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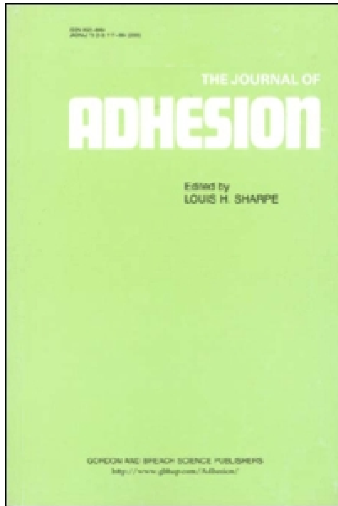

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Monocyte Attachment to Native and MGO-Treated Laminin. Differences Between Healthy Volunteers and Diabetic Patients

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Note

Monocyte Attachment to Native and MGO-Treated Laminin. Differences Between Healthy Volunteers and Diabetic Patients

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Monocytes and oxidative stress are central in atherosclerosis. Monocyte attachment to laminin-1 derived from either healthy volunteers or patients with diabetes mellitus type II has been evaluated by three different assays: the myeloperoxidase assay, Hemacolor kit, and crystal violet staining. Monocytes derived from diabetic patients showed an increased ability to attach to native laminin-1 as compared with controls. In addition, monocytes derived from both healthy donors and diabetics attached at a higher degree to laminin-1 carbonylated by methylglyoxal in comparison with the native molecule. The results indicate an increased interaction between monocytes and laminin-1 in the case that the protein is carbonylated.

Keywords: Attachment; Diabetes mellitus; Laminin-1; Methylglyoxal (MGO); Monocytes

1. INTRODUCTION

The interactions between monocytes and the basement membranes (BM) have been implicated in atherosclerosis pathophysiology. Reaction of free radicals with the side chains of lysine, arginine, proline, and threonine

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residues of proteins leads to the formation of carbonyl derivatives [1]. Moreover, reaction of reactive carbonyls (RCS), such as methyglyoxal (MGO), with the nucleophilic sites of proteins as well as the oxidative cleavage of proteins also leads to the formation of carbonyl derivatives [2]. Carbonylation is a key factor in the development and progression of many human diseases, such as diabetes and atherosclerosis [2]. The levels of methylglyoxal have been reported to be elevated in serum and certain tissues of diabetic patients and in experimental animal models of diabetes [3,4], while they induce extensive protein modifications under the above pathological conditions [5,6]. MGO levels were also found to be elevated in subjects that followed the Atkins' diet, as a result of ketosis, as well as in patients that suffered from Alzheimer's disease [7,8]. MGO also serves as an important precursor for advanced glycation end products (AGEs) formation, while it mediates extensive tissue damage [7,8].

Laminins are structural and functional components of the BM that play a key role in many biological functions such as cell adhesion [9]. Previous studies performed in our laboratory showed that laminin phosphorylation and oxidation strongly influence its biological properties, such as monocyte attachment, which is associated with the initiation of atherosclerosis [10,11]. Moreover, it has been reported that diabetic monocytes carbonylate and attach to oxidized laminin at a higher degree, as compared with controls [11]. Accordingly, laminin carbonylation by RCS may also change some of the functional properties of the protein, thus, affecting its interactions with monocytes. Monocytes have a key role in the early phase of atherosclerosis, which is a frequent complication of diabetes mellitus [12,13]. Previous studies performed in our laboratory indicated a possible link between monocytes from diabetic patients and the initiation of atherosclerosis [11,14].

Monocyte attachment to several substrates has been studied through the estimation of myeloperoxidase (MPO) [15,16] and crystal violet staining [17,18] in previous studies. To our knowledge monocyte attachment through the use of a Hemacolor kit has never been studied.

In an attempt to investigate the biological significance of laminin carbonylation to the initiation of atherosclerosis, monocyte attachment to MGO-treated laminin-1 was studied using monocytes derived from both healthy donors and patients with diabetes mellitus type II by the use of three different approaches.

