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Influence of Macrophages and Macrophage-Modified Collagen I on the Adhesion and Proliferation of Vascular Smooth Muscle Cells in Culture

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Summary

The adhesion, proliferation and morphology of rat vascular smooth muscle cells (VSMC) in cocultures with macrophages or in cultures on type I collagen modified by activated macrophages were evaluated. In the first set of experiments, rat alveolar macrophages were added to 24-hour-old VSMC cultures. Between days 2 and 5 after VSMC seeding, the population densities and doubling times of cells were similar in both VSMC-macrophage and pure VSMC cultures. However, from day 5, the cocultures proliferated about two times more rapidly and on day 7, they reached higher cell population density by 40%. The pure macrophage cultures did not proliferate. In the second set of experiments, rat alveolar macrophages were activated by non-toxic TiO2 dust to produce reactive oxygen species and incubated for 120 min with collagen I. The collagen was then adsorbed on plastic culture dishes and seeded with VSMC. The collagen exposed for 10 min only, the unmodified collagen and pure culture dishes were used as control growth supports. On all four tested substrates, the number of initially adhered cells was similar, but on the collagen modified for 120 min, the cells were less spread. Moreover, on day 2 to 3 after seeding, some cells on this collagen became vacuolated and detached spontaneously from the growth support. The remaining VSMC, however, rapidly proliferated, so that on day 9, the cell population density on 120-min-modified collagen was similar as on both control collagens and significantly higher compared to that on uncoated dishes. Our results suggest that 1. The delayed growth-stimulating effect of macrophages on VSMC-macrophage mixed population is probably due to autocrine production of mitogens by both cell types rather than due to an acute effect of short-living oxygen radicals released from macrophages immediately after adding to VSMC cultures. 2. The effect of collagen I exposed to activated macrophages for 120 min is slightly cytotoxic, which could, however, stimulate a release of mitogens from damaged as well as surviving VSMC.

Key words

Remodeling of vascular wall • Cell adhesion • Cell growth • Spontaneous cell detachment • Reactive oxygen species • Activated macrophages • Rat aortic smooth muscle cells • Coculture • Extracellular matrix

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Introduction

The main cause of hypoxic pulmonary hypertension is the remodeling of peripheral pulmonary arteries which results from proliferation of vascular smooth muscle cells (VSMC) and from increased fibroproduction (Reid 1986). According to our hypothesis, the structural remodeling of the pulmonary vasculature results from injury to the pulmonary vascular wall, which may originate from increased radical production (Herget *et al.* 1997). The production of NO (Hampl *et al.* 1995) and oxygen-derived radicals (ROS) (Nakanishi *et al.* 1995) is enhanced in chronic hypoxia and reduced production of these radicals inhibits the structural remodeling of pulmonary vasculature (Hampl *et al.* 1993, Hoshikawa *et al.* 1995).

Cells of the monocyte/macrophage lineage play an important role in the development of vascular injury. The monocyte-derived macrophages adhere to the activated endothelium through adhesion molecules of the immunoglobulin, selectin and α_2 integrin families (Fan *et al.* 1993). Activated endothelial cells as well as underlying smooth muscle cells produce chemokines, particularly the monocyte chemoattractant protein-1, which enable the penetration of adherent macrophages into the vessel wall (Fan *et al.* 1993, Wenzel *et al.* 1995, Rollins 1997, Takahara *et al.* 1997). Inside the vascular wall, the macrophages can divide and proliferate (Rosenfeld *et al.* 1992, Gordon and Rekhter 1997), synthesize growth factors for VSMC (Kaufmann *et al.* 1990, Inoue *et al.* 1993, Ozer *et al.* 1995), digest and remodel the extracellular matrix (for review see Kaufmann *et al.* 1990). Activated macrophages are important sources of ROS and NO. These radicals and namely the product of their interaction – peroxynitrite and its metabolites – alter matrix proteins (Kato *et al.* 1997) and activate the tissue collagenolytic activity (Frears *et al.* 1996, Rajagopalan *et al.* 1996).

