSA-induced IL-8 and MCP-1 secretion revealed dose-dependent hyperbolic kinetics (r = 0.99). The EC_{50} values for half-maximal stimulation of IL-8 and MCP-1 were 1101 and 71 μg/mL, respectively. The EC_{50} value for MCP-1 induction was below the normal plasma GHSA concentration range (350–500 μg/mL) [25, 26]. With GHSA concentration at 500 μg/mL, production of MCP-1 reached a maximum, while the peak for IL-8 secretion occurred at the concentrations higher than 2000 μg/mL.

Effects of glucose and insulin on GHSA stimulation

To test the effects of glucose on GHSA-induced IL-8 and MCP-1 production, two concentrations of glucose, 5 mM (90 mg/dL) and 20 mM (360 mg/dL), were selected. The lower concentration represents the glucose levels maintained in standard hRPE growth medium and in normal plasma. The higher concentration approximates the glucose levels found in poorly controlled diabetic patients [27]. Elevating ambient glucose in the medium to 20 mM for 24 h before adding 500 μg/mL GHSA significantly (P< 0.01) enhanced GHSA-induced IL-8 production by 1.8-fold (**Fig. 3**). GHSA-induced MCP-1 production was increased 1.3-fold by pretreatment with 20 mM glucose but this increase was not significant (Fig. 3). Glucose added alone did not affect IL-8 and MCP-1 secretion (data not shown).

In the fasting state, plasma insulin concentrations range from 0.2 to 0.8 ng/mL (5–20 μU/mL) [28]. As with glucose, exposure of hRPE cells to insulin alone did not alter IL-8 and MCP-1 secretion (data not shown). However, co-incubation of GHSA with insulin (0.5 ng/mL), led to 44% inhibition of the GHSA-induced IL-8 secretion, but did not affect GHSA-induced MCP-1 secretion (Fig. 3). Further increase of insulin concentrations (6 and 12 ng/mL) did not cause additional reductions in GHSA-induced hRPE IL-8 and the MCP-1 secretion remained unchanged.

Effects of actinomycin D and cycloheximide on GHSA-stimulated IL-8 and MCP-1 secretion

To examine whether new protein synthesis was involved in the GHSA-mediated IL-8 and MCP-1 induction, 10 μg/mL cycloheximide or 1 μg/mL actinomycin D was added before GHSA challenge. As shown in **Table 2**, cycloheximide and actinomycin D alone did not affect the IL-8 and MCP-1 secretion by hRPE cells. The presence of cycloheximide or actinomycin D inhibited GHSA-induced levels of IL-8 and MCP-1 by >90%, suggesting that induction of these chemokines by GHSA was dependent upon de novo mRNA as well as de novo protein synthesis.

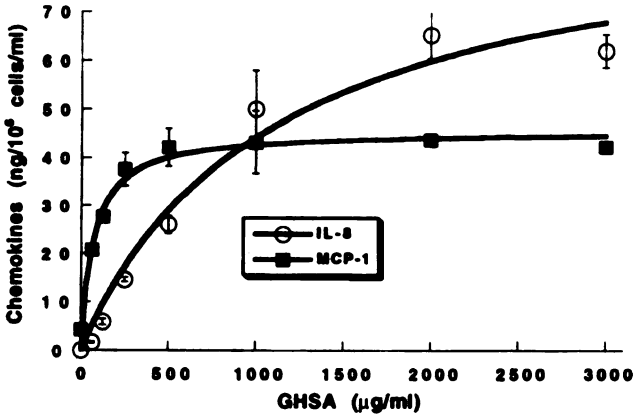

Fig. 2. Dose-dependent IL-8 and MCP-1 production by GHSA-stimulated human RPE cells. Media overlying RPE cells treated with various concentrations of GHSA (62.5–3,000 μg/mL) were collected after 24-h incubations and chemokines were measured by ELISA.