2. MATERIALS AND METHODS

2.1. Materials

Laminin-1 was isolated from EHS (Engelbreth-Holm-Swarm) tumor. Iscove's Modified Dulbecco's Medium (IMDM), fetal calf serum

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(FCS), and Percoll were purchased from Biochrom (Cambridge, UK). O-dianisidinedihydrochloride tablets, hexadecyltrimethylammonium bromide, crystal violet, and methylglyoxal solution (MGO) were from Sigma (St. Louis, MO, USA). Hydrogen peroxide (30%) and Hemacolor staining kit were purchased from Merk (Darmstadt, Germany). Acetic acid was from Applichem (Darmstadt, Germany).

2.2. Participating Subjects

Monocytes were prepared from blood samples taken either from six patients with type II diabetes mellitus or from six age-matched healthy volunteers (Table 1). All participants were informed about the aims of the study. All the blood samples were tested for each parameter/experiment. For each sample ten separate measurements were performed. The reported investigations have been carried out in accordance with the principles of the Declaration of Helsinki as revised in 2000.

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2.3. Monocyte Preparation

Monocytes were isolated from blood samples as previously described [19]. A total of 1–2 million cells/ml were isolated each time with a purity >85%. Flow cytometry using a CD14 monoclonal antibody labelled with phycoerythrin and a CD36 monoclonal antibody labelled with fluorescein isothiocyanate (FITC) revealed that more than 98% of the cells were monocytes (Beckman Coulter Epics, Inc., Fullerton, CA, USA). The $CD14^+$ $CD36^+$ population was homogenous indicating that all monocytes were in a similar stage of differentiation.

2.4. Laminin Preparation

Laminin-1 was carbonylated by methylglyoxal (MGO) as described elsewhere [20]. In brief, laminin was placed on 96 well plates in phosphate buffered saline (PBS) at room temperature overnight. Plates were washed once with PBS and incubated in the same buffer with

TABLE 1 Clinical Parameters of Healthy and Diabetic Participants

	Age (years)	BMI	Glucose levels (mg/dl)	Glycated haemoglobin
Healthy volunteers	67.3 ± 4	<25	$91.3 \pm 4^*$	$4.1 \pm 0.4\%^*$
Diabetic patients	66.9 ± 5	<25	168.2 ± 9	$8.52 \pm 0.8\%$

*Indicates statistically significant difference from the respective diabetic value.

and without 2 mM MGO solution at 37°C, overnight and in the dark. Plates were then washed twice with PBS.

2.5. Monocyte Adhesion Assays

Monocyte attachment was measured on microwells. 80,000 cells in IMDM (10% FCS) were placed in each well of polystyrene plates that had been precoated with 37.5 μg/ml carbonylated or non-carbonylated laminin-1. The cells were incubated in the wells for 30 min at 37°C and, after incubation, non-adherent cells were discarded by aspiration and the wells were rinsed with sterile PBS (pH 6.0). Aspiration was done carefully and was mild so that no considerable shear stress was expected. It should be also mentioned that the above method has been extensively used in our and other laboratories. Monocyte binding was quantified using three different methods: the myeloperoxidase (MPO) assay; the Hemacolor kit, which stains DNA, and the crystal violet, which stains proteins.

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2.5.1. Myeloperoxidase Assay

The Myeloperoxidase assay was used as previously described [15]. In brief, after monocyte attachment to laminin the cells were lysed using 0.5% (w/v) hexadecyltrimethylammonium bromide in PBS (pH 6.0) for 30 min at 37°C. After lysis, 50 μL 0.2 mg/mL dianisidinedihydrochloride in PBS (pH 6.0) containing 0.4 mM H_2O_2 were added to each well. After 15 min, the MPO activity of the lysate was measured spectrophotometrically at 405 nm, using an ELISA reader (Stat-Fax-2100-Awareness Technology Inc., Palm City, FL, USA).

2.5.2. Hemacolor Kit

After monocytes attached to laminin they were fixed and stained with the Hemacolor staining kit and left to dry for 10 min at RT. 200 μL 10% acetic acid were then added to each well in order to remove the bound dye and after gentle shaking for 3–4 min, the optical density was measured at 590 nm in an ELISA reader.

