

# Glycation of Matrix Proteins in the Artery Inhibits Migration of Smooth Muscle Cells from the Media to the Intima

(glycation / advanced glycation end products / collagen / elastin / myocytes / atherosclerosis)

A. KUZAN<sup>1</sup>, O. MICHEL<sup>1</sup>, A. GAMIAN<sup>1,2</sup>

<sup>1</sup>Department of Medical Biochemistry, Wrocław Medical University, Wrocław, Poland

<sup>2</sup>Laboratory of Medical Microbiology, Ludwik Hirsztfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland

**Abstract.** Formation and growth of atherosclerotic plaques have serious clinical consequences. One mechanism that occurs during atherogenesis is migration of smooth muscle cells from the middle layer of the artery to the intima, where they proliferate and are transformed into foam cells. This degenerative process is accompanied by glycation, by which proteins are modified and change the biomechanical and biochemical properties. The aim of the study was to determine whether glycation of collagen and elastin building the walls of blood vessels alters the adhesion and rate of myocyte migration. *In vitro* experiments included migration assays and immunocytochemical staining with anti  $\alpha$ -actin,  $\beta$ -catenin anti-collagen type IV antibodies. It turns out that there is a tendency to decrease the number of cells that had migrated through the barrier consisting of glycated proteins as compared to the control. Adversely, the morphology of the cells cultured in the presence of glycated substrates is changed. The lower intensity of  $\beta$ -catenin staining indicates lower adhesiveness of such cells. It is proposed that glycation inhibits migration of smooth muscle cells from the media to the intima, which represents part of the anti-atherogenic mechanism.

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Corresponding author: Aleksandra Kuzan, Department of Medical Biochemistry, Wrocław Medical University, Chałubińskiego 10, Wrocław 50-368, Poland. Phone: (+48 71) 784 13 87; e-mail: [aleksandra.kuzan@umed.wroc.pl](mailto:aleksandra.kuzan@umed.wroc.pl)

Abbreviations: AGE – advanced glycation end products, ECM – extracellular matrix, EGF – epidermal growth factor, FGFb – basic fibroblast growth factor, G6P – glucose-6-phosphate, MGO – methylglyoxal, MMP – matrix metalloproteinases, MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, PBS – phosphate-buffered saline, PDGF – platelet-derived growth factor, RGD – tripeptide Arg-Gly-Asp, SMC – smooth muscle cells.

## Introduction

Smooth muscle cells (SMC) are predominantly located in the middle layer of the artery, where they play a crucial role: they determine the contractility of the tissue, hereby adjusting the blood flow through the artery. In such a case, they are called contractile smooth muscle cells, which are characterized by the richness in actin filaments. They may, however, undergo phenotype switching and migration and become cells that reside in the intima. Then, they are called synthetic type SMC, are rich in rough endoplasmic reticulum and characterized by active metabolism (Stary et al., 1992, 1994; Tukaj, 2010). Such a change in the phenotype is associated with a number of impacts: intensive proliferation of these cells occurs in the intima, secretion of inflammatory mediators and apoptosis rate are increased, matrix protein synthesis is disturbed (Tukaj, 2010); all this increases atherogenesis. Smooth muscle cells, similarly to macrophages, after the uptake of large amounts of lipids become foam cells. After their death, the lipid content is poured into the intercellular space to form a fatty core (Stary et al., 1992, 1994).

Glycation is a non-enzymatic process, spontaneously occurring in the body or outside of a living organism, which involves reaction of reducing sugars (glucose, fructose, glucose-6-phosphate (G6P), and other) with the amino groups of proteins. Due to the fact that only a small part of the sugars is present in the form of a chain having a free aldehyde group under physiological conditions, the process occurs very slowly, over many weeks or months (Slatter et al., 2008). Therefore, the problem of malfunction of the glycated protein mainly concerns long half-life proteins, such as collagen and other extracellular matrix proteins (Xiao et al., 2007).

It is noteworthy that among modifications undergone by Amadori products arising from glycation is also condensation. The reaction of these compounds with amino acids of other proteins leads to the formation of crosslinks between proteins and induces formation of large protein complexes. The result of this phenomenon in the case of fibrous proteins is increased thickness of

the fibrils, and consequently, increased stiffness of the fibre, reduced solubility and lower susceptibility to enzymatic digestion (Avery and Bailey, 2008). Furthermore, modifications of the side chains of amino acids building scleroproteins entail not only structural changes, but also induce alterations in the location of electrostatic charges in the molecules, which in turn can affect interactions with other proteins (Jabłońska-Trypuć and Czerpak, 2007). As a result, this contributes to the limitation of binding with specific integrins, which considerably weakens the interaction of collagen with the cells. This is particularly important, inter alia, in the development of vascular damage or in wound healing in diabetes (Avery and Bailey, 2006).

In collagen glycation, attention should also be paid to the fact that different types of collagen may vary in susceptibility to this non-enzymatic process. As previously mentioned, the process concerns only long half-life proteins. The half-life, however, is not equal for different types of collagen. The turnover of type II collagen present in the cartilage is approx. 100 years, whereas for type I collagen occurring in the skin reaches approx. 10 years, for collagen building bone it amounts only to 1–2 years, and the turnover of collagen from periodontal ligaments takes only one day (Avery and Bailey, 2006).

