Structural and Functional Changes of Laminin and Type IV Collagen After Nonenzymatic Glycation

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Laminin and type IV collagen are two major basement membrane glycoproteins; they are large multidomain macromolecules that are involved in two types of functions. First, they provide the structural framework of all basement membranes, and second, they interact with cell-surface molecules and are key to adhesion, spreading, and proliferation of cells. We summarize experimental evidence that nonenzymatic glucosylation of these two macromolecules in vitro alters their structure, their ability to polymerize, and their ability to promote cell adhesion. Additional studies are needed to document these changes in situ and therefore extend these conclusions to intact basement membranes. *Diabetes* 41 (Suppl. 2):49–51, 1992

Basement membranes are specialized areas of the extracellular matrix. They differ from the rest of the extracellular matrix in at least two respects: their characteristic localization and their macromolecular composition. Basement membranes are found at the basal surface of polarized cells and surround certain cell types. In the microvasculature, basement membranes underlie endothelial cells and surround pericytes. In people with diabetes, the basement membrane of the microvasculature exhibits thickening and increased permeability to plasma proteins.

The isolation and characterization of basement-membrane macromolecules was not an easy task, mainly due to their low solubility resulting from extensive crosslinking. In the late 1970s, the development of model systems that produce—in large amounts and with little crosslinking—almost exclusively basement-membrane macromolecules allowed identification and detailed structural analysis of these proteins. The most widely used of these model systems and the one we use in most of our studies is the Engelbreth-Holm-Swarm tumor, a noninvasive murine tumor (1). The basement-membrane macromolecules can be classified in three groups: collagenous glycoproteins, of which type IV collagen is by far the most abundant; noncollagenous glycoproteins, of which laminin is best studied; and proteoglycans. Because our studies focus on the effect of nonenzymatic glucosylation of laminin and type IV collagen on basement membrane structure and function, we will discuss in more detail some features of these two macromolecules.

Laminin has a molecular weight of 850,000 and consists of three polypeptide chains: B1, B2, and A. The exact amino acid sequence of all three chains is known (2–4). These chains share many similarities in their domain organization, despite differences in their sequence. The technique of rotary shadowing allowed the visualization of the laminin molecule and the appreciation of its multidomain arrangement (5). In the electron microscope, laminin appears as an asymmetric cross with three short arms (35 nm long) and one long arm (75 nm). Laminin can polymerize in a temperature-, time-, and concentration-dependent fashion (6). During this process, dimers and small oligomers are first formed, and eventually a large polymer can be detected. Laminin dimers are preferentially formed by the interaction of two long arms (7).

Type IV collagen has a molecular weight of about 500,000 and consists of three polypeptide chains of known amino acid sequence, forming to a large extent a triple-helical rod. It is a heterodimer, composed of two identical a1(IV) chains and one a2(IV) chain (8). The carboxyl terminal end of type IV collagen forms the most prominent, globular, noncollagenous domain, known as

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EHS, Engelbreth-Holm-Swarm; NC1, carboxy terminal end of type IV collagen; K_d, dissociation constant.

NC1. At the electron microscopic level, type IV collagen appears as a flexible rod with an average length of ~400 nm. Type IV collagen can polymerize by a series of complex interactions: the final product appears as a semiregular hexagonal network, consisting of laterally associated molecules (9). Domain NC1 appears to play a crucial role in this process, because isolated NC1 domain can bind specifically at four sites along the length of type IV collagen ~100-nm apart, antibodies to NC1 domain can very effectively block lateral association and the presence of isolated NC1 inhibits type IV collagen polymerization (10).

Laminin and type IV collagen are involved in two types of interactions: first, self-association (6) and interaction with each other (11), both of which may be crucial for the structural integrity of all basement membranes; and second, interaction with cell-surface components on the basal surface of endothelial cells crucial for the adhesion of these cells to the underlying basement membrane. We present data suggesting that nonenzymatic glucosylation will interfere with both types of interactions of both laminin and type IV collagen.

In vitro nonenzymatic glucosylation of laminin demonstrated a concentration-dependent incorporation of glucose to laminin, which, at 200 mM glucose, for a 6-day incubation resulted in about 14 mol of glucose per laminin molecule. Formation of cross-links, predominantly intramolecular, was observed by gel electrophoresis. We examined the structure of laminin with the electron microscope, and we observed that nonenzymatic glucosylation created remarkable shape changes and deformations. The shape alterations could be largely, but not totally, prevented by aminoguanidine in the incubation mixture. The effect of aminoguanidine provided additional suggestive evidence that shape alterations may be related to cross-link formation (12). We then examined the effect of nonenzymatic glucosylation of laminin polymerization. To see any effect on the initial stages, we examined the type of long-arm to long-arm dimers formed. Glucosylation dramatically reduced this specific association. To examine the effect on the final product, we examined the development of turbidity of laminin solutions incubated with increasing glucose concentrations. We observed a glucose concentration-dependent reduction in the plateau value of the turbidity. In both stages of polymerization, equimolar amounts of aminoguanidine in the incubation mixture could prevent the effect of nonenzymatic glucosylation (12). These data suggest that nonenzymatic glucosylation of laminin reduces its ability to polymerize and raise the possibility that other interactions between laminin and basement membrane macromolecules may be compromised.