2.5.3. Crystal Violet Staining

After monocyte attachment to laminin, 50 μL absolute ethanol were added to each well in order to fix cells. Ethanol was then discarded by aspiration and 200 μL 0.1% crystal violet solution were added to each well for 30 min at RT. After washing twice with water, 100 μL 10% Triton-X solution (1% ethanol) were added to each well. After lysis, the protein content of the lysate was measured spectrophotometrically at 540 nm, using an ELISA reader.

2.6. Statistical Analysis

For the statistical evaluation, the statistical software GraphPad InStat version 3.00 for Windows was used (GraphPad Software Inc., San Diego, CA, USA). Values are expressed as means ± standard errors of means (SEMs). The statistical significance of the differences between the sets of data was estimated by the Student's t-test (paired or un-paired).

3. RESULTS

3.1. Monocyte Attachment to Carbonylated and Non-Carbonylated Laminin-1

Monocyte attachment by the use of three different methods, MPO assay, crystal violet assay, and Hemacolor kit was estimated. Our results showed that monocytes derived from diabetic patients attach to native laminin at a higher degree as compared with those from healthy donors, in the case that the MPO assay ($p < 0.0001$), the Hemacolor kit ($p < 0.0001$) or the crystal violet assay ($p = 0.032$) was used. In the case of the MPO assay, diabetic monocytes also showed an increased ability to attach to laminin carbonylated by MGO as compared with the non-carbonylated molecule ($p < 0.0001$). Increased attachment to MGO-treated laminin as compared with the native molecule was not observed for diabetic monocytes when either Hemacolor kit or crystal violet staining was used. Moreover, the results revealed from either MPO assay or Hemacolor kit showed that control monocytes attached to MGO-treated laminin at a higher degree as compared with the native molecule ($p < 0.0001$ for MPO assay, $p = 0.0293$ for Hemacolor kit). The above results could not be attributed to differences in the rinsing procedures followed, as the rinsing of the cells after aspiration was performed similarly in each assay used. Moreover, aspiration was done carefully and mildly and no considerable shear stress was expected.

On the contrary, in the case that crystal violet assay was used, initially, control monocytes seemed to attach at a lower degree to MGO-treated laminin as compared with the native molecule ($p = 0.0239$). The above results were reversed in the case that the optical density (OD) of the background of the assay was subtracted from that of the respective samples (absorbance of native and carbonylated laminin samples alone: native laminin $A_{540\,nm} = 0.38 \pm 0.0048$, carbonylated laminin $A_{540\,nm} = 0.326 \pm 0.035$, $p = 0.0071$). It should be noted that the background was also measured when either MPO assay or Hemacolor kit was used without indicating any significant differences

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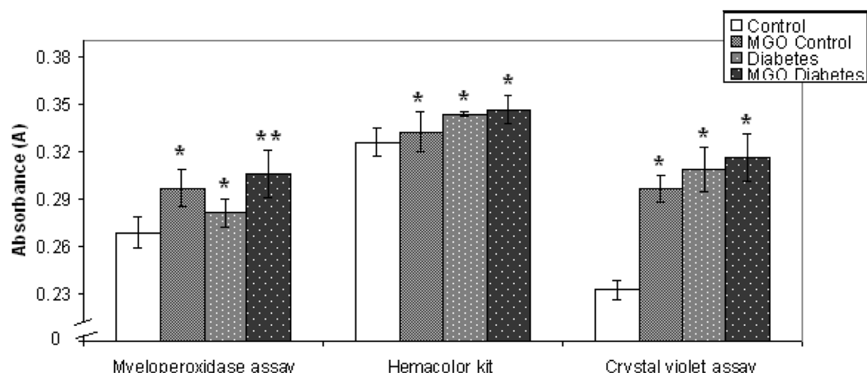

FIGURE 1 Monocyte attachment to either native or MGO-treated laminin-1. Monocytes were isolated from either healthy donors or patients with diabetes mellitus type II. Bars represent mean value ± standard errors of means of at least six independent experiments. * indicates significant difference with the respective control value: ** indicates significant difference with both the control and the diabetic value.

