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Advanced glycation end products (AGEs) on the surface of diabetic erythrocytes bind to the vessel wall via a specific receptor inducing oxidant stress in the vasculature: A link between surface-associated AGEs and diabetic complications

(vascular complications)

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ABSTRACT **Vascular complications are an important cause of morbidity and mortality in patients with diabetes. The extent of vascular complications has been linked statistically to enhanced adherence of diabetic erythrocytes to endothelial cells (ECs) and to the accumulation of a class of glycated proteins termed advanced glycation end products (AGEs). We hypothesized that formation of AGEs on the surface of diabetic erythrocytes could mediate their interaction with ECs leading to binding and induction of vascular dysfunction. Enhanced binding of diabetic erythrocytes to ECs was blocked by preincubation of erythrocytes with anti-AGE IgG or preincubation of ECs with antibodies to the receptor for AGE (RAGE). Immunoblotting of cultured human ECs and immunostaining of normal/diabetic human tissue confirmed the presence of RAGE in the vessel wall. Binding of diabetic erythrocytes to endothelium generated an oxidant stress, as measured by production of thiobarbituric acid-reactive substances (TBARS) and activation of the transcription factor NF-κB, both of which were blocked by probucol or anti-RAGE IgG. Erythrocytes from diabetic rats infused into normal rats had an accelerated, early phase of clearance that was prevented, in part, by antibody to RAGE. Liver tissue from rats infused with diabetic erythrocytes showed elevated levels of TBARS, which was prevented by pretreatment with anti-RAGE IgG or probucol. Thus, erythrocyte surface AGEs can function as ligands that interact with RAGE on endothelium. The extensive contact of diabetic erythrocytes bearing surface-associated AGEs with vessel wall RAGE could be important in the development of vascular complications.**

Nonenzymatic glycation of proteins, such as hemoglobin, has been shown to provide a useful index for management of patients with diabetes (1). The ultimate result of the nonenzymatic glycation and oxidation of proteins is formation of advanced glycation end products (AGEs), whose presence in plasma and tissues has been linked to the development of complications in diabetics (2–5). The cellular interactions of AGEs are mediated by receptors/cell surface binding proteins identified on endothelial cells (ECs) and mononuclear phagocytes (MPs), engagement of which leads to perturbation of cellular functions (3, 6–8). Our studies have characterized an integral membrane protein, receptor for AGE (RAGE), a newly discovered member of the immunoglobulin superfamily, which has a central role in mediating the interactions of AGEs with cellular surfaces (7–9).

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We previously showed that erythrocytes from diabetic patients exhibited enhanced binding to cultured endothelium (10). We hypothesized that, dependent on the duration of exposure of erythrocytes to plasma hyperglycemia, AGE modification of erythrocyte surface membrane proteins could occur, allowing them to bind and thereby to modulate properties of RAGE-expressing vessel wall cells. Our studies demonstrate that the molecular basis of the increased adherence of diabetic erythrocytes results largely from AGEs on the erythrocyte surface interacting with EC RAGE. This results in the induction of oxidant stress in the endothelium, potentially modulating expression of a spectrum of genes that could contribute to the pathogenesis of vascular complications.

MATERIALS AND METHODS

Subjects. The group of patients (n = 18 each for the normal and diabetic subjects) was comparable in age, duration of diabetes, fasting blood glucose, and hemoglobin A_{1c} levels. Erythrocytes from two patients homozygous for sickle cell disease were also studied.

Erythrocyte Adhesion Assay. Cultured human umbilical vein ECs were prepared and assayed as described (10–12). The specific activity of ^{51}Cr-labeled erythrocytes for normal and diabetic erythrocytes was 3750 ± 260 and 3820 ± 253 cpm per mg of hemoglobin, respectively. The adhesion ratio (AR) was calculated as follows: AR = (cpm of diabetic erythrocytes)/(cpm of normal erythrocytes). An AR value of 1 represents the adhesion observed with normal erythrocytes. Where indicated, either erythrocytes or ECs were preincubated with soluble RAGE (sRAGE) or antibodies and EC nuclear extracts were prepared (13).

