

Growth of Vascular Smooth Muscle Cells on Collagen I Exposed to RBL-2H3 Mastocytoma Cells

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Key Words

Pulmonary hypertension • Vascular remodeling • Mast cells • Proteases • Collagen degradation • Smooth muscle cell proliferation • High performance liquid chromatography • Mass spectrometry

Abstract

Remodeling of the peripheral pulmonary vasculature during chronic hypoxia is characterized by accelerated collagenolysis and thickening of the vascular wall. Low molecular weight peptides, products of cleavage by interstitial collagenase and muscular layer in the peripheral pulmonary vessels, are typically present. The aim of this “in vitro” study was to verify that mast cells (RBL-2H3) as a potent source of a variety of biomolecules which can affect vessel wall remodeling are capable of splitting collagen and then facilitating the growth of vascular smooth muscle cells (VSMC). Collagen I was exposed to RBL-2H3 cells cultured 48 hours under normoxic or hypoxic (3% O₂) conditions and then seeded with VSMC. The VSMC proliferated with the shortest doubling time and reached the highest cell population density on the collagen pre-modified with hypoxic RBL-2H3 cells.

This increased growth activity of VSMC was probably due to the fragmentation of collagen by proteases released from RBL-2H3 cells. Absolute amount of collagen fragments was similar in samples exposed to normoxic and hypoxic RBL-2H3 cells, but the concentration of at least one collagen fragment was significantly higher under hypoxic conditions. Mast cells exposed to hypoxia are more capable to split collagen and facilitate the growth of VSMC.

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Introduction

Both pulmonary and systemic hypertension are serious diseases associated with remodeling of the vascular wall structure, which involves proliferation of vascular smooth muscle cells (VSMC) and their migration from the *tunica intima* to the *media*. This can result in thickening of the vessel wall, stenosis of the vascular lumen, increased vascular resistance and subsequent heart failure [1, 2]. A prerequisite for activation of the migratory and proliferative response of VSMC is degradation of

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the basement membrane and other components of extracellular matrix (ECM) by proteases, particularly tissue metalloproteases (MMPs) 2, 9 and 13 [3]. In addition, metalloproteases cleave the extracellular portions of cell adhesion receptors, and thus weaken the cell-matrix and cell-cell adhesion that is necessary for maintaining the quiescent non-proliferative state of the cells [4]. As reported earlier in our studies and also in studies by other authors, proteases can be produced by cells physiologically resident in the vascular wall, such as VSMC themselves, endothelial cells and fibroblasts [5], and also by inflammatory cells infiltrating the vascular wall under pathological conditions, such as leucocytes, macrophages and particularly mast cells. The latter have been called “forgotten cells” [6], because their role in vascular pathology has often been underestimated, though the ratio between mastocytes and VSMC in a pathologically changed vessel wall can reach 1:5, and thus they become a considerable component of the diseased vascular wall [7].

Mast cells are potent producers of proteases, such as chymase, tryptase, carboxypeptidase A and metalloproteases [8, 9]. These enzymes not only degrade the ECM and facilitate migration and proliferation of VSMC, but they can also aggravate vascular disease by converting angiotensin I into angiotensin II [10].

In addition, mast cells secrete a wide range of other proinflammatory, reparative and vasoactive substances, which can influence the growth of VSMC, particularly cytokines, e.g., tumor necrosis factor- α , interleukin-1 [6], growth factors e.g. transforming growth factor- β_1 , platelet-derived growth factor [11], nerve growth factor [12], vascular endothelial growth factor [13], proteoglycans (heparan sulphate, chondroitin sulphate [14], histamine [15] and prostanoids, especially leukotrienes, which further increase the activity of metalloproteases [16]. Mast cells also participate in the process of heterotopic bone formation, compensatory neovascularization and lipid accumulation in vascular lesions [17]. Studies dealing with the pathogenesis of pulmonary hypertension [8, 9] as well as myocardial infarction [15], show that the accumulation of mastocytes in the vessel wall and secretion of biomolecules, including proteolytic enzymes, by these cells can be markedly enhanced under hypoxic conditions. Hypoxia had stimulatory effects on pulmonary vascular remodeling (characterized by hyperplasia of pulmonary arterial smooth muscle cells), although direct stimulation of VSMC by hypoxia did not lead to an increase in the proliferation activity of these cells [5].