It is undoubtable that smooth muscle cells play a significant role in the pathogenesis of atherosclerosis (Tukaj, 2010). Numerous studies in the last decade focused on this aspect of atherogenesis (Butoi et al., 2014; Chiong et al., 2014; Wall and Bornfeldt, 2014; Chelopenko, 2015; Monk and George, 2015, and many others). Nevertheless, only few studies are devoted to the issue of migration of smooth muscle cells in the context of the influence of advanced glycation end products on this process. It is generally postulated that advanced glycation end products (AGE) stimulate migration of SMC within atherosclerotic plaques (Dan et al., 2004; Chung et al., 2013).

Research in this stream focuses predominantly on the investigation of the mechanism leading to such effect and relates mainly to low molecular weight of AGE or small glycated proteins (mostly on glycated bovine serum albumin). Meanwhile, the question of the influence of glycation of long, fibrous, long half-life proteins from extracellular matrix on the migration of SMC appears to be still unexplored. From the studies concerning the glycation of these proteins it follows that these structures are stiffer than the protein unmodified by glycation. Presumptively, in this way they will provide a more inaccessible barrier for SMC, thereby slowing their migration. This would constitute an anti-atherogenic mechanism, in contrast to many other mechanisms stimulating growth of the atherosclerotic plaque, which is attributed to AGE.

## Material and Methods

Smooth muscle cells derived from arteries were Primary Aortic Smooth Muscle Cells, Normal, Human

(ATCC, Dziekanów Leśny, Poland). The cell culture was maintained in a dedicated medium augmented with special supplements contained in the Vascular Smooth Muscle Growth Cell Kit (ATCC).

### *Glycation of the protein-sugar mixtures*

Mixtures of proteins, collagen and elastin were subjected to the glycation process with simultaneous microwave stimulation. The protein-sugar compounds (glucose, glucose-6-phosphate (G6P) and proteins – collagen and/or elastin in specified concentration) were exposed to microwave irradiation at 640W, in cycles: heating (30 s microwave action) and cooling (1 min at  $-20^{\circ}\text{C}$ ). Cumulative length of the microwave action was 10 and 30 min.

The protein mixture included collagen type I (Sircol Bovine Collagen Reference Standard, acid soluble type I, Biocolor, Carrickfergus, UK), type II collagen (collagen from chicken sternal cartilage, type II, Sigma-Aldrich, Poznan, Poland), type IV collagen (collagen from human placenta, Bornstein and Traub Type IV, Sigma) and elastin (elastin from bovine neck ligament, Sigma). An equal volume of glucose (Sigma) or G6P sodium salt (Sigma) in concentration 400 mM was added to the obtained mixture. Subsequently, the mixture was partitioned into three parts, the first of which was irradiated for 10 min, the other for 30 min, and the third was left as a control.

The amount of advanced glycation products was measured using specific spectrophotometric and fluorescent properties: absorbance at 440 nm and fluorescence at an excitation wavelength of 370 nm and emission 400–480 nm. Exemplary fluorescence spectra can be found in Fig. 1. It may be concluded that glycation occurs with much higher efficiency with the involvement of glucose-6-phosphate than in the presence of glucose.

### *MTT assay – cytotoxicity test of the collagen/collagen-elastin mixtures*

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed in order to exclude the influence of the mixture components on the viability of smooth muscle cells derived from an artery. Cells were grown in a 96-well plate (5000 cells/well in 200  $\mu\text{l}$ ). After 24 h post seeding, the medium was replaced with a medium containing glucose, G6P, collagen, or their mixtures. After an additional 24 and 48 h, the culture medium was removed and 100  $\mu\text{l}$  of MTT solution at a concentration of 0.5 mg/ml was added to each well. After 2 h of incubation, 100  $\mu\text{l}$  of isopropanol acidified with HCl was added to each well in order to dissolve formazan crystals. The results were read with the use of an EnSpire reader (Perkin Elmer, Waltham, MA) at 550/630 nm.

### *The migration test using ThinCert™ membranes*

The test was carried out using ThinCert™ inserts (Greiner Bio-One, Krensmünster, Austria) with an inte-