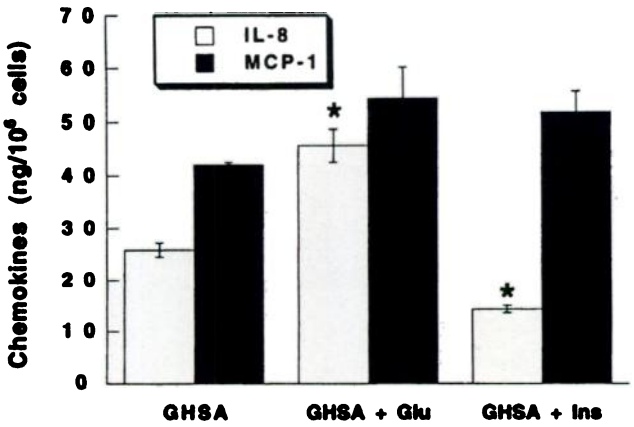

Fig. 3. Effect of glucose and insulin on GHSA-stimulated IL-8 and MCP-1 secretion by hRPE cells. hRPE cultures were pretreated with 15 mM glucose (total = 20 mM, 360 mg/dL) and insulin (0.5 ng/mL) for 24 and 1 h, respectively, prior to adding GHSA (500 μg/mL). *Significant ($P < 0.01$) difference of IL-8 levels compared with corresponding GHSA control.

GHSA-induced steady-state mRNA expression of hRPE IL-8 and MCP-1

Steady-state IL-8 and MCP-1 mRNA expression was minimal in unstimulated hRPE cells by Northern blot analysis. Following stimulation by GHSA, IL-8 and MCP-1 mRNA expression in hRPE was markedly increased. Enhancement in IL-8 and MCP-1 mRNA expression was time-dependent (**Fig. 4**). MCP-1 mRNA expression was substantially elevated by 4 h, whereas that of IL-8 was found to be substantially increased only at 24 h. Dose-dependent IL-8 and MCP-1 mRNA levels were also observed over GHSA levels ranging from 250 to 1,000 μg/mL, with steady-state mRNA levels for IL-8 and MCP-1 increasing 2.0- and 1.4-fold, respectively, over the GHSA concentration range (**Fig. 5**). The dose-dependent increases in IL-8 and MCP-1 mRNA levels both appeared to peak by 1,000 μg/mL GHSA. Either glucose or insulin alone did not induce detectable levels of IL-8 and MCP-1 mRNA expression (data not shown). The steady-state IL-8 mRNA levels, as determined by laser densitometry, increased 72% in the presence of glucose and reduced by 41% when insulin was present during hRPE exposure to GHSA (**Fig. 6**).

GHSA-induced secretion of other hRPE chemokines

In addition to IL-8 and MCP-1, chemokines NAP-2, GRO-α, RANTES, IP-10, MIP-1α, MIP-1β, and ENA-78 were examined in GHSA-treated hRPE growth media. As summarized in **Table 3**, 500 μg/mL of GHSA induced a 10-fold increase in hRPE cell to secrete NAP-2. No constitutive production of either GRO-α or RANTES was found in media overlying unstimulated hRPE cells, while both chemokines appeared after 24-h incubations with GHSA. Our ELISA did not show detectable secretion of IP-10, MIP-1α, MIP-1β, or ENA-78 by hRPE cells either constitutively or in the presence of GHSA.

DISCUSSION

Protein glycation is a nonenzymatic posttranslational modification reaction in which the hydroxyl group of the sugar binds covalently to the α or ε-amino group of proteins. Serum protein glycation can occur under normal physiological conditions. The levels of glycation, however, are rapidly increased in diabetics [25]. Because glucose is the major plasma monosaccharide, plasma protein glycation is predominately formed via covalently linked glucose. During this process, glucose nonenzymatically attaches to proteins, forms a labile Schiff base intermediate, and undergoes Amadori rearrangement, leading to relatively stable early adducts, ketoamine, or fructosamine [29, 30]. The rate of formation of such adducts is about 1/60 that of the spontaneous dissociation to glucose and protein [31].