Preparation of AGE-Modified Proteins, AGE Binding Proteins, and Antisera. AGE albumin was prepared and characterized as described (3, 7, 8). AGE binding proteins were purified from bovine lung (7) and consisted of RAGE, a lactoferrin-like polypeptide (LF-L), and a 20-kDa polypeptide (7). The form of RAGE purified from lung has a mass of ≈35 kDa and consists of the N-terminal two-thirds of the molecule; it is termed sRAGE (7). Monospecific, blocking

Abbreviations: AGE, advanced glycation end product; RAGE, receptor for AGE; sRAGE, soluble RAGE; LF-L, lactoferrin-like AGE binding protein; EC, endothelial cell; TBARS, thiobarbituric acid-reactive substance(s); MP, mononuclear phagocyte; TM, thrombomodulin.

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polyclonal antibodies to the above polypeptides were prepared in guinea pigs and rabbits (7, 8). Immunolocalization of RAGE in human vasculature employed polyclonal antibody to RAGE and was performed on formalin-fixed (3.5%)/paraffin-embedded samples obtained from National Disease Research Interchange (Philadelphia) as described (14). Affinity-purified anti-AGE antibody was prepared and characterized as described (8, 15). Antisera were prepared by standard methods (16) to purified mouse thrombomodulin (TM) (17) and were reactive with rat TM (data not shown).

Electrophoretic Gel Mobility-Shift and Thiobarbituric Acid-Reactive Substance (TBARS) Assays. Cultured ECs ($\approx 10^7$ cells) were treated as indicated and exposed for 1 hr to erythrocytes (2.7×10^{10}), and nuclear extracts were prepared. Complementary 27-bp oligonucleotide probes representing the consensus murine NF-κB site (18, 19) were synthesized, and oligonucleotides were annealed and 3'-end-labeled using Klenow fragment of DNA polymerase via standard procedures (20). Binding reactions were performed by preincubating nuclear extract protein (1.8 μg) in 20 mM Hepes, pH 7.9/60 mM KCl/1 mM $MgCl_2$/0.1 M EDTA/10% (vol/vol) glycerol/0.5 mM dithiothreitol/2 μg of poly(dI-dC) on ice for 10 min, followed by addition of the double-stranded ^{32}P-labeled oligonucleotide ($\approx$0.2 ng) and a second incubation at room temperature for 20 min. Samples (1.8 μg per lane) were loaded directly onto nondenaturing 6% polyacrylamide gels and electrophoresis/autoradiography was performed. Experiments were also performed with the GelShift kit purchased from Stratagene. Induction of oxidant stress on cultured endothelium or in livers of normal rats was determined by assessment of TBARS by measuring adsorbance at 532 nM (21, 22).

Transfusion of Diabetic and Normal Rat Erythrocytes into Normal Rats. Male Wistar rats (Iffa Credo) weighing $\approx$200 g initially were treated with streptozotocin (45 mg/kg; Sigma) intravenously and animals were maintained for 11 weeks prior to experiments. Glycemia in diabetic rats was 4–6 times higher than that of controls. Blood was collected and centrifuged to remove plasma and buffy coat, and the packed erythrocytes were washed and, for certain experiments, labeled with ^{51}Cr (final specific radioactivity, 1.5×10^6 cpm/ml) (10). Animals were pretreated with antibodies or probucol i.v., and 15 min later they were infused with either unlabeled or ^{51}Cr-labeled erythrocytes. Blood (0.1 ml) was sampled at 0.5 hr and once every 24 hr thereafter for up to 20 days. At the indicated times, animals were sacrificed, organs were harvested, and tissue samples were weighed or prepared for scintillation counting. For determination of TBARS, livers were harvested from rats 4 hr after the erythrocyte infusion and processed as described above.