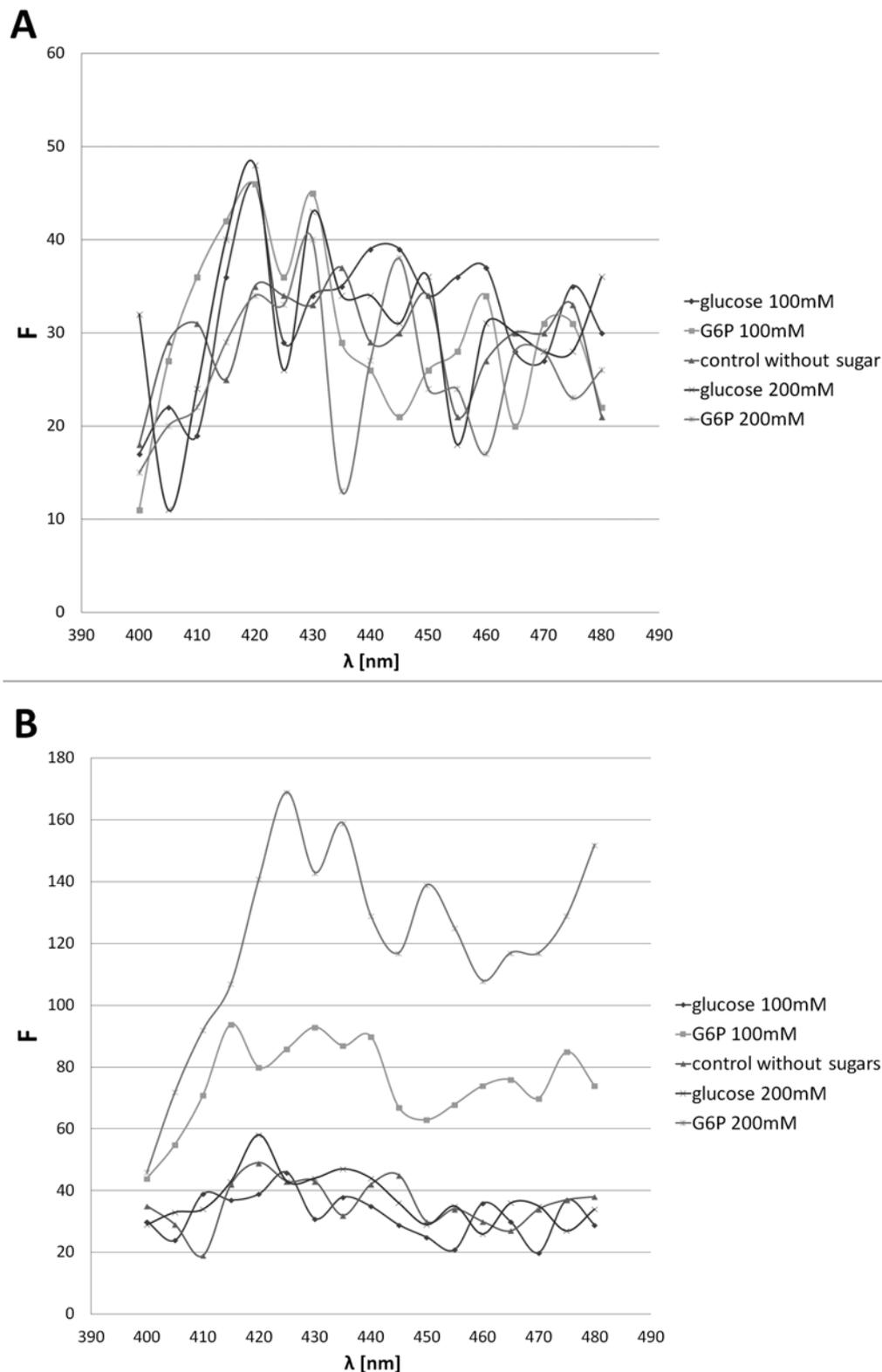


Fig. 1. AGE content in a solution of collagen (1 mg/ml) as an increase in fluorescence in the presence of glucose-6-phosphate and glucose, before (A) and after (B) exposure to microwaves

rior surface of  $0.336 \text{ cm}^2$ ,  $8 \mu\text{m}$  pore size and pore density of  $0.15 \times 10^6/\text{cm}^2$ . Inserts were rehydrated by immersion in sterile phosphate-buffered saline (PBS) for 2 h in a  $\text{CO}_2$  incubator. Forty  $\mu\text{l}$  of pre-glycated protein mixture was applied on the insert surface and subsequently dried. Next, the inserts were transferred to the

wells of a 24-well tissue plate containing full culture medium for vascular smooth muscle cells (Vascular Cell Basal Medium, ATCC + Vascular Smooth Muscle Cell Growth Kit, ATCC). Ten thousand cells/well were seeded on the surface in a basal medium for vascular smooth muscle cell culture (Vascular Cell Basal Medium,

ATCC) without serum, epidermal growth factor (EGF), and basic fibroblast growth factor (FGFb) in order to provide conditions for chemotaxis. The plate was maintained in standard conditions (37 °C, 5.5% CO<sub>2</sub>). After 18 h, non-migrating cells were removed from the surface by means of sticks wrapped with cotton, the insert was transferred to a solution of DAPI for 1 h, and afterwards translated into PBS. The nuclei of the migrating cells were counted using a fluorescence microscope Olympus BX53 with fluorescence attachment X-Cite® series 120PC Q, at 10× magnification. Five representative fields of view were analysed and the results were transposed to the total surface of the insert.