Serum albumin, a nonglycoprotein, constitutes approximately one-half of total plasma protein and is the major target of glycation. Because glycated albumin is a natural byproduct that is elevated in diabetics [32], the levels of fructosamine in glycated serum albumin as well as that in hemoglobin are commonly used to clinically monitor the

TABLE 2. Effect of Actinomycin D and Cycloheximide on GHSA-Induced IL-8 and MCP-1 Secretion by hRPE Cells[a]

	IL-8		MCP-1	
	ng/10⁶ cells/mL	Inhibition (%)	ng/10⁶ cells/mL	Inhibition (%)
Control	0	—	0.99 ± 0.00	—
AD	0	—	0.72 ± 0.12	—
CHX	0	—	0.93 ± 0.10	—
GHSA	26.01 ± 1.77	0	40.34 ± 1.26	0
GHSA + AD	0.42 ± 0.08[b]	98.4	0.62 ± 0.11	98.5
GHSA + CHX	2.56 ± 0.12[b]	90.2	0.55 ± 0.06	98.6

[a]The hRPE cells were preincubated with actinomycin D (AD) or cycloheximide (CHX) for 1 h. Glycated human albumin (GHSA) was then added and IL-8 and MCP-1 were measured in media collected after 24 h of hRPE exposure to GHSA. The GHSA– induced secretion of IL-8 and MCP-1 were calculated by subtracting the corresponding values in the absence of GHSA from the total values in the presence of GHSA. The concentrations of AD, CHX, and GHSA were 1, 10, and 500 μg/mL, respectively.
[b]$P < 0.001$ as compared with GHSA stimulation.

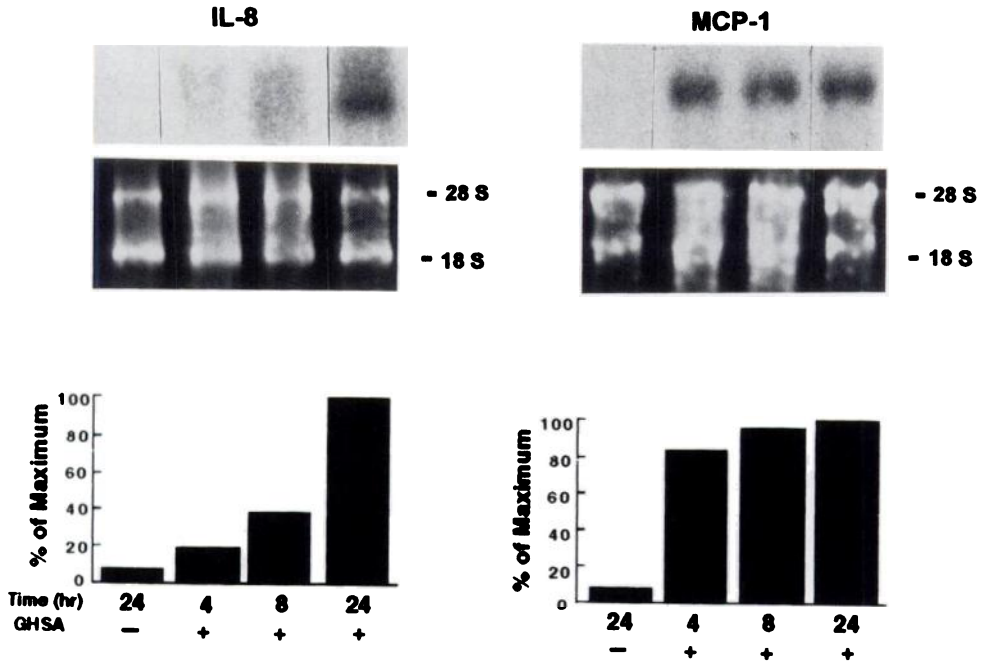

Fig. 4. Time-dependent induction of human RPE IL-8 and MCP-1 mRNA expression by GHSA. Human RPE cells were exposed to GHSA (500 μg/mL) and harvested at the times indicated. RNA was isolated from the hRPE cells and prepared for Northern blots. Top, autoradiographs of Northern blots probed for IL-8 and MCP-1 mRNA; middle, 18s and 28s rRNA of the same blots; bottom, relative density of corresponding mRNA signals determined by laser densitometry. Unstimulated (control) hRPE cells are denoted as 24-