RESULTS

Effect of AGE Albumin and Anti-AGE IgG on the Interaction of Diabetic Erythrocytes with Endothelium. When erythrocytes were incubated with cultured ECs, the enhanced binding of erythrocytes from diabetic patients was blocked by addition of increasing amounts of AGE albumin (AGE-BSA; Fig. 1*A*). Although levels of glycated albumin necessary for inhibition of diabetic erythrocyte–EC binding are considerably greater than those estimated to be present in diabetic plasma (23, 24), the inhibitory effect was selective: (*i*) AGE albumin had no effect on the binding of erythrocytes obtained from controls (Fig. 1*A*); (*ii*) AGE-modified IgG had a similar effect (Fig. 1*B*); and (*iii*) neither native albumin nor IgG altered the binding of erythrocytes to endothelium (data not shown; note that native albumin was present in all buffers). Further supporting the concept that AGEs were involved in the interaction of diabetic erythrocytes with endothelium was the finding that preincubation of diabetic erythrocytes with anti-AGE IgG resulted in inhibition of erythrocyte–EC interaction (Fig. 1*C*).

Engagement of Endothelial RAGE by Cell Surface-Bound Diabetic Erythrocytes. When ECs were preincubated with anti-AGE binding proteins (7), only anti-RAGE IgG inhibited diabetic erythrocyte–EC interaction (Fig. 1*D*). The effect of anti-RAGE IgG on diabetic erythrocyte–EC binding was specific: (*i*) anti-RAGE IgG had no effect on normal erythrocyte binding to ECs; (*ii*) anti-RAGE IgG did not alter increased EC adhesion of erythrocytes from patients with sickle cell anemia (Fig. 1*E*); (*iii*) anti-ICAM-1 (cell adhesion molecule) (CD54) IgG, which blocks binding of malaria-infected erythrocytes to endothelium (25), had no effect (Fig. 1*F*). Inhibition of the interaction of diabetic erythrocytes with endothelium was dependent on the concentration of anti-RAGE IgG (Fig. 1*G*). Furthermore, diabetic erythrocytes preincubated with sRAGE resulted in dose-dependent inhibition of erythrocyte–EC interaction (Fig. 1*H*).

These data, which implied that RAGE was present on human ECs, were confirmed by immunoblotting, where anti-RAGE IgG detected a band of $\approx$35 kDa (Fig. 2*A*, lane 2), comigrating with purified bovine lung RAGE (lane 1). The latter bands were due to anti-RAGE IgG binding to EC-derived RAGE, as shown by their disappearance when sRAGE was added to reaction mixtures during incubation of blots with anti-RAGE IgG (lanes 3 and 4). Immunocytochemical studies with cultured human ECs demonstrated staining for RAGE (Fig. 2*B*), whereas nonimmune IgG under the same conditions was negative (Fig. 2*C*). Immunostaining of human tissues with anti-RAGE IgG indicated RAGE in ECs of the coronary arteries from normal (Fig. 2*D*) and diabetic (Fig. 2*E*) individuals. Nonimmune IgG or samples of anti-RAGE IgG preadsorbed with the antigen showed no staining in the same cell cultures and tissue sections (data not shown). Examination of multiple human vessels demonstrated RAGE to be distributed throughout the vasculature (14).

Induction of EC Oxidant Stress by Diabetic Erythrocytes: Role of AGE–RAGE Interaction. Studies with soluble AGE ligands, such as glycated albumin, have shown that their binding to the cell surface modulates properties of target cells (8, 26, 27). In view of the association of AGEs with generation of reactive oxygen intermediates and its relevance to the pathogenesis of diabetic complications (28–35), we considered whether exposure of ECs to diabetic erythrocytes would lead to induction of oxidant stress. Compared with ECs treated with erythrocytes from normal individuals, diabetic erythrocytes led to a significant increase in TBARS, which was blocked by preincubation with probucol or by anti-RAGE IgG (Fig. 2*F*). Generation of TBARS was specifically due to the induction of oxidant stress in ECs as (*i*) the number of diabetic erythrocytes bound to the EC monolayer would make only a minor contribution to the total TBARS observed; (*ii*) AGEs are nonreactive in the TBARS assay (15); (*iii*) generation of TBARS was time dependent after binding of diabetic erythrocytes to endothelium; and (*iv*) probucol inhibited the appearance of TBARS, which would not have occurred if the lipid peroxides had been entirely preformed on the diabetic erythrocytes.