### Immunocytochemical staining

β-Catenin staining was performed to assess the adhesive abilities of the examined cells, α-actin was stained in order to visualize the protein marker for smooth muscle cells, and collagen type IV was stained for detailed morphological assessment. Ten-well microscope slides (Thermo Scientific Diagnostic Slides, Thermo Scientific, Waltham, MA) were coated with protein-sugar mixtures, previously glycosylated or non-glycosylated. The mixture was dried, and arterial smooth muscle cells were seeded on the surface (4000 cells/well of 0.34 cm<sup>2</sup> area). After 24 h of culture under standard conditions, cells were washed in PBS, fixed with paraformaldehyde (4%, 20 min), and rinsed again. Then the slides were incubated with proteinase K (Dako, Santa Clara, CA) for 10 min at 37 °C to expose the antigen. After that, Real Peroxidase-Blocking Solution (Dako) for 10 min and Protein Block (Dako) for 15 min were applied. Solutions of rabbit polyclonal anti-collagen type IV antibodies (Sigma), anti α-actin (Abcam, Cambridge, UK) and β-catenin (Abcam) were then spotted. Incubation was performed overnight at 4 °C in humid glass chambers. Then the procedure was performed using the DAKO LSAB kit + System-HRP, successively with Biotinylated Link Universal (Dako), Streptavidin-HRP (Dako), DAB + substrate buffer with DAB + chromogen, and following

the general method guidelines. Delafield's haematoxylin was used as the counterstain. The slides were then immersed and closed with a glass coverslip using DPX (Aqua Medica, Łódź, Poland).

## Results

### *Analysis of the impact of substrate glycation on the proliferation and cell survival*

To exclude the potential effect of glycation of the substrates on the cell viability and proliferation, a number of MTT assays were performed after 24 and 48 h of incubation in the presence of collagen/ glucose/ glucose-6-phosphate blends of a/m compounds. No cytotoxicity was shown with regard to the smooth muscle cells; some of the components, however, accelerated proliferation, in particular G6P at 100 mM (500 % of control), glucose at 25 mM (200 % of control), and collagen at 200 µg/ml (350 % of control) and 50 µg/ml (250 % of control). To avoid bias associated with extended proliferation, all migration tests were performed with the use of equal sugar concentrations (100 and 25 mM) and equal protein content (0.125 mg/ml or 0.03 mg/ml; for migration tests converted to µg/cm<sup>2</sup>).

### *The results of migration tests*

A series of migration tests were performed to investigate the influence of glycation on the rate of smooth muscle cell migration through the protein mixture. Twelve distinct mixtures were tested, as shown in Table 1.

It is noteworthy that mixtures 10, 11 and 12 represent the most precise reproduction of the actual composition of extracellular matrix (ECM) present in the human artery (according to data obtained in a preliminary study on arterial specimens collected *post mortem* (Kuzan, unpublished data).

Each of the mixtures was divided into three parts – the first non-irradiated, the second irradiated for 10 min, the third irradiated for 30 min. The cells that migrated

Table 1. Composition of the mixtures coating inserts in the migration tests

Sample	Collagen type I	Collagen type II	Collagen type IV	Elastin
1	50% (1.84 µg/cm <sup>2</sup> )			50% (1.84 µg/cm <sup>2</sup> )
2	75% (2.94 µg/cm <sup>2</sup> )			25% (0.92 µg/cm <sup>2</sup> )
3	100% (3.68 µg/cm <sup>2</sup> )			
4				100% (3.68 µg/cm <sup>2</sup> )
5	50% (1.84 µg/cm <sup>2</sup> )	50% (1.84 µg/cm <sup>2</sup> )		
6	75% (2.94 µg/cm <sup>2</sup> )	25% (0.92 µg/cm <sup>2</sup> )		
7	50% (1.84 µg/cm <sup>2</sup> )		50% (1.84 µg/cm <sup>2</sup> )	
8	75% (2.94 µg/cm <sup>2</sup> )		25% (0.92 µg/cm <sup>2</sup> )	
9			100% (3.68 µg/cm <sup>2</sup> )	
10	45% (6.59 µg/cm <sup>2</sup> )	1% (0.11 µg/cm <sup>2</sup> )	9% (1.29 µg/cm <sup>2</sup> )	45% (6.59 µg/cm <sup>2</sup> )
11	65% (9.53 µg/cm <sup>2</sup> )		10% (1.47 µg/cm <sup>2</sup> )	25% (3.65 µg/cm <sup>2</sup> )
12	25% (3.65 µg/cm <sup>2</sup> )		10% (1.47 µg/cm <sup>2</sup> )	65% (9.53 µg/cm <sup>2</sup> )

Table 2. The results of migration tests – the migration rate of 10,000 smooth muscle cells migrating through the membrane (surface 0.336 cm<sup>2</sup>) coated with glycated ECM protein mixtures