degree of blood glucose control in diabetics [25]. The Amadori rearrangement in albumin that forms GHSA occurs about five times more rapidly than that in hemoglobin [33] and contains cyclic glycosylamine rearrangement products [18]. A close correlation between the serum glucose content and the degree of albumin glycation has been reported [25]. As glucose levels on the average rise twofold from normal (96 mg/dl) to diabetic (205 mg/dl), the percentage of early albumin glycation products also increases from 1.1 to 2.8% [25]. Assuming the normal serum albumin at 42 ± 3.5 mg/mL, this percentage shift in albumin glycation corresponds to a rise from 462 to 1176 μg/mL [26]. The albumin glycation percentage, therefore, is a good indicator of recent persistent hyperglycemia or multiple episodes of hyperglycemia. Accordingly, the levels of early glycation adducts may reflect the average blood sugar concentrations over the past 2–3 weeks [29].

As nonenzymatic glycation proceeds, irreversible AGEs are formed [5]. The formation of AGEs occur in vitro only after a long period (>3 weeks) of albumin incubation with high concentrations of glucose (>50 mM). AGEs can be found in vivo under pathological conditions such as diabetic mellitus or with senescence [7]. Protein glycation, especially the formation of AGEs, is thought to underlie many diabetic microvascular complications [4, 34–36]. The relative risk for developing any diabetic retinopathy, PDR, and progression of diabetic retinopathy is well correlated with elevated levels of glycated hemoglobin [1]. However, once large areas of retina are nonperfused due to microvascular damage, even perfect normoglycemia, as may be achieved by pancreatic transplantation, is unable to protect against the development and progression of PDR. This suggests a point of no return once severe diabetic retinopathy is established [37]. It is, therefore, important to delineate possible etiologic roles of protein glycation in the pathogenesis of PDR for early intervention.

The impact of protein glycation on cellular functions and its link to tissue remodeling have been demonstrated in several studies with AGEs [3–7]. These studies showed two major roles of AGEs. First, AGEs stimulate secretion of various cytokines and growth factors, including TNF-α, IL-1β, IL-6 , IFN-γ, platelet-derived growth factor, and IGF in macrophages/monocytes [38–41], endothelial cells [42], lymphocytes [43], and renal cell carcinoma cells [44]. Second, excessive deposition of AGEs in tissue result in macrophage/monocyte chemotaxis [40, 45]. Although AGEs are chemotactic for monocytes, AGEs-induced chemokine secretion by cells has not been reported. In this study we demonstrate that GHSA stimulates hRPE to secrete C-X-C family (IL-8, NAP-2, and GRO-α) and C-C family (MCP-1 and RANTES) chemokines. Stimulation of GRO-α secretion by GHSA may explain observations that small but significant increases in GRO-α secretion occur when hRPE cells are exposed to serum-containing media [46]. This serum-dependent stimulation may be due to low levels of glycated albumin that exists in serum.

The GHSA used in our study was made less than 1 week prior and purified to exclude residual contamination with AGEs as described by Baynes et al. [18]. GHSA products, therefore, represent albumin early glycation adducts (Dr.