As another index of cellular oxidant stress, activation of the transcription factor NF-κB was studied in ECs exposed to diabetic erythrocytes by electrophoretic gel mobility-shift assay (19). Compared with ECs treated with normal erythrocytes (Fig. 2*G* I, lane 1), nuclear extracts prepared from ECs incubated with diabetic erythrocytes showed a much stronger gel-shift band (Fig. 2*G* I, lane 2; II, lane 1). The specificity of the gel-shift band for interaction of DNA binding proteins with the NF-κB sequence was shown by competition studies (Fig. 2*G* II, lanes 2 and 3). Diabetic erythrocyte-induced activation of endothelial NF-κB was due to interaction with RAGE, as anti-RAGE IgG blocked

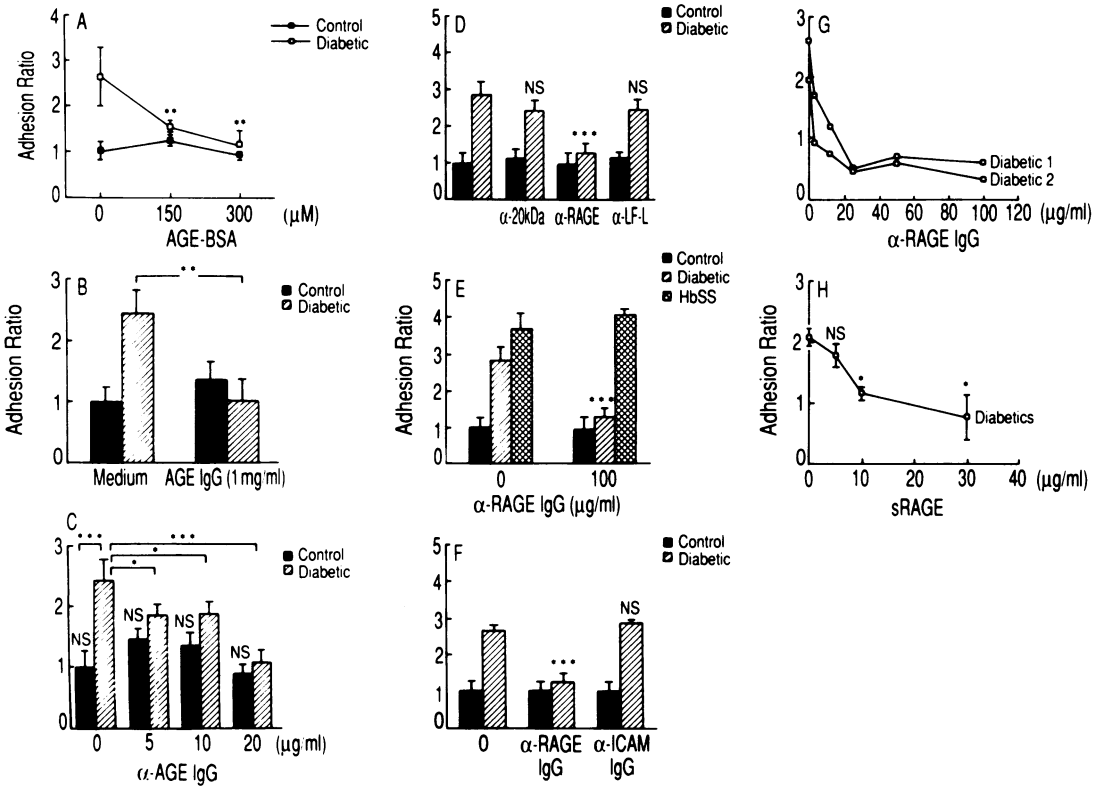

FIG. 1. Enhanced binding of diabetic erythrocytes to endothelium is mediated by AGEs on the erythrocyte and RAGE on the endothelium. (*A–C*) Effect of AGE albumin (*A*), AGE IgG (*B*), or anti-AGE IgG (*C*). Confluent ECs (9.6 cm^2 growth area/2.5 × 10^5 cells) were incubated with erythrocytes from normal subjects or diabetic patients (2.5 × 10^5 cells per well) in the presence of the indicated concentration of AGE albumin (*A*) or AGE IgG (1 mg/ml; *B*) for 30 min at 37°C, wells were washed five times, and adherent cells were quantitated based on radioactivity associated with the monolayer. (*C*) Erythrocytes were preincubated for 60 min at 37°C with anti-AGE IgG at the specified concentration and washed; then a binding assay was performed. In each case, the mean ± SEM of triplicate determinations is shown, and the experiment was repeated three times with erythrocytes from at least three patients. (*D–F*) Effect of anti-RAGE IgG on the binding of erythrocytes to endothelium. (*D*) Confluent EC monolayers were preincubated with medium alone (0) or with medium containing anti-20-kDa AGE binding protein IgG, anti-RAGE IgG, or anti-LF-L IgG (100 μg of immune IgG per ml was added in each case) for 60 min at 37°C; cultures were washed; and then a binding assay was performed by adding erythrocytes from normal subjects (control; $n = 7$) or diabetic patients ($n = 7$) as in *A–C*. (*E*) The same EC binding assay was done except that erythrocytes from two patients with sickle cell anemia (HbSS) were also included, along with control or diabetic erythrocytes. (*F*) The binding assay used diabetic erythrocytes as described above, except that certain EC monolayers were also preincubated with a blocking monoclonal antibody to cell adhesion molecule