We have shown that alveolar macrophages harvested from the lung of hypoxic rats are primed to produce more oxygen-derived radicals *in vitro* after stimulation than macrophages from normoxic animals (Wilhelm *et al.* 1996). Recently, we observed that VSMC grew more rapidly *in vitro* on a collagen surface which was oxidized by UV irradiation (Bačáková *et al.* 1997c). In chronic hypoxia, the structure of collagen in peripheral pulmonary arteries is substantially modified (Novotná and Herget 1998). It was previously shown (Vilím *et al.* 1987) that alveolar macrophages can be activated by several mineral dusts to produce reactive oxygen species and this activation can be detected by measurement of luminol-dependent chemiluminescence.

In the present study, we investigated the direct and indirect influence of rat alveolar macrophages, activated by non-toxic dust (TiO_2), on the adhesion and proliferation of rat aortic smooth muscle cells *in vitro*. We cultured the VSMC together with macrophages or on type I collagen exposed to activated macrophages before seeding the VSMC. We evaluated the number of initially adhered cells, their shape and degree of spreading, resistance to the detachment by trypsin, doubling time and cell population density.

Materials and Methods

Isolation, identification and culture conditions of VSMC

Smooth muscle cells (VSMC) were isolated from the intima-media complex of the thoracic aorta of four young male Wistar-Kyoto rats (age 8 weeks, body weight 220-240 g, Inst. Physiol. AS CR, Prague) by the explantation method (Bačáková *et al.* 1997a,b,c). At confluence, the cells displayed a hill-and-valley pattern characteristic for VSMC (Chamley-Campbell *et al.* 1979) and positive immunocytochemical staining for smooth muscle specific α-actin according to Skalli *et al.* (1986) (data not shown). For the experiments in cocultures with macrophages as well as in cultures on macrophage-modified collagen, adhesion and growth were assayed in cells at passages 7 to 10. They were grown in a Dulbecco-modified Eagle minimum essential medium (DMEM, SEVAC, Prague, CR) supplemented with 10 % fetal calf serum (Sebak GmbH, Aidenbach, Germany) and gentamicin (40 μg/ml, LEK, Ljubljana, Slovenia).

Isolation of alveolar macrophages and their activation in vitro

Rat alveolar macrophages were isolated according to a procedure described in our previous study (Wilhelm *et al.* 1996). The cells were counted under a microscope and their viability was assayed on the basis of Trypan Blue exclusion. The typical preparation contained more than 98% viable cells, 95% of which were positive for non-specific esterase, a marker of macrophage cell type. The isolated macrophages were activated by the addition of non-toxic mineral dust (TiO_2 in crystalline form, , particle size around 2 μm) in a concentration of 5 mg/ml/10^6cells. The production of reactive oxygen species was assayed on the basis of luminol-dependent chemiluminescence as reported previously (Vilím *et al.* 1987).

Growth of VSMC in cocultures with macrophages

The VSMC were seeded in polystyrene Nunclon Multidishes (diameter 1.5 cm, NUNC, Roskilde, Denmark) in 1.0 ml of the above-mentioned medium at the density of 30 000 cells per well (i.e.17 000 cells/cm^2). One day after seeding, the medium was discarded to remove unattached cells. In the first group of samples, the adhered VSMC were detached by 0.2 % trypsin in phosphate-buffered saline (PBS) and counted in a Bürker hemocytometer. To the second parallel group of samples, 1.5 ml of the fresh medium containing 30 000 macrophages was added. On days 2, 5 and 7 of the experiment, the adhered cells were trypsinized, counted and the growth curves were constructed. The doubling time (DT) was calculated according to the equation:

$$DT = (t\text{-}t_o)\log 2/\log Nt\text{-}\log Nt_o ,$$

where Nt_o and Nt were the numbers of cells at two selected time intervals t_o and t of the culture.

Separate cultures of pure VSMC or macrophages served as the control samples. For each experimental group, four independent samples were evaluated.