Curelaru, Sigma Chemical Co., personal communication). In contrast to AGEs [38], GHSA did not induce detectable levels of TNF-α and IL-1β secretion. We also demonstrated that the GHSA-induced hRPE secretion of IL-8, but not MCP-1, is significantly enhanced in the presence of high ambient glucose. Because equimolar quantities of 3-*O*-methyl glucose and mannitol did not enhance chemokine expression [47], we believe the additive effects of GHSA and glucose on the induction of IL-8 were not due to a hypertonic effect. Our data are consistent with a previous report showing that glucose enhances AGE-BSA-stimulated IL-6 and TNF-α production in monocytes [48]. Because normal plasma GHSA concentrations (about 400 μg/mL) are sufficient for saturating MCP-1 production (EC_{50} = 71 μg/mL), the additional rise in glycation due to elevated glucose concentrations in diabetes mellitus should be effective for IL-8 (EC_{50} = 1101 μg/mL) induction only. Because IL-8 has angiogenic properties [49], the high EC_{50} value and sustained secretion for GHSA-induced IL-8 secretion could be involved in retinal neovascularization in diabetes.

Significantly increased levels of IL-8 and MCP-1, in fact, have been detected in vitreous from patients with PDR [14]. Local production of these chemokines by resident cells within the eye, including RPE cells [9, 10], may be important to leukocytic infiltration and neovascularization that occur in PDR.

We have shown that IL-8 and MCP-1 are major hRPE chemokines that are induced by GHSA but not HSA. They are also inducible by IL-1β , TNF-α, and LPS [9, 10]. IL-1β and TNF-α-mediated IL-8 and MCP-1 secretion was very sensitive to inhibition by specific neutralizing antibodies, whereas similar levels of IL-8 and MCP-1 induced by GHSA were not affected. The absence of IL-1β and TNF-α in the GHSA-stimulated hRPE growth medium was confirmed by sensitive ELISA that were able to detect concentrations (10–50 pg/mL), much lower than those required for stimulating hRPE IL-8 and MCP-1 secretion to the level induced by 500 μg/mL of GHSA (Table 1). Our data thus exclude the possibility of autocrine mechanisms mediated by IL-1β or TNF-α. We also found substantial inhibition of LPS-induced IL-8 and MCP-1 secretion by polymyxin B. That polymyxin B did not affect the GHSA inductions of hRPE chemokines, rules out LPS contamination as an inducer of the responses we obtained with GHSA. These data, taken together, point to the essential role of glycation in the GHSA-induced chemokine secretion.

Even though moderate levels of GHSA exist in normal plasma [25], penetration of large molecules, including albumin, from retinal vessels and choriocapillaris into the extracellular space surrounding the RPE cells is restricted. The tight junctions in retinal vascular endothelium make retinal vessels impermeable even to some smaller molecules. In contrast, the choriocapillaris is characterized by a fenestrated structure and is permeable to plasma proteins. In fact, the choriocapillaris is about five times more permeable than the kidney [50]. Direct contact of plasma proteins with RPE cells is largely prevented by Bruch's membrane, which behaves very much like a vascular base-