In the present study, we have evaluated the initial adhesion and subsequent growth of rat aortic VSMC in cultures on collagen I, an important component of the vascular extracellular matrix, pre-exposed to a mast cell line RBL-2H3, stimulated by hypoxia. In our earlier study, the RBL-2H3 cells proved to be a potent source of MMPs 2, 9 and 13, as well as chymase and tryptase, especially under hypoxic conditions [9]. Therefore, the collagenolytic activity of RBL-2H3 cells is also examined, using High Performance Liquid Chromatography combined with mass spectrometry (HPLC/MS) and SDS-PAGE electrophoresis.

Materials and Methods

Types, sources and pre-cultivation of cells

A rat basophilic leukemia (mastocytoma) cell line RBL-2H3 (CRL-2256, ATCC Manassas, VA, U.S.A.) was used as a model of mast cells infiltrating the walls of pulmonary blood vessels. These cells retain most characteristics of mature mast cells in peripheral tissues *in situ* as well as the characteristics of freshly isolated serosal or perivascular mast cells *in vitro*, such as the ability to produce hydrogen peroxide, to degranulate and to release various bioactive molecules, namely histamine, cytokines, growth factors, prostanoids or proteases [18]. After being purchased, the cells were expanded in Eagle Minimum Essential Medium (E-MEM; Sevapharma, Prague, Czech Republic) with 5 or 10% of fetal bovine serum (FBS; Sebak GmbH, Aidenbach, Germany) and 50 $\mu\text{g}/\text{mL}$ of Gentamicin (LEK Pharmaceuticals, Ljubljana, Slovenia), and were used in the 9th passage.

Vascular smooth muscle cells (VSMC) were isolated from the intima-media complex of the thoracic aorta of 8-week-old male Wistar SPF rats (Inst. Physiol., Acad. Sci. CR) by an explantation method described earlier [19]. The cells were expanded in polystyrene flasks (TPP, Trasadingen, Switzerland) in Dulbecco's modified Eagle's Minimum Essential Medium (DMEM; Sigma, U.S.A., Cat. N° D5648) supplemented with 10% of FBS (Sebak GmbH, Aidenbach, Germany) and gentamicin (40 $\mu\text{g}/\text{mL}$, LEK, Ljubljana, Slovenia). The cells displayed a hill-and-valley pattern typical for VSMC, and as revealed by immunofluorescence they contained bundles of alpha-isoform of actin, a marker of VSMC identity and differentiation [20]. For the experiments, the VSMC were used in the 5th passage.

Preparation of modified collagen substrates

Type I collagen (Sigma-Aldrich, Cat. No. C-8919) was diluted in sterile distilled and deionized water (designed for preparation of cell culture media) in a concentration of 0.05 mg/mL. The bottoms of the wells in polystyrene 24-well multidishes (NUNC, diameter 1.5 cm, culture area of $\sim 1.8 \text{ cm}^2$) or (NUNC 6-well multidishes, diameter 3.4 cm, culture area of $\sim 9.1 \text{ cm}^2$) were then coated with 200 or 1020 μL of the collagen solution, respectively. The collagen was then allowed to adsorb

at 4°C for 72 hours. Non-adsorbed collagen was then removed by rinsing in a phosphate-buffered saline (PBS; 137 mM NaCl; 2.7 mM KCl; 4.3 mM Na₂HPO₄; 1.47 mM KH₂PO₄), pH adjusted to 7.3. For the experiments, six plates of 24-well multidishes and four plates of 6-well multidishes were prepared.

The culture wells were then supplemented with polycarbonate membrane inserts (NUNC) with 3 µm pores in their bottom (insert diameter of 1 cm and 2.5 cm for the 24-well and 6-well multidishes, respectively). The cell culture inserts were seeded with RBL-2H3 cells at a density of 5 × 10⁴ and 25 × 10⁴ cells per smaller and larger insert, respectively, and into 0.5 and 2 mL of chemically-defined serum-free medium supplemented with epidermal growth factor, fibroblast growth factor and insulin (BD Biocoat, Cat. No. 355160). The serum-free medium was used in order to minimize the additional adsorption of molecules influencing cell adhesion, such as vitronectin, fibronectin and albumin, present in the serum, over the investigated collagen coating.