Time of exposure (min)	0	10	30	0	10	30	0	10	30
<b>Mixture No.</b>	<b>1</b>			<b>2</b>			<b>3</b>		
Mean	1219.2	1046.4	1084.8	883.2	739.2	316.8	979.2	960.0	499.2
Deviation	339.4	13.6	556.6	162.9	40.7	13.6	135.8	108.6	190.1
P rANOVA	0.73			0.16			0.27		
P Friedman	1.000			0.071			0.250		
<b>Mixture No.</b>	<b>4</b>			<b>5</b>			<b>6</b>		
Mean	1296.0	595.2	307.2	556.8	240.0	326.4	1056.0	1238.4	412.8
Deviation	203.6	298.7	81.5	190.1	95.0	108.6	271.5	312.3	149.3
P rANOVA	0.21			0.36			0.19		
P Friedman	0.14			0.25			0.25		
<b>Mixture No.</b>	<b>7</b>			<b>8</b>			<b>9</b>		
Mean	1526.4	1344.0	441.6	1113.6	960.0	710.4	844.8	816.0	393.6
Deviation	692.4	787.4	162.9	0	27.2	108.6	298.7	583.8	40.7
P rANOVA	0.25			0.12			0.37		
P Friedman	0.14			0.14			0.25		
<b>Mixture No.</b>	<b>10</b>			<b>11</b>			<b>12</b>		
Mean	729.6	643.2	691.2	864.0	777.6	412.8	480.0	508.8	384.0
Deviation	108.6	13.6	108.6	570.2	176.5	95.0	27.2	95.0	81.5
P rANOVA	0.57			0.71			0.49		
P Friedman	0.50			0.25			0.50		

through the membranes coated with the mixtures were counted and the results are presented in Table 2 and in the form of graphs for the two exemplary mixtures in Fig. 2.

Analysis of the data obtained in the assay reveals that cells migrating through a glycated-protein barrier are likely to present lower migration potential in comparison to a barrier consisting of non-glycated proteins. Furthermore, there is a relationship between migration and time of exposure – the longer the exposure, the less cells migrated through the membranes coated with pro-

teins. Statistical analysis (Table 2) was carried out by two methods; however, too large standard deviations did not allow for undeniable demonstration of statistically significant correlation. Closest to achieve statistical significance is the result following the application of a mixture consisting of 75 % type I collagen and 25 % elastin.

#### Immunocytochemical analysis

$\alpha$ -Actin,  $\beta$ -catenin, and collagen type IV immunocytochemical staining was performed simultaneously with ThinCert migration tests for exhaustive migration anal-