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in absorbance values between native and carbonylated laminin samples alone (data not shown). Moreover, the data presented here resulted from background subtraction. Consequently, after the subtraction of background our results were in accordance with those from Hemacolor and the myeloperoxidase assay. More precisely, control monocytes attached to MGO-treated laminin at a higher degree as compared with the native molecule ($p = 0.0081$) (Fig. 1).

4. DISCUSSION

In the present study, monocyte attachment to laminin-1 after its modification by the reactive carbonyl compound MGO in normal and diabetic monocytes was estimated. Previous studies performed in our laboratory showed that monocytes derived from diabetic patients carbonylate laminin-1 at a higher degree as compared with those from healthy volunteers, while laminin oxidation by specific oxidants affects monocyte interactions, such as attachment to the protein [11]. Metal-catalyzed oxidation of another BM protein, collagen IV, was also reported to affect monocyte attachment [14]. The oxidative burst of monocytes was previously measured in our laboratory using dihydroethidium, that detects superoxide anions and dihydrorhodamine 123, that detects hydrogen peroxide. Our results indicated that diabetic monocytes produce increased levels of both superoxide anions and hydrogen peroxide in relation to controls [11]. Furthermore, the role

of reactive carbonyl species in podocyte, mesangial, and endothelial cell interactions with extracellular matrix (ECM) proteins was previously studied [20]. However, to our knowledge, this is the first time that laminin-1 specific carbonylation by MGO is associated with monocyte attachment and atherosclerosis.

This work is a part of a general study conducted in our laboratory that was focused on the investigation of monocyte attachment to BM proteins after oxidative modification and their potential role in the initiation and pathogenesis of atherosclerosis. The main candidate for this study is laminin-1, the prototype laminin, which is widely used for the study of interactions between blood cells and laminins [10,11] and is easily isolated from the Engelbreth-Holm-Swarm (EHS) tumour.

The data of the present study indicate a modified interaction between monocytes and BM in diabetes mellitus in the cases that laminin is carbonylated. More precisely, our results showed that monocytes derived from diabetic patients attach to laminin-1 at a higher degree as compared with those from healthy volunteers, while control monocytes attach to carbonylated laminin at a higher degree in relation to the native molecule. Previous studies performed in our laboratory also showed an increased attachment of diabetic monocytes to laminin [11]. In that case, monocytes were labelled with 35S-methionine in order to estimate monocyte attachment, while in the present study three other assays were used and confirmed the previously reported results. Moreover, when the MPO assay was used, diabetic monocytes showed an increased ability to attach to carbonylated laminin as compared with the native molecule, while similar results have not been observed when either Hemacolor kit or crystal violet staining used. The slight differences observed between the three assays used could be attributed to their different features. Crystal violet stains protein and is less specific and sensitive as compared with the other two methods, while the Hemacolor kit measures DNA and the MPO assay estimates monocyte attachment through measurement of a specific monocyte enzyme, myeloperoxidase. Considering the fact that the purity of our samples was $>85\%$, the most accurate and reliable method for the estimation of monocyte attachment to laminin is the myeloperoxidase assay, as the Hemacolor kit could stain, apart from monocytes, any other type of cells and crystal violet could stain not only cells but also laminin.

The differences observed in monocyte attachment between the native and carbonylated laminin could be attributed to differences in integrin receptors. Previous studies performed in our laboratory indicated that the $\alpha 2$ integrin subunit plays a role in the increased attachment of diabetic monocytes to either laminin-1 or collagen IV as