Nonenzymatic glucosylation of isolated domain NC1 of type IV collagen leads to minor changes that could be detected with the electron microscope. A small percentage of NC1 globules, ~6%, appear larger than usual. This change could be attributed to the formation of intermolecular cross-links; we observed such cross-links after nonenzymatic glucosylation of domain NC1 (13). Glucosylated domain NC1 failed to interact in a specific way with the triple-helical rod of type IV collagen, as observed with the electron microscope. Furthermore, modified NC1 is not able to compete lateral association of intact type IV collagen. This change is observed in turbidimetry experiments and is confirmed with the electron microscope, in which a lack of network formation is striking (13). These experiments demonstrate that nonenzymatic glucosylation of domain NC1 impairs its ability to interact with intact type IV collagen and mediate lateral association, presumable by a primary modification. We attempted to map precisely this important function of NC1 domain to a specific amino acid sequence. We identified the sequence TAGSCLRKFSTM (Hep I), representing positions 49–60 of the amino acid sequence of chain a1(IV). This peptide can bind to intact type IV collagen in a specific manner with an apparent K_d of 1.66 nM (14). Peptide Hep I can inhibit lateral association of type IV collagen in a concentration-dependent fashion; however, nonenzymatic glucosylation of this peptide renders it unable to inhibit type IV collagen polymerization (C.S. Haitoglou et al., unpublished observations). These data suggest that peptide Hep I, and, more specifically, lysine number 56 of the a1 (IV) chain, is a potentially important target of nonenzymatically attached glucose and can lead to functional impairment of type IV collagen. The data described so far on type IV collagen were generated with either domain NC1 (a proteolytically derived fragment) or peptide Hep I (a synthetic peptide) as substrates for nonenzymatic glucosylation. We recently developed a system in which intact type IV collagen can be used for in vitro nonenzymatic glucosylation in the presence of 0.5 M NaCl to avoid precipitation due to polymer formation during the incubation. Using this system, we confirmed the previous findings. We observed that type IV collagen incubated with 50 mM glucose for 3 days loses 55% of its ability to laterally associate, and if the incubation takes place in 500 mM glucose, the loss of the ability to polymerize is 86% (C.S. Haitoglou et al., unpublished observations). These data suggest that nonenzymatic glucosylation may interfere with the ability of type IV collagen to polymerize.

As mentioned earlier, laminin and type IV collagen are both considered very strong adhesive macromolecules. Consequently, glycation-mediated structural changes may lead to changes in their ability to interact with cells and eventually influence cellular phenotype. We used bovine aortic endothelial cells as a model system and examined their adhesion on plastic-coated, nonenzymatically glucosylated laminin and type IV collagen. For these experiments, it was necessary to precisely quantitate the effect of nonenzymatic glucosylation on the coating efficiency of laminin and type IV collagen. Indeed, we found that glycation produced a differential coating, and we adjusted the results of our experiments to the effective coated concentration in each case. The adhesion of metabolically labeled bovine aortic endothelial cells was measured on control and glycated laminin and type IV collagen. Nonenzymatic glycosylation resulted in a small but consistent decrease in the ability to adhere to both macromolecules. This decrease ranged from 10–40%, depending on the coating concentration of each macromolecule and the time interval used. This

phenomenon was more prominent in early time intervals (15–20 min), but persisted in longer time intervals (45–60 min) (C.S. Haitoglou et al., unpublished observations).

In people with diabetic retinopathy, increased proliferation of endothelial cells and disappearance of pericytes have been observed. One or both of these phenomena could be influenced at least to some extent by altered adhesion on nonenzymatically glycated basement membrane macromolecules. We used isolated retinal endothelial cells and pericytes and measured their proliferation on control and glycated laminin and type IV collagen coated on plastic, as described above. Retinal endothelial cells had an increase in their proliferation rate after 3 days on glycated versus control laminin or type IV collagen. Retinal pericytes had a decrease of proliferation after 36 h on glycated macromolecules. These data suggest that nonenzymatic glucosylation of basement membrane macromolecules may be one of the contributing factors in the pathogenesis of retinal microvascular complications of diabetes.

Our observations presented above support the hypothesis that nonenzymatic glucosylation alters structurally and functionally basement membrane macromolecules, and these changes may contribute to diabetic microangiopathy. However, we are far from understanding in depth the mechanism(s) by which basement membrane alterations occur. The model systems used so far have yielded important information; however, one should always keep in mind certain drawbacks. First, we used basement membrane macromolecules extracted from the matrix of the EHS tumor. There is growing evidence suggesting that specific basement membranes can contain various isoforms of either laminin (15–17) or type IV collagen (18) exhibiting structural variations. Second, the association studies with isolated macromolecules do not take into account the importance of the cellular environment, and its influence may be critical for the final assembly product. Third, nonenzymatic glucosylation of isolated macromolecules affects available amino groups of lysine residues. However, some of these residues may be present in critical sites for interactions and therefore not available for modifications in vivo. As a result, in basement membranes, the amino acid residues that undergo nonenzymatic glucosylation may differ from those of extracted macromolecules. This situation was encountered with hemoglobin, for which significant differences were observed between the in vitro and the *in vivo* glucosylation sites (19). For these reasons, in future studies our group will use intact isolated basement membranes and examine the structural and functional alterations due to nonenzymatic glycosylation.

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