The cells were allowed to recover in an air atmosphere with 5% CO₂ for 24 h at 37°C. Then the sets of multidishes with inserts were divided into two groups and put in the Modular Incubator Chambers (Billups-Rothenberg, Inc.) purged with a normoxic (21% O₂+5% CO₂) or hypoxic (3% O₂+5% CO₂) mixture for 10 min with flow rate 5 L/min, and cultivated for the next 48 h at 37°C. Gas mixtures were prepared by Linde a.s., Czech Republic. The O₂ concentrations inside the chambers were tested (MiniOX 3000, Cheirón s.r.o., Czech Republic). As control samples, collagen-coated wells not exposed to the cell cultures were used (both groups being exposed either to normoxia or hypoxia).

After completion of the 48-hour cultivation of RBL-2H3 cells in the inserts, the inserts with cells were removed from the wells, the cultivation media were collected and frozen, and the wells were rinsed in PBS. The 24-well multidishes were used for cultivating VSMC on the modified collagen substrates, while the 6-well multidishes served for biochemical analysis of the collagen substrates.

Culture of VSMC on collagen substrates

The VSMC were seeded on the bottoms of the wells in 24-well multidishes; (1) uncoated wells, (2) coated with unmodified collagen I and (3) coated with collagen I modified with RBL-2H3 cells (all three groups of samples being exposed either to normoxia or hypoxia). Each well contained 5000 cells (i.e., 2830 cells/cm²) and 1 mL of the serum-free BD Biocoat medium mentioned above. The cells were grown for 1 and 4 days at 37°C in a standard humidified atmosphere of 95% of air and 5% CO₂. The cells were then rinsed with PBS, fixed in 70% cold ethanol (-20 °C) for 10 min and visualized with a combination of the following fluorescence dyes diluted in PBS: (1) Texas Red C₂-maleimide (Molecular Probes, Invitrogen, Cat. No. T6008; concentration 20 ng/mL), which conjugates with proteins of the cell membrane and cytoplasm and stains the cells red [21], and (2) Hoechst # 33342 (Sigma, U.S.A.; 5 µg/mL), which counterstains the cell nuclei blue. The cells were incubated in the mixture of both dyes for 2 hours at room temperature, rinsed in PBS (twice for 3 min) and mounted under round coverslips in a fluorescence-preserving medium Gel-

mount™ (Biomedica Corp., CA, U.S.A.).

The cell number and morphology were evaluated on microphotographs taken under an epifluorescence IX 51 microscope (obj. 20) equipped with a digital DP 70 camera (both from Olympus, Japan). For each experimental group and time interval, three wells were used, and each well was evaluated in 9 randomly selected fields (0.136 mm²) homogeneously distributed on the well bottom. The cell numbers (cells per cm²) were used for constructing growth curves and for calculating the cell population doubling time (DT) according to the formula $DT = (t - t_0) \log 2 / \log N_t - \log N_{t_0}$, where t_0 and t represent earlier and later time intervals after seeding, respectively, and N_{t_0} and N_t the number of cells at these intervals.

On day 1 after seeding, the size of the cell spreading area (i.e., the cell area projected on the adhesion substrate) was also measured, using Atlas software (Tescan s.r.o., Brno, Czech Republic).

Biochemical analyses of collagen

High Performance Liquid Chromatography and Mass Spectrometry (HPLC/MS). The collagen from collagen-coated surfaces of wells (diameter 3.4 cm) was analyzed after being exposed to mast cells. Four sample groups were set up: (1) collagen exposed to normoxic RBL-2H3 cells, (2) collagen exposed to hypoxic RBL-2H3 cells, (3) collagen exposed to normoxia without cells, (4) collagen exposed to hypoxia without cells. The sample of each group contained 6 wells (i.e., one 6-well plate). The collagen on the surface of the wells was dissolved in 1 mL of 0.5% acetic acid, lyophilized and dissolved in 0.1 mL of 0.05 mol/L NH₄HCO₃. All samples were digested with trypsin (1:50, enzyme/substrate ratio) at 37° C for three hours.

Chromatographic separation was carried out in a C12 RP Jupiter Proteo 90 A column, 250x2 mm, particle size: 4 µm, pore size: 9 nm (Phenomenex, Torrance, CA, USA).