ICAM-1 (100 μg/ml). In each case (*D–F*), the mean ± SEM of seven determinations is shown, and the experiments were repeated four times. (*G* and *H*) Dose-dependent inhibition of diabetic erythrocyte–EC binding by anti-RAGE IgG (*G*) or sRAGE (*H*). (*G*) Confluent EC monolayers were preincubated with the indicated concentration of anti-RAGE IgG for 60 min at 37°C and washed, and then a binding assay was performed with diabetic erythrocytes from two different patients. (*H*) Diabetic erythrocytes were preincubated for 60 min at 37°C with the indicated concentration of sRAGE, the mixture was added to confluent ECs, and a binding assay was performed. (*G* and *H*) Mean ± SEM of three determinations is shown and the experiment was repeated three times. All data were analyzed by the Student *t* test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

appearance of the band (lane 4), as did probucol (data not shown).

Diabetic Erythrocyte–RAGE Interaction *in Vivo*: Effect on Disappearance of Infused Diabetic Erythrocytes and Induction of Oxidant Stress. Erythrocyte survival, as evaluated by ^{51}Cr labeling, was shortened when erythrocytes from diabetic rats, compared with normal erythrocytes, were transfused into syngeneic animals (36). The diminished survival of ^{51}Cr-labeled erythrocytes from diabetic rats was significantly reversed when normal rats were pretreated with anti-RAGE IgG (Fig. 3*A*). Infusion of anti-TM IgG (15) had no effect (Fig. 3*A*). These experiments were repeated with erythrocytes from 3 different diabetic rats transfused into 12 normal animals. The results showed interanimal variation in normal rats receiving erythrocytes from different diabetic rats, although anti-RAGE IgG consistently delayed the removal of transfused diabetic erythrocytes from the blood. Transfused erythrocytes from diabetic rats demonstrated accumulation of ^{51}Cr in the spleen, lung, kidney, and liver. Anti-RAGE IgG reduced by ≈3.5-fold the diabetic erythrocyte sequestration compared with anti-TM IgG (data not shown). These antibodies had no effect on tissue sequestration of erythrocytes from normal rats.

Animals infused with diabetic erythrocytes also showed evidence of oxidant stress, as indicated by the increase in TBARS in the liver (Fig. 3*B*), which was blocked by pretreatment with anti-RAGE IgG or probucol (Fig. 3*C*). Activation of NF-κB was also observed in nuclear extracts from livers of animals infused with diabetic erythrocytes (data not shown).