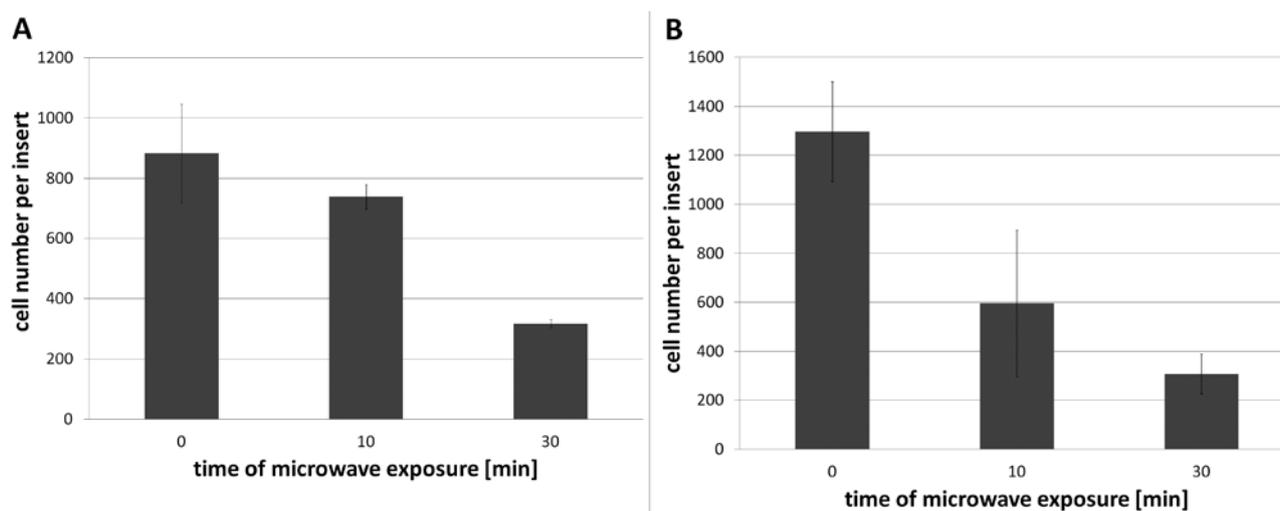


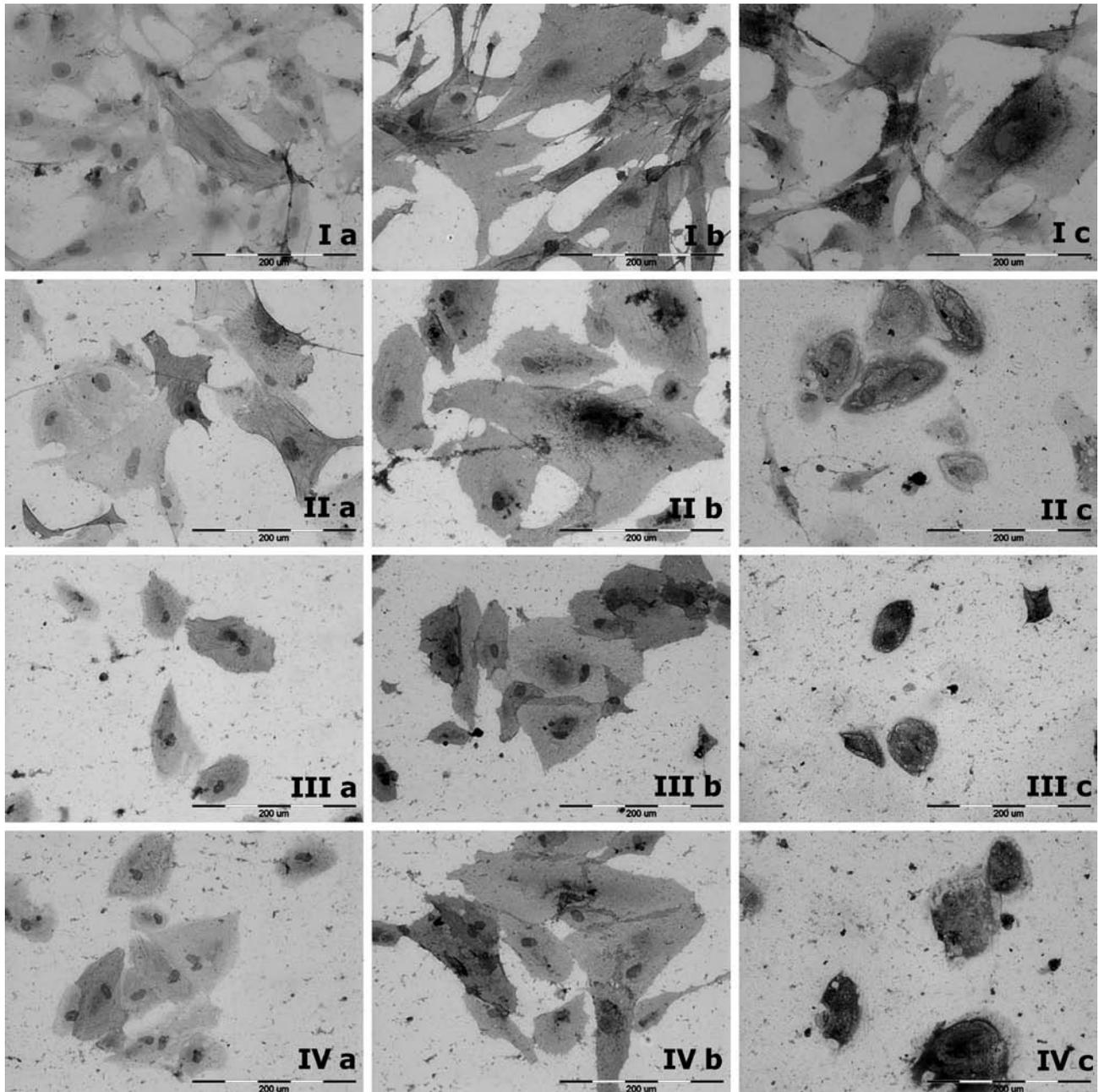
Fig. 2. The results of migration tests; the mixture coating the insert was represented by number 2 (panel A) and 4 (panel B).

ysis. The results of experiments with protein mixtures most congruous to the actual composition of the ECM are shown in figure and summarized table (Fig. 3, Table 3).

Interestingly, only part of the SMC from an artery stained for the presence of  $\alpha$ -actin. In certain cases, visible protein filaments can be seen in a form of stress fibres. It may be assumed that only this part of the cell represents the contractile phenotype. The rest of the cell arguably transformed into synthesizing phenotype due to the non-physiological culture conditions (limitation of *in vitro* methods, the lack of ability to perform coordinated contractions).

Furthermore, the morphological and numerical changes were observed under the influence of the substratum. Apparently, cells grown on non-glycated substrates function similarly to control cells, but with increasing degree of glycation of the substrate, there are fewer cells, on account of slower proliferation and/or death. Cellular debris were visible between the cells. Cells that were fixed as living tended to be smaller, with fewer protrusive structures and shape approaching the oval.

The more glycated was the substrate on which the cells were cultured, the more intensely they stained for type IV collagen. This may be associated with greater



*Fig. 3.* Immunocytochemical staining of smooth muscle cells derived from artery walls for the presence of  $\alpha$ -actin (a)  $\beta$ -catenin (b), and collagen type IV (c); I – control cells; II, III, IV – cells cultured on the substratum coated with protein mixture number 10; II – a mixture not exposed to microwave action; III – a mixture previously exposed to microwaves for 10 min; IV – a mixture previously treated with microwaves for 30 min, 200 $\times$  magnification.

Table 3. Results of immunocytochemical  $\alpha$ -actin staining of SMC grown on a substratum coated with glycated proteins

The mixture covering the substratum	Microwave exposure (min)	Size of the cells (0–3)	Number of cells (0–3)	Number of protrusive structures (0–3)	Percentage of cells stained for the presence of $\alpha$ -actin	Colour intensity (0–3)	Other characteristics
Control	-	3	3	3	10 %	1	Protein in the cytoplasm, partly dispersed, partly in the form of a stress fibre
Mixture No. 10	0	2		2	25 %	2	Cells with a tendency to cluster and overlapping; stress fibres are pronounced
	10	2	1	0	15 %	2	Cells with oval shape
	30	2	1	0	15 %	1	Cells with oval shape

expression of the protein. It is probable that this also might be due to the density of the cytoplasm, as the morphological changes associated with the decreasing cell surface were observed at the same time.