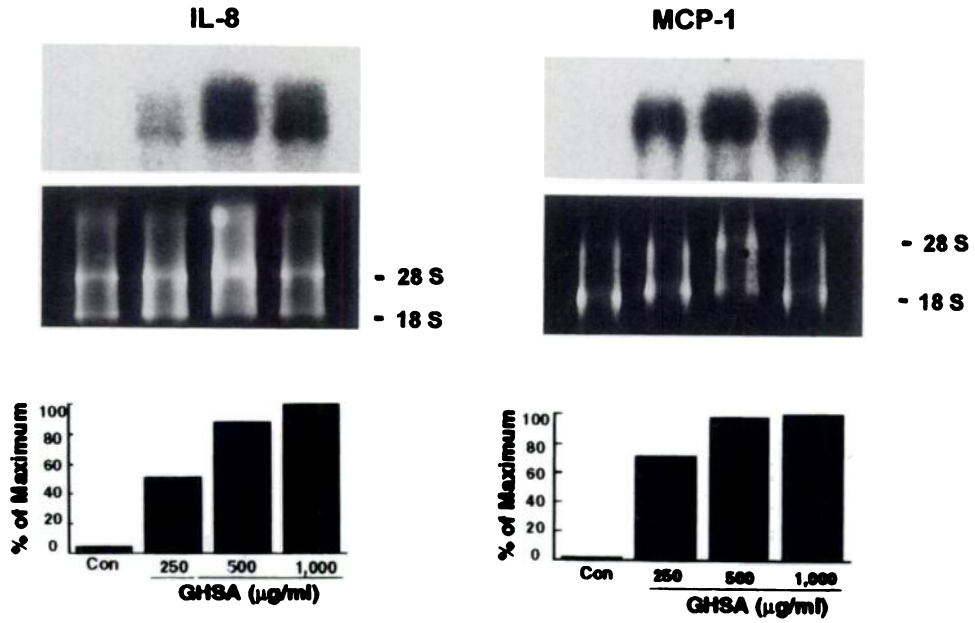

Fig. 5. Dose-dependent induction of IL-8 and MCP-1 mRNA expression by GHSA. Human RPE cells were exposed to 0, 250, 500, and 1,000 μg/mL GHSA for 24 h, harvested, and RNA isolated and prepared for Northern blots. Top, autoradiographs of Northern blots probed for IL-8 and MCP-1 mRNA; middle, 18s and 28s rRNA of the same blots; bottom, relative density of corresponding mRNA signals determined by laser densitometry.

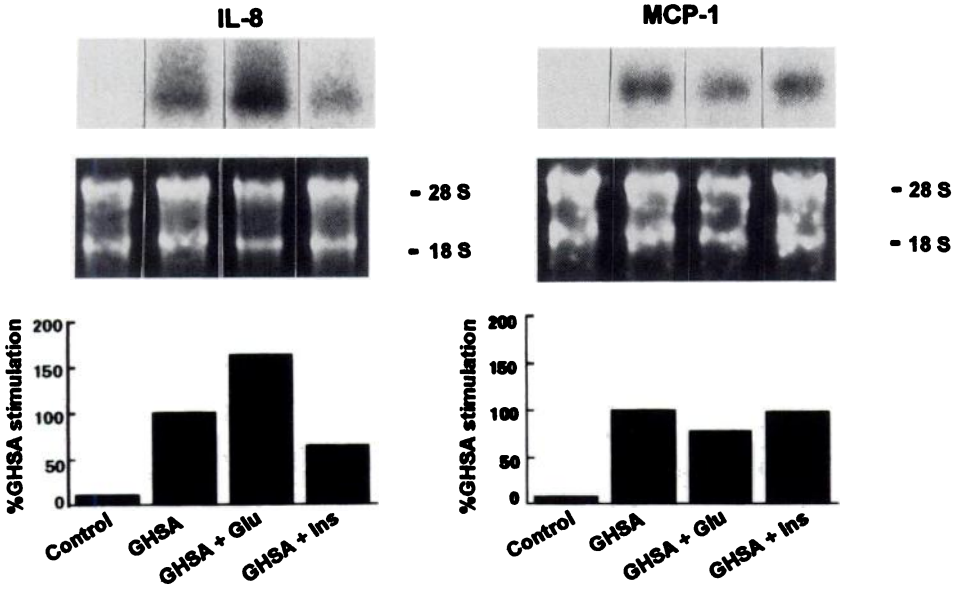

Fig. 6. Effects of glucose and insulin on the GHSA-stimulated IL-8 and MCP-1 mRNA expression. HRPE cultures were pretreated with 15 mM glucose (total = 20 mM, 360 mg/dL) and insulin (0.5 ng/mL) for 24 and 1 h, respectively, prior to adding GHSA (500 μg/mL). Top, autoradiographs of Northern blots probed for IL-8 and MCP-1 mRNA; middle, 18 and 28s rRNA of the same blots; bottom, relative density of corresponding mRNA signals determined by laser densitometry.