DISCUSSION

We previously observed a correlation between HbA_{1c} levels and erythrocyte adhesion in patients with diabetes mellitus (10). These data demonstrate that AGEs formed *in vivo* on the surface of diabetic erythrocytes interact with EC RAGE, which serves as receptor/binding protein for this class of glycated compounds. We speculate that enhanced diabetic erythrocyte interaction with endothelial RAGE has its effects, at least in part, through induction of oxidant stress, potentially perturbing multiple cellular properties, such as induction of cytokines (19).

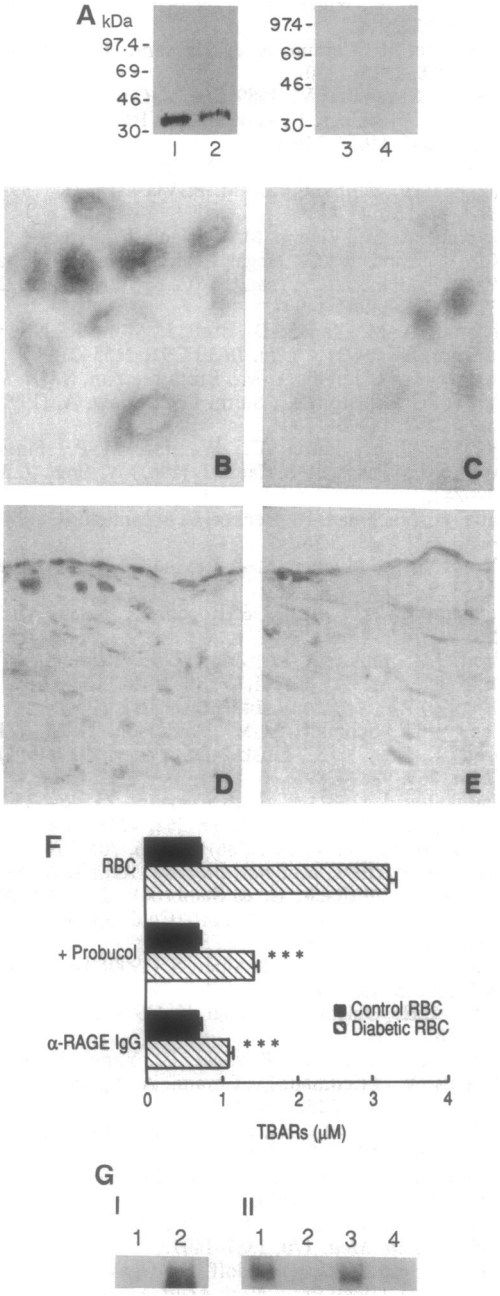

FIG. 2. RAGE is present in human ECs in culture and *in vivo*, and engagement of endothelial RAGE by diabetic erythrocytes leads to induction of oxidant stress. (*A–E*) Identification of RAGE in human endothelium. (*A*) Immunoblotting of cultured EC extracts for RAGE using anti-RAGE IgG (5 μg/ml). Samples were as follows: lane 1, purified lung RAGE (1.5 μg); lane 2, EC extract (5 μg); lanes 3 and 4, purified RAGE and EC extract as in lanes 1 and 2 with immunoblotting done in the presence of excess free sRAGE (65 μg/ml) added during incubation of blots with the primary antibody. (*B* and *C*) Immunostaining of cultured ECs with anti-RAGE IgG (*B*) or nonimmune IgG (*C*). (*D* and *E*) Immunostaining of a coronary artery from a normal (*D*) and a diabetic (*E*) individual with anti-RAGE IgG. (*B–E*, ×260.) (*F* and *G*) Diabetic erythrocytes induce oxidant stress in ECs leading to generation of TBARS (*F*) and activation of NF-κB (*G*). (*F*) Generation of TBARS. ECs (2×10^4 cells per plate) were exposed to either diabetic or normal erythrocytes (2×10^{10} cells in each case) for 4 hr at 37°C. Either probucol (50 μM) or anti-RAGE IgG (50 μg/ml) was included along with the diabetic erythrocytes (RBC). Results shown are means ± SEM of two sets of experiments, each done in triplicate. ***, $P < 0.001$ based on the Student *t* test. (*G*) Electrophoretic gel mobility-shift