For  $\beta$ -catenin, a very slight tendency to decrease the staining intensity along with increasing substrate glycation was noticeable. This result indicates lower adhesiveness of the cells that are in contact with glycated proteins.

## Discussion

The present state of knowledge on the role of AGE in the pathogenesis of atherosclerosis leads to the conclusion that the amount of glycation end products grows with age and is associated with diabetes (Aronson, 2003; Monnier and Taniguchi, 2016). Furthermore, monocytes overexpress the main receptor of glycated lipids – scavenger receptor CD36, which enhances collection of lipoproteins from the blood and their absorption by the cells forming the atherosclerotic plaque (Meerwaldt et al., 2008). Additionally, glycation of the RGD motif of fibronectin in the inner layer of the artery hinders adhesion of endothelial progenitor cells to the damaged locations, hereby blocking endothelium reconstruction (Yamagishi, 2011).

The research that led to the above-mentioned conclusions did not consider the issue of cell migration. In atherosclerosis, this process is a crucial stage in the development of atherosclerotic plaque – smooth muscle cells in the middle layer are stimulated to migration by chemoattractants released from monocytes, macrophages and lymphocytes in the outer layer of the artery with primary atherosclerotic lesions. Following partial digestion of the internal elastic membrane by MMP (matrix metalloproteinases) released from cells of the immune system, the smooth muscle cells migrate from the media to the intima, where they express the ECM proteins and proliferate, contributing to the growth of the atherosclerotic plaque. Are the proteins modified by glycation hindering migration of these cells? This question has not yet been answered.

The hypothesis proposed in this study implies that glycation inhibits cell migration. It is based on the know-

ledge of formation of the so-called crosslinks during glycation. These links not only may form a “denser” network to which lipoproteins, immunoglobulins and other proteins are bound (Jabłońska-Trypuć and Czerpak, 2007), but may also provide a difficult to cross and digesting barrier for SMC.

Overall, it appears that glycation of collagen inhibits migration of multiple cell types. This is evidenced by the reports of Bartling et al. (2009), Francis-Sedlak et al. (2009), Liao et al. (2009), or Haucke et al. (2014), the brief description of which is given below. However, there is no data consistency, as there are also reports that glycation of ECM proteins accelerates cell migration (Yuen et al., 2010).

Analysis of the results obtained by the team of Francis-Sedlak et al. (2009) on glycated collagen type I led to the conclusion that the glycation of the substrate on which the cell is grown results in stimulated proliferation and reduction of invasiveness. However, these studies were carried out on fibroblasts, which do not reflect the research model for atherosclerosis; therefore, it cannot be concluded that smooth muscle cells will act similarly in the course of atherogenesis. Moreover, these studies included only collagen type I, not reflecting the composition of the extracellular matrix proteins, which is much more complex.

Based on other *in vitro* experiments, it was found that glycated collagen is characterized by modified interactions with fibroblasts (altered morphology, adhesiveness, proliferation and migration capacity), which may have implications for wound healing in diabetic patients (Haucke et al., 2014). Collagen isolated from human blood vessels collected during bypass operations was glycated using methylglyoxal, and subsequently the migration, adhesion and cytoskeleton of T lymphocytes (Jurkat T-cells) were evaluated. It appeared that collagen slightly modified by glycation reduced cell migration by 30 %, whereas collagen greatly modified by glycation inhibited cell migration by 60 %. The reduction in the migratory potential was accompanied by a change of actin polymerization. The conclusion of the study was that extracellular matrix proteins modified by glycation inhibit migration and adhesion of T lymphocytes (Haucke et al., 2014).

The same author also postulated that glycation of collagen type IV using methylglyoxal alters the structure of the RGD motif (Arg-Gly-Asp) recognized by integrin, which results in unhitching of endothelial cells from the substrate (Haucke et al., 2014). Hence it can be concluded that glycation also weakens cell adhesion.

For *in vivo* murine setting, it has been demonstrated that glycated collagen inhibits wound healing (Liao et al., 2009), which may be associated with a reduced migration potential in order to enable epithelialization. Experiments on lung cancer cells show that cells modified by glycation are less invasive (Bartling et al., 2009), which also indicates smaller migration potential.

As can be seen from the above summary, the impact of glycation on cell migration was tested on several models: fibroblasts, lymphocytes and cancer cells. Nevertheless, according to our knowledge, no research has addressed the impact of glycation of ECM proteins on the migration of SMC in the context of the development of atherosclerosis.

The hypothesis that glycation inhibits cell migration is based on the claim that glycation stiffens the ECM proteins. However, this hypothesis is sometimes being questioned.