Preparation of macrophage-modified collagen

The purified collagen of type I (Sigma, St. Louis, MO, U.S.A.) was dissolved in Tris-HCl buffer (pH 8.0) at the concentration of 0.5 mg/ml. One milliliter of dust-activated macrophages (1.10^7 cells/ml) were added to 20 ml of collagen and incubated for 30 min. Then the cells and dust were sedimented by centrifugation and a new batch of activated cells was added. This procedure was then repeated twice so that the total time of exposition of collagen to activated macrophages was 120 min. When measuring the fluorescence of collagen (excitation 280 nm, emission 350 nm), we observed an interference caused by released proteins from activated macrophages. In order to measure these effects quantitatively, the fluorometer (Perkin Elmer LS-5) was calibrated with the manufacturer's standard No. 2 and the fluorescence intensity was expressed in relative fluorescence units (RFU). The results were expressed as the mean of triplicate measurements. To take into consideration the possible effect of released proteins on the proliferation of VSMC, we used control collagen which had been exposed to activated macrophages for 10 min. This time was sufficient for accumulation of macrophage-derived proteins while the exposition to oxidative environment was relatively short. As additional control samples, the normal unmodified collagen type I and standard tissue culture polystyrene Petri dishes (GAMA, České Budějovice, CR) were used.

Growth of VSMC on macrophage-modified collagen of type I

The collagen prepared according to the above described procedures was further diluted to the concentration of 5 mg/ml in Tris-HCl buffer (pH 8.0) sterilized by filtration (cellulose acetate filters, pore size 0.2 μm, Sartorius AG, Göttingen, Germany). Three milliliters of this collagen solution was added to polystyrene Petri dishes GAMA (diameter 5 cm, České Budějovice, CR) and adsorbed for 24 h at room temperature. After adsorption, the remaining collagen solution was removed, the dishes were rinsed in PBS (twice for 3 min) and seeded with the above mentioned rat aortic VSMC (30 000 cells and 3 ml of DMEM with 10 % FCS and 40 μg/ml of gentamicin per dish). As control samples, dishes coated with control collagen I and uncoated dishes were used. On days 1 and 2 after seeding, the number of adhered cells and their morphology were evaluated using an inverted microscope with an ocular grid. For each experimental group, six dishes were used (cells were observed in 10 randomly selected fields of each dish). On days 5, 7 and 9 after seeding, when the cell population densities were higher, the trypsinized cells were counted in a Bürker hemocytometer. In this case, three dishes for each day and experimental group were evaluated.

Statistics

The data were presented as means ± S.E.M. and compared by the Student's test for unpaired data.

Results

Adhesion and proliferation of VSMC in separate cultures

One day after seeding, only 52 % of VSMC (15 700 from 30 000 cells seeded per Nunclon dish) adhered to the dish bottom. However, they were well spread and spindle or polygonal in shape (Fig 1. A). Between days 1 to 7, they rapidly proliferated and reached a final population density of 608 000 ± 25 000 cells/cm^2 (Fig. 2).

Adhesion and proliferation of macrophages in separate cultures

Macrophages adhered in the initial number of 25 200 cells per dish (i.e. 84 % out of 30 000 cells seeded). In contrast to VSMC, most of the adhered macrophages retained their round shape and their adhesion area was much lower during all seven days of the experiment (Fig. 1 B). Despite this lower degree of spreading, the macrophages were more resistant to the

detachment by trypsin. After 10 min of treatment with 0.2 % trypsin in PBS, all VSMC but only 31 % of initially adhered macrophages (i.e. 7 900 out of 25 200 per dish) were detached from the dish bottom (the non-detached macrophages were counted using an inverted microscope with an ocular grid). As far as the proliferation of macrophages in separate cultures is concerned, they did not change their initial number significantly during the whole experiment (Fig. 2).