The HPLC apparatus used here was an HP 1100 LC system (Agilent, Palo Alto, CA, USA) consisting of a degasser, a binary pump, an autosampler, a thermostated column compartment, and a diode array detector. It was coupled to an ion-trap (IT) mass spectrometer (Agilent LC-MSD Trap XCT-Ultra). For peptide identification and selection experiments, separation of peptides was achieved via a linear gradient between mobile phase A (water/formic acid, 100:0.1 v/v) and B (ACN/formic acid, 100:0.085 v/v). The water used in the analytic experiments was MilliQ; acetonitrile (HPLC gradient grade) and formic acid were obtained from Merck (Darmstadt, Germany). Trypsin (type IX-S, lot 51K72501) and NH₄HCO₃ was obtained from Sigma (St. Louis, MO, USA).

Separation was started by running the system isocratically for two minutes with 2% mobile phase B, followed by gradient elution to 35% B at 40 min. Finally, the column was eluted with 100% B for 10 min. Equilibration before the next run was achieved by washing with 2% mobile phase B for 10 min. For analyses of selected peptides (quantification), the gradient was changed, as follows, in order to achieve separation within a shorter period of time: separation was started by running the system isocratically for two minutes with 3 % mobile phase B, followed by gradient elution to 12% B at 15 min. Finally, the

Peptide Sequence	Specific production (m/z)	Precursor ion MH ⁺
GIpGPVGAAGATGAR	634.4	1251.6
GFpGSpGNIGPAGK	644.3	1255.6
GSAGPpGATGFpGAAGR	730.3	1427.7

Table 1. List of collagen peptides selected for the experiment (p-hydroxyproline).

culture conditions / adhesion substate	Plast	Coll	Coll + RBL
Normoxia	17619 ± 1440*	27742 ± 1311	26464 ± 1441
Hypoxia	16891 ± 1164*	30299 ± 1658	23454 ± 2298**

Table 2. Size of the cell spreading area of VSMC on day 1 after seeding on uncoated standard polystyrene cell culture wells (plast), wells coated with unmodified collagen I (coll) and wells coated with collagen I pre-exposed to mastocytes under normoxic or hypoxic conditions (coll + RBL). Mean ± SE from 63-95 cells for each experimental group. Differences were compared using ANOVA and Fisher's post hoc test, values $p < 0.05$ were considered significant. * $p < 0.0001$ plast groups vs. all collagen coated groups. ** $p < 0.0027$ coll + RBL hypoxic group vs. unmodified collagen hypoxic group.

column was eluted with 100% B for 10 min. Equilibration before the next run was achieved by washing with 3% mobile phase B for 10 min. The flow rate for both types of experiments was 0.25 mL/min, the injection volume was 40 μ L, and the column temperature was held at 25°C.

API-ESI positive mode IT MS was used under the following operating conditions: drying gas (N₂), 10 L/min; drying gas temperature, 350°C; nebulizer pressure, 1.7 x 10⁵ Pa (25 psi).

For peptide identification and selection experiments, ions were observed over the mass range m/z 100-2200 (MS: standard mode, MS/MS: enhanced mode). The analysis was performed in auto MS/MS mode (three precursor ions, see Table 1), excluded after two spectra for 0.5 min. The fragmentation amplitude was set to 1.14 V. Analysis of MS/MS data (peptide/protein identification and searching for possible post-translational modifications - hydroxylation of proline and lysine) was carried out using SpectrumMill software (v.3.02, Agilent). Searches were performed in the SwissProt full protein database. To identify peptides with multiple hydroxylations, searches were performed on the data extracted from this database.

Three peptides specific for collagen of type I, obtained by tryptic digestion, were selected for measuring their intensity in collagen samples (Table 1). Trypsin digested samples were used directly for HPLC/MS analyses. Each of these marker peptides had to meet all of the following criteria at the same time: (a) they had to be detected in all samples, (b) they had to be part of the collagenous domain, (c) their peak area in HPLC-MS/MS chromatograms of the samples had to be reproducible, and (d) they had to be unique to collagen type I, i.e., the peptide could not be part of any other protein structure in the SwissProt/NCBI databases.