assay for NF-κB. ECs (8×10^6 cells per plate) were incubated for 4 hr at 37°C with either normal or diabetic erythrocytes (2×10^{10} cells in each case). Samples were prepared as follows (^{32}P-labeled NF-κB) oligonucleotide probe was also present in each lane; a control with the latter probe alone showed no band). I: lane 1, nuclear extracts from ECs exposed to control/normal erythrocytes; lane 2, nuclear extracts from ECs exposed to diabetic erythrocytes. II: lane 1, nuclear extracts from ECs exposed to diabetic erythrocytes; lanes 2 and 3, same as lane 1 except samples contained 100-fold excess unlabeled NF-κB (lane 2) or CRE (lane 3); lane 4, nuclear extracts from ECs pretreated with anti-RAGE IgG (50 μg/ml) and then exposed to diabetic erythrocytes. Nonimmune IgG (50 μg/ml) had no effect on NF-κB activation.

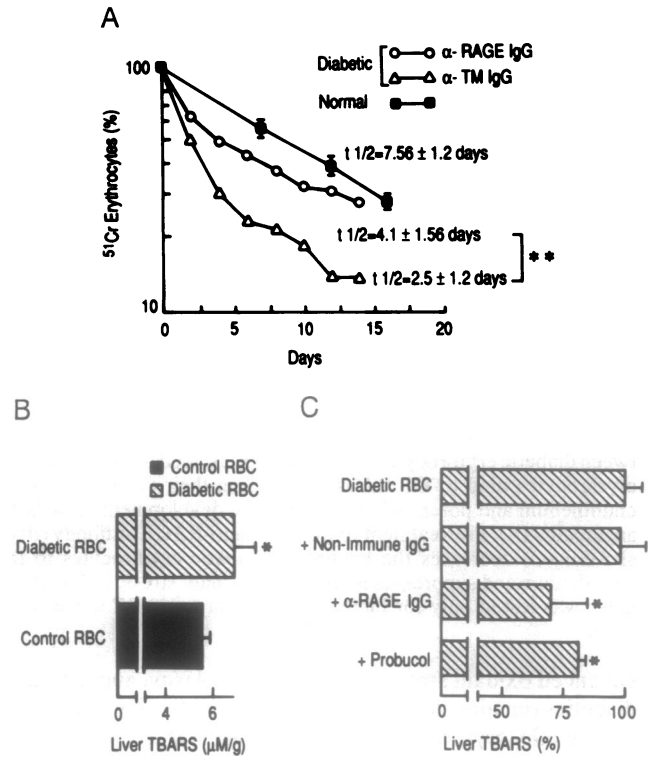

FIG. 3. Infusion of diabetic/normal erythrocytes into normal rats: Effect of anti-RAGE IgG on their clearance (*A*) and induction of oxidant stress in the liver (*B* and *C*). (*A*) Erythrocytes were isolated from diabetic or normal rats ($n = 2$ in each case), labeled with ^{51}Cr, and transfused into normal rats as follows (7.5×10^5 cpm per rat, which corresponded to 2.66×10^9 erythrocytes): normal erythrocytes alone or diabetic erythrocytes in animals preinfused with anti-RAGE IgG (170 μg/100 g) or with anti-TM IgG (170 μg/100 g). **, $P < 0.01$ by the Mann–Whitney rank test. (*B*) Erythrocytes (RBC) were isolated from diabetic and normal rats as described above and infused into normal rats (unlabeled). Liver was harvested and the TBARS assay was performed. *, $P < 0.05$ by the Wilcoxon test. (*C*) Erythrocytes were isolated from diabetic rats as described above and infused into normal rats in the presence of anti-RAGE IgG (as described above), nonimmune IgG (170 μg/100 g), or probucol (50 μM). Liver tissue was harvested and TBARS was determined as described in the text. *, $P < 0.05$ by the Wilcoxon test.