ment membrane. However, the permeability of the retinal vessels and Bruch's membrane can be dramatically altered under many pathological conditions. For example, breakdown of the inner BRB at retinal vascular endothelium has been demonstrated in diabetic retinopathy [51] and other diseases [52]. Bruch's membrane damage can be found in age-related macular degeneration [53], hypertensive choroidopathy [54], and photocoagulation [55]. It is generally believed that the increased BRB permeability may lead to plasma protein leakage. Therefore, serum albumin has been selected as a marker to evaluate the BRB permeability. One study has demonstrated a positive correlation between appearance of extravascular albumin in eye tissues and the severity of retinopathy [56]. In this report, extravascular albumin in retina or RPE was detected in 89% of PDR cases. Our data suggest that direct contact of GHSA with hRPE cells may lead to secretion of potent chemokines. That this event may occur in vivo is also supported by fact that concentrations of early glycated proteins in vitreous of diabetic patients is threefold higher than that measured in normal human vitreous [9]. In addition to GHSA, chemokine secretion by hRPE cells may also be stimulated by IL-1β and TNF-α [9, 10], which may also be present in diabetic vitreous [57]. The hRPE production of leukocyte chemokines, therefore, may also be expected to be more pronounced as hRPE cells migrate into the vitreous [58] and become cellular components of proliferative membranes in PDR and PVR. Under such conditions hRPE cells could be exposed to a variety of stimuli that induce them to produce chemokines.

The GHSA-induced production of the chemokines by the neural-derived RPE may initiate leukocyte-mediated microvascular injury leading to further GHSA leakage and the propagation of microvascular disease leading to neovascularization. It has been emphasized that vascular permeability and hemorrhage per se do not transport chemokines from the blood into local tissue because significant serum levels of IL-8 and MCP-1 have not been detected in normal patients or in patients with diabetes mellitus [14]. It is therefore reasonable to conclude that serum-derived modulators can induce chemotactic factors in affected tissues. GHSA thus may function as a component of natural immunity. It is possible that other mediators in plasma may exert effects similar to GHSA on hRPE cells.

It is well known that exudation of plasma and tissue infiltration of leukocytes are two concomitant events that accompany increased microvascular permeability. Our finding that GHSA, a normal plasma component, can elicit

TABLE 3. Stimulation of GRO-α, RANTES, and NAP-2 secretion by GHSA in hRPE cells[a]

Chemokines	Control (ng/10^6 cells/mL)	GHSA (ng/10^6 cells/mL)
GRO-α	0	34.12 ± 1.26
RANTES	0	1.82 ± 0.13
NAP-2,	0.65 ± 0.09	7.08 ± 0.55

[a]Induction of chemokine expression was determined as described in Materials and Methods after challenge with 500 μg/mL of GHSA for 24 h.

chemotactic agent secretion by hRPE cells, resident neural supportive cells of the retina, may provide a pathophysiological link between these two events.

The mechanisms underlying the GHSA-induced chemokine gene expression require further investigation to explain the different kinetic patterns and, hence, regulation of GHSA-induced IL-8 and MCP-1 gene expression. It is possible that the GHSA induction of IL-8 and MCP-1 is a receptor-mediated process as shown for AGEs that bind to specific and nonspecific cellular binding sites [59, 60]. Interestingly, the number and affinity of the AGE-receptors on mononuclear phagocytes are inversely correlated with ambient insulin levels [61]. The down-regulation of GHSA-stimulated hRPE IL-8 expression may also occur via similar mechanisms. In contrast to levels of secreted IL-8, GHSA-stimulated steady-state IL-8 mRNA achieved maximal levels at lower concentrations of GHSA. It is unclear whether GHSA has effects on IL-8 mRNA stability or protein turnover. As reported earlier, multiple control mechanisms exist for sustained IL-8 production [62]. Studies of GHSA binding at the RPE cell membrane and the effects of GHSA on other cell types in resident tissues may provide further insight into its role in the processes of inflammation, microvascular damage, and neovascularization.

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