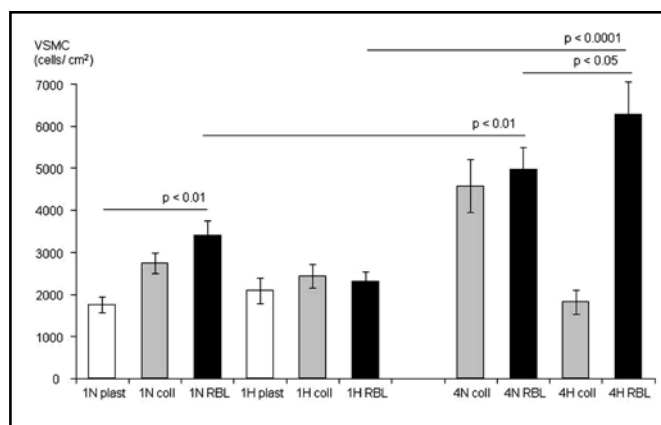


Fig. 1. Cell population density (cells/cm²) of rat aortic smooth muscle cells (VSMC) in 1-day-old and 4-day-old cultures on dishes. Dishes were non-coated (plast), coated with collagen I alone (coll) or with collagen I pre-modified with mastocytoma cells cultured under normoxic (N RBL) or hypoxic (H RBL) conditions. Differences were compared using ANOVA and Fisher's post hoc test, values $p < 0.05$ were considered significant.

Collagen peptides were characterized by their specific m/z (molecular mass of peptide/charge of the peptide).

SDS-PAGE electrophoresis. Separations of collagen peptides were performed by SDS-PAGE electrophoresis on a discontinuous slab gel using 4% stacking gel and 7.5% separating gel. The samples were dissolved in sample buffer in concentration 4 mg/mL, and 30 μ g of collagen fraction was loaded per line. The electrophoresis separation was run in the Tris-glycine buffer system without reduction in Mini-PROTEAN

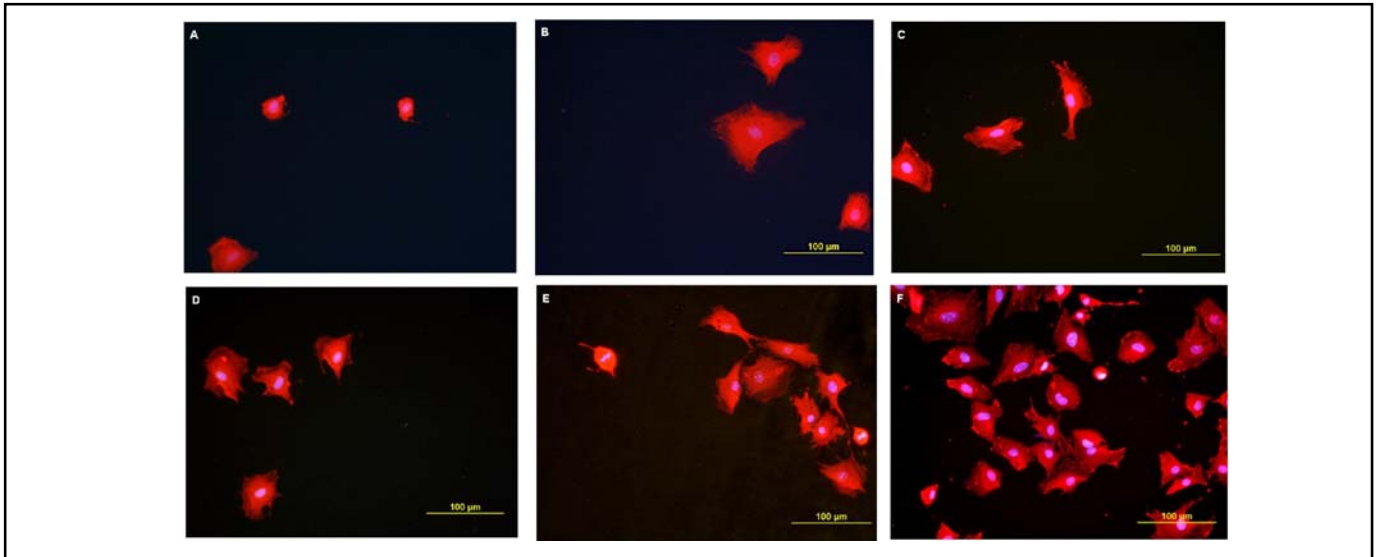


Fig. 2. Morphology of rat aortic smooth muscle cells in cultures on day 1 (A-D) and 4 (E, F) after seeding on standard cell culture polystyrene dishes (A), dishes coated with collagen I (B), and dishes coated with collagen I pre-modified with RBL-2H3 mastocytoma cells cultured in polycarbonate dish inserts under normoxic (C, E) or hypoxic (D, F) conditions. Cells stained with Texas Red C₂-maleimide (red fluorescence) and Hoechst 33342 (blue fluorescence). Olympus epifluorescence microscope IX 51, obj. 20, digital camera IX 70, bar=100 μm.