It is noteworthy to analyse the study conducted by Stephen and colleagues, who examined the biomechanical properties of glycated collagen and elastin in the tissue. It has been shown that the strength and flexibility of porcine aortic slices remain unchanged regardless of whether they have been subjected to glycation (5–10 mM glucose, 48 h) or not (Stephen et al., 2014). This result is contradictory to many other studies reporting that glycation stiffens the proteins (Greenwald, 2007; Avery and Bailey, 2008, Kohn et al., 2015). Moreover, it has been shown that the clippings from aortas deprived of collagen (NRDC digestion in formic acid) under the influence of glycation reduce their rigidity, which also seems to be illogical in the light of the data obtained by other researchers (Tatoń, 1996; Greenwald, 2007; Avery and Bailey, 2008, Kohn et al., 2015). The results were interpreted as meaning that a short duration treatment (2 days) with very high glucose concentrations (5–10 mM) may have different consequences for the biomechanics and biochemistry of the tissue than the physiological process, which is long but runs at a much lower concentration. Lowering the flexibility of the tissue would be related to fragmentation of the elastin fibres, which was observed using a confocal microscope (Stephen et al., 2014).

A different hypothesis has also appeared in the literature. Kohn et al. (2015) postulated that the increase in rigidity of the ECM enhances proliferation and migration induced by platelet-derived growth factor (PDGF) released by endothelial cells and macrophages in the process of atherogenesis. Assuming that the stiffness, as repeatedly confirmed, is potentiated by glycation, it can be inferred that in this manner, glycation accelerates migration.

A more direct arguments in this matter can be found in the study by Yuen et al. (2010), who deduced that collagen modified by methylglyoxal (MGO) accelerates migration of myofibroblasts. Cardiac fibroblasts grown on such collagen were characterized by increased expression of  $\alpha$ -actin, fibronectin and cadherins; therefore, they differentiated into myofibroblasts faster than control cells. Migration tests, similarly to our study, demonstrated enhanced cell migration through the membrane coated with collagen modified by methylglyoxal. Another feature of cells grown on collagen-MGO was a smaller amount of adhesion and migration regulators – vinculin and paxillin; hereby, these cells display lower adherence. It was concluded that the rapid migration results from the inability to create proper adhesion complexes with glycated collagen (Yuen et al., 2010).

Apart from material, the main methodological difference between our study and the study carried out by Yuen et al. (2010) was the factor used for collagen modification. Here, we decided to use glucose 6-phosphate instead of methylglyoxal. It has been reported that it is even more reactive than glucose; nevertheless, its level in the blood is much lower than that of glucose or its direct metabolite – G6P.

A certain advantage of our research is also based on the well-defined protein composition of the glycated substrate, in particular the fact that we respected the relationships between the individual components of ECM, which was as close as possible to the actual composition.

During our work we assessed cell adhesiveness via immunocytochemical staining using  $\beta$ -catenin antibody. Visibly, there was a small downward trend in the staining intensity in cells cultured on a modified substrate as compared to the cells cultured on glass slides coated with a mixture of non-glycated matrix proteins. It is assumed that the more catenin is in the cytoplasm, the higher adhesion strength the adhesion complexes possess, and hence adhesion is increased and the migration capacity reduced. Theoretically, this result suggests a decrease in the capacity of adhesion, and thus an increase in the potential for migration proportionally to the degree of the modification of proteins that are in contact with arterial smooth muscle cells. However, the morphology of the cells should also be taken into account. Fewer protrusive structures – lamellipodia and filopodia – indicate lower migratory potential in the cells grown on the glycated substrates. A significant drop in the amount of SMC that are in contact with the modified ECM proteins is also evident. The cell membrane in cells seeded on a substrate subjected to 30 min glycation in the microwave is damaged, frayed. These observations provide evidence that cells that are in contact with glycated proteins are weakened and have poorer metabolism, proliferation and survival. Since cell migration requires energy input for efficient cytoskeleton rearrangements and other biochemical mechanisms, it may be suspected that cells also present inferior migration potential.

The results of our study do not unequivocally settle the issue of the influence of collagen glycation on the migration potential of smooth muscle cells; however, there is visible tendency to decrease the number of cells along with extension of the glycation of proteins used by the cells to migrate.

As pointed out in the introduction and at the beginning of this section – it is postulated that in general, AGE stimulate atherogenesis, mainly by the effect on the development of inflammatory conditions, facilitating binding of low density lipoproteins to the cells of the artery, activation of leukocytes and platelets, stimulating changes in the expression level of growth factors, MMP adhesion molecules and other proteins. The vast majority of literature data indicates that glycation of ECM proteins inhibits migration of cells in the matrix. The results obtained in the presented study confirm the validity of this hypothesis.

It is known that migration of SMC contributes to the growth of the atherosclerotic plaque. Assuming that artery myocytes indeed migrate more slowly from the media to the intima through glycosylated ECM proteins, an anti-atherogenic mechanism takes place, the only one that can be attributed to glycation.

However, it should be expected that the significance of this mechanism is not able to compensate for the negative role of AGE in the development of atherosclerotic plaques. Therefore, it is worth continuing research directed towards the possibility of reducing the intensity of glycation and to develop therapeutic strategies rectifying the effects of the presence of AGE in the body.

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