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compared with healthy volunteers, as well as in the increased attachment of control monocytes to laminin or collagen oxidized by 2,2′ methylpropionamidine dihydrochloride (ABAP) or metals as compared with the native molecule [11,14]. Previous studies performed in our laboratory showed through a sensitive ELISA assay that both ABAP and metals carbonylate laminin (data not shown). It should also be mentioned that the metals used for the oxidation of either laminin or collagen have the ability to carbonylate also other substrates, such as bovine serum albumin [11]. Accordingly, and considering the fact that carbonylation is a type of oxidation, we may assume that the $\alpha 2$ integrin subunit plays a role in the increased monocyte attachment to laminin-1 carbonylated by MGO. It could also be conjectured that the insertion of carbonyl groups into laminin, which leads to the addition of negatively charged residues to the protein, may influence the conformation of interacting cell surface proteins, such as integrins, leading to modified interactions with the cells. Carbonylation by reactive carbonyl compounds could also release some new integrin binding sites on the surface of laminin which were cryptic (*i.e.*, hidden) under non-oxidative conditions. The involvement of other cell surface molecules on the described phenomenon cannot be excluded.

Furthermore, the modified interactions between monocytes and substrata in the case that proteins are carbonylated could be related to increased surface energy and its consequences. Previous studies showed that when carbonyl sites from model proteins were either exposed or masked, differences in surface-hydrogen-bonding were observed [21]. Adhesion of the cells can also be a direct function of the solid surface energy. The introduction of amide groups into a polymer surface was found to increase the free surface energy [21]. Accordingly, the introduction of carbonyl groups into laminin could also increase free surface energy, thus promoting monocyte attachment. Moreover, several substituents, such as phenyl, -OH, -COOH, -SH, or $-NH_2$ were reported to promote adhesiveness through increase of surface tension [22]. Accordingly, it could be assumed that carbonyl groups also enhance laminin's surface tension, leading to increased adhesion with cells. Based on the above, the addition of negatively charged residues to laminin through carbonylation may influence cell surface proteins by increasing laminin surface tension and solid surface energy, thus changing integrin affinity for the protein. However, this phenomenon seems to be specific for laminin or other cell adhesion molecules since the carbonylation of serum albumin does not influence cell attachment on this molecule [11].

Our data are in accordance with previous studies where laminin-1 and collagen IV were oxidized by either 2,2′methylpropionamidine-dihydrochloride (ABAP) or metals and, subsequently, monocyte

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attachment was estimated [11,14]. On the other hand, our results are in contrast to a previous study where ECM proteins were treated by reactive carbonyls and, subsequently, podocyte, mesangial cell, and endothelial cell attachment was estimated [20]. According to their results, MGO seemed to inhibit cell adhesion *via* modification of critical arginine residues in ECM proteins [20]. The differences between Pedchenko *et al.* [20] and our study may be attributed to the different types of cells used. These types of cells (human umbilical vein endothelial cells, mesangial cells, and podocytes) possess a different panel of integrin receptors in relation to monocytes and, consequently, may have a different attachment capacity to carbonylated laminin. More precisely, monocytes possess some specific types of integrins which are present only on leukocytes; they are known as the "leukocyte integrins" or "$\beta 2$ integrins" and are involved in adhesion-dependent processes (Arnaout, [23]). $\beta 2$ integrins were reported to mediate MCP-1-stimulated monocyte attachment to laminin [17]. Furthermore, previous studies performed in our laboratory showed that the alphaL and beta2 integrin subunits mediate the increased attachment of diabetic monocytes to laminin as compared with controls, as well as the increased attachment of control monocytes to oxidized laminin as compared with the native molecule (unpublished data). Consequently, the exclusive presence of the above integrin subunits on monocytes could explain the differences observed between the present study and Pedchenko's. On the other hand, in our study three different methods were used, while Pedchenko *et al.* used only the crystal violet staining, which is the least specific and the subtraction of the background from the samples is required.

The data of the present study indicate that in diabetes mellitus, where the levels of reactive carbonyl species are elevated, laminins may be carbonylated and this carbonylation may induce monocyte attachment to the protein. The increased monocyte attachment to BM proteins could be associated with increased monocyte accumulation in the sub-endothelial space and initiation of atherosclerosis. The scope of the present paper was to study monocyte cell attachment to carbonylated laminin, a phenomenon that is related to the early stages of atherosclerosis. However, the study of monocytes in various stages of atherosclerosis might be the scope of future studies.

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