II Electrophoresis Cell (Bio-Rad Laboratories, USA). The gels were stained for proteins with 0.25% Coomassie Brilliant Blue R in methanol - acetic acid - water (40:10:50 v/v/v). Destaining was performed with methanol - acetic acid - water (40:10:50 v/v/v).

Statistics

The results are presented as mean ± SE. They were evaluated by an unpaired t-test or by one-way ANOVA with subsequent Fisher's post hoc test (StatView 5.0, SAS Institute, Cary, NC, USA). Differences were considered significant when $p < 0.05$.

Results

Initial adhesion and morphology of VSMC on collagen I modified by RBL-2H3 mastocytoma cells

On day 1 after seeding, the VSMC on collagen samples non-modified by mast cells adhered in similar numbers, which tended to be on an average higher than those on non-coated standard polystyrene dishes, though these differences were not significant. Interestingly, on collagen exposed to normoxic RBL-2H3 mastocytoma cells, the average number of initially adhering VSMC was slightly but non-significantly higher than that on collagen modified with hypoxic RBL-2H3 cells (Fig. 1).

Most of the VSMC on all studied samples were of similar polygonal morphology, though some cells remained rounded and non-spread even up to day 4 after seeding

(Fig. 2). On day 1, the average cell spreading area was significantly lower in both collagen-uncoated groups in comparison with all collagen-coated groups. The spreading area of cells on collagen pre-exposed to mastocytes was significantly lower than the area on unmodified collagen under hypoxic conditions (Table 2).

Growth of VSMC on collagen I modified by RBL-2H3 mastocytoma cells

From day 1 to 4, the VSMC on the investigated collagen samples varied in their growth activity (Fig. 1). The VSMC on the collagen samples non-exposed to RBL-2H3 mastocytoma cells did not proliferate at all or proliferated with a relatively long cell population doubling time (98 hours). Similarly, the doubling time of VSMC grown on collagen exposed to normoxic RBL-2H3 cells was also very long, reaching 138 hours. In contrast, VSMC on collagen modified with hypoxic RBL-2H3 cells proliferated with the shortest doubling time (49 hours), and on day 4 they attained the highest cell population density. This value was significantly higher than the corresponding values on collagen modified with normoxic RBL-2H3 cells (Fig. 1).

Fragmentation of collagen I exposed to RBL-2H3 mastocytoma cells

The HPLC/MS used in the present study revealed about twenty collagen peptides cleaved from the collagen coating of dishes pre-exposed to RBL-2H3 mastocytoma

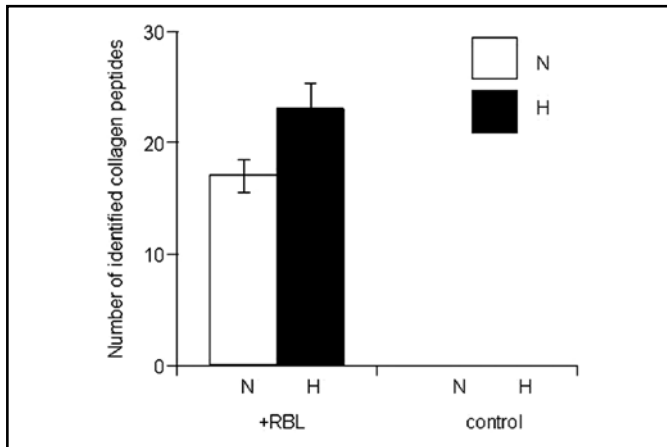


Fig. 3. Number of collagen peptides identified by HPLC/MS. +RBL: collagen samples after exposure to RBL-2H3 cells under normoxic (N) or hypoxic (H) conditions (unpaired t-test, $p = 0.06$); control: collagen samples without exposure to RBL-2H3 cells kept under normoxic (N) or hypoxic (H) conditions (no collagen peptides were identified).

cells under normoxic or hypoxic conditions, while collagen peptides were not detectable in the collagen samples non-treated with RBL-2H3 cells (control) (Fig. 3). In the RBL-2H3 cell-treated collagen, most of the analyzed peptides (i.e., approx. 80%) were cleaved on the cleavage site typical for the group of enzymes related to trypsin (e.g., trypsin, tryptase). We found only collagen peptides in both RBL-2H3 cell-treated groups and one specific peptide of interstitial collagenase in one sample from the hypoxic group. The average amount of collagen peptides tended to be slightly higher in collagen treated with hypoxic RBL-2H3 cells, but this difference was not statistically significant (Fig. 3). Using three marker peptides (Table 1), we found a significantly higher concentration of collagen peptides at least in one case, i.e. in collagen treated with hypoxic RBL-2H3 cells (Fig. 4).