Nonenzymatic glycation and oxidation are closely linked in terms of the formation of AGEs, as well as the known capacity of AGEs prepared *in vitro* to form reactive oxygen intermediates (28–35). The ability of diabetic erythrocytes to induce oxidant stress in ECs, which was blocked by preventing AGE–EC interaction with anti-RAGE IgG or by probucol, suggests that one role of RAGE is to localize an oxidizing stimulus to the cell surface, allowing it to perturb cellular properties (Fig. 4). The source of reactive oxygen intermediates is likely AGEs on the erythrocyte surface, as ligation of EC RAGE by antibodies does not induce oxidant stress, and AGE-induced oxidant stress is blocked by the addition of oxygen free radical scavenger enzymes to culture medium (15). The contribution of erythrocyte-associated AGEs to

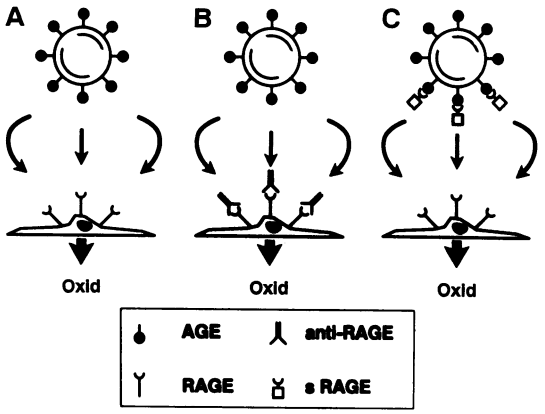

FIG. 4. Schematic depiction of AGE-mediated interactions between diabetic erythrocytes and vascular ECs. AGE-bearing diabetic erythrocytes bind to EC RAGE, increasing their adherence to the endothelium and potentially facilitating the development of diabetic angiopathy (*A*). Pretreatment of ECs with anti-RAGE antibody (*B*) or sRAGE (*C*) decreases the binding of diabetic erythrocytes to the endothelium and suppresses induction of oxidant stress, although the latter was tested only for inhibition of erythrocyte–EC adhesion *in vitro*.

enhanced oxidant stress observed in the plasma and tissues of diabetics remains to be analyzed, but the extensive interaction of diabetic erythrocytes with the vascular cells that bear RAGE (ECs, MPs, etc.) suggests that this could be responsible for the correlation between erythrocyte–EC adherence and diabetic complications. Thus, by virtue of the ligand, AGE–RAGE interaction focuses an oxidant stress on cells bearing the receptor, thereby activating intracellular pathways not traditionally associated with other scavenger receptors (37–39).

The cellular receptor for AGEs, as characterized on ECs and MPs, consists of two components, both of which independently bind soluble AGE ligands, such as AGE albumin: RAGE, an integral membrane protein, and a protein likely identical to lactoferrin, LF-L (7). In previous studies, we have found that these two proteins can associate *in vitro* (40) and appear to interact on the cell surface to form the binding site for AGE albumin, as antibodies to either polypeptide blocked the binding of the AGE ligand to ECs and MPs and prevented AGE-induced modulation of MP migration (7, 8). Our observation that only antibody to RAGE, but not anti-LF-L IgG, blocked binding of diabetic erythrocytes to endothelium suggests that there may be a different spectrum of AGE structures recognized by each of the cell-associated AGE binding proteins.

These data indicate that AGEs either form on the diabetic erythrocyte surface or become attached to it, allowing interaction of the diabetic erythrocyte with a vessel wall receptor not recognized by erythrocytes from normal individuals. The consequences of the binding of diabetic erythrocytes to RAGE on the vessel surface for diabetic complications remain to be elucidated, but the potential of erythrocytes bearing AGEs to perturb function of the vessel wall merits further study.

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