Fragmentation of collagen I by the action of the interstitial collagenase contained in the lysate of RBL-2H3 cells was also confirmed by SDS-PAGE electrophoresis. This effect was more apparent in collagen samples treated by the lysate from RBL-2H3 cells exposed to hypoxia, as documented by $\frac{3}{4}$ fragments A1 and A2 and $\frac{1}{4}$ fragments B1 and B2 of $\alpha 1$ and $\alpha 2$ collagen type I chains (Fig. 5).

Discussion

The aim of this study was to verify whether mast cells are capable of splitting collagen on the same

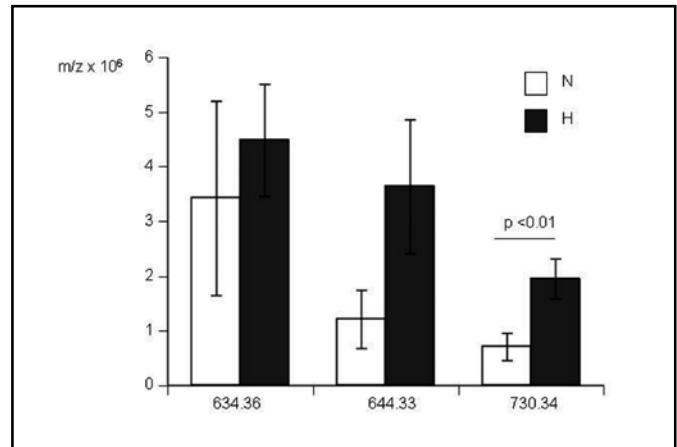


Fig. 4. Peptidic collagen fragments of various molecular weights, released by treatment with 0.5 % acetic acid from the collagen coating of dishes after previous exposure of the collagen to RBL-2H3 mastocytoma cells cultured in polycarbonate inserts in these dishes under normoxic (N) and hypoxic (H) conditions. The number of each peptide refers to its characteristic m/z (i.e., the molecular mass of the peptide/charge of the peptide). The statistical analysis made use of the unpaired t-test, values $p < 0.05$ were considered significant.

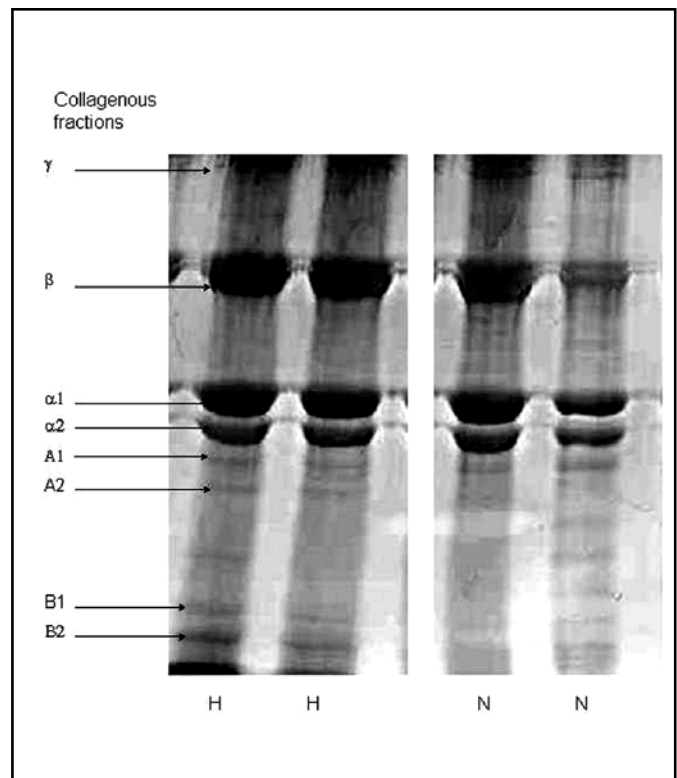


Fig. 5. Gel electrophoresis profile of collagen I. Collagen I was exposed to lysates from RBL-2H3 cells cultured under normoxic (N) or hypoxic (H) conditions. This effect was more apparent in collagen samples treated by the lysate from RBL-2H3 exposed to hypoxia, as documented by $\frac{3}{4}$ fragments A1 and A2 and $\frac{1}{4}$ fragments B1 and B2 of $\alpha 1$ and $\alpha 2$ collagen type I chains.

fragments as were found in the peripheral pulmonary arteries of chronically hypoxic rats [1], and whether the collagen modified by mast cells stimulates the growth of VSMC.

We found an increased growth activity of VSMC on day 4 after seeding on collagen I exposed to RBL-2H3 mastocytoma cells cultured under hypoxic conditions compared to collagen I exposed to RBL-2H3 cells in normoxia. This behaviour of VSMC could be explained by degradation of collagen I by hypoxia activated proteolytic enzymes released from RBL-2H3 cells. Our earlier study has shown that RBL-2H3 cells are a potent source of metalloproteases 2, 9 and 13, chymase and tryptase, and the production of these enzymes is enhanced by hypoxic conditions. In the present study, we also found collagen peptides cleaved from the collagen coating of culture dishes pre-exposed to RBL-2H3 cells. Some of these peptides were present predominantly in collagen samples exposed to RBL-2H3 cells cultured under hypoxic conditions, which further confirmed that the production of proteolytic enzymes by RBL-2H3 cells is stimulated by hypoxia.

The adhesion of VSMC to collagen fragments can stimulate their proliferation activity. It has been shown that the adhesion of VSMC or pulmonary fibroblasts to collagen monomers activated the proliferation of these cells, whereas polymerized collagen maintained both cell types in a quiescent non-proliferative state [22, 23]. On polymerized fibrillar collagen, cyclin E-associated kinase and cyclin-dependent kinase 2 (cdk2) phosphorylation were inhibited, while the levels of cdk2 inhibitors (p27Kip1 and p21Cip1/Waf1) are increased compared with those in VSMC on monomer collagen [22]. Also in renal mesangial cells, polymerized type I collagen prevented the DNA synthesis and cell replication by down-regulation of cyclins D1 and E, reduced cyclin E-associated kinase activity and by the expression of cdk-inhibitor protein p27Kip1 [24].

The effects of collagen on cell proliferation are mediated by signaling through collagen-binding integrin adhesion receptors on the cell membrane. Interaction of β_1 integrins with polymerized collagen suppressed fibroblast proliferation by the formation of a β_1 integrin-protein phosphatase 2A (PP2A)-tuberous sclerosis complex 2 (TSC2) complex that represses the activity of S6K1 kinase, a key enzyme for the transition from the G1 to S phase of the cell cycle [23]. In our earlier study, VSMC in cultures on collagen III oxidized and degraded by ultraviolet light irradiation showed a lower

concentration of β_1 integrin per mg of protein, and also less apparent organization of these adhesion molecules, as well as their associated proteins talin and vinculin, into focal adhesion sites. At the same time, these cells exhibited higher proliferation activity than VSMC on non-modified collagen [25].

Another cause for the enhanced growth of VSMC on collagen modified by RBL-2H3 cells, especially hypoxic cells, could be the production of cytokines and growth factors by these cells. For example, RBL-2H3 mastocytoma cells are capable of synthesizing and releasing interleukins, tumor necrosis factors, transforming growth factor [26], nerve growth factor [27], vascular endothelial growth factor, macrophage chemoattractant protein - 1 (MCP-1) [28] or serotonin [29], which have been reported to stimulate the proliferation of VSMC [29, 30]. Although we have not evaluated the presence of these factors in the mastocytoma cell-modified collagen coating, it cannot be excluded that some of these factors may be bound to or retained in the collagen coating and released during VSMC cultivation.

Conclusion

We found that vascular smooth muscle cells in cultures on collagen I pre-exposed to RBL-2H3 mastocytoma cells incubated under hypoxic conditions grew more rapidly and reached a higher cell population density than VSMC on non-modified collagen or on collagen exposed to RBL-2H3 cells under normoxic conditions. This behaviour of VSMC may be explained by degradation of collagen by proteases released from RBL-2H3 cells. Although the total amount of collagen fragments was similar after exposure to normoxic or hypoxic RBL-2H3 cells, but the concentration of at least one collagen fragment was significantly higher under hypoxic conditions.

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