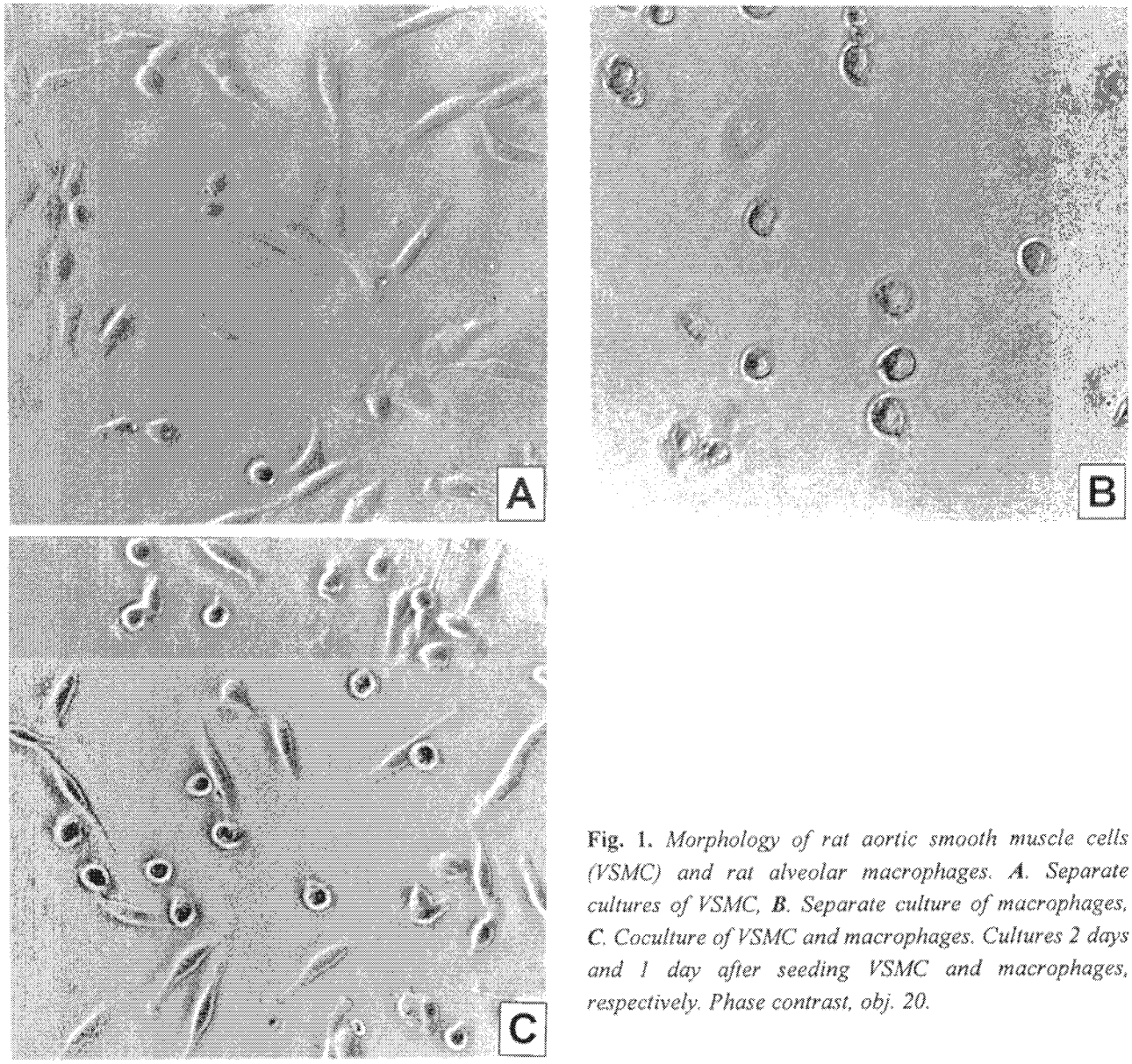

Fig. 1. *Morphology of rat aortic smooth muscle cells (VSMC) and rat alveolar macrophages.* ***A****. Separate cultures of VSMC,* ***B****. Separate culture of macrophages,* ***C****. Coculture of VSMC and macrophages. Cultures 2 days and 1 day after seeding VSMC and macrophages, respectively. Phase contrast, obj. 20.*

Adhesion and proliferation of VSMC and macrophages in cocultures

The morphology of attached and spread VSMC was similar to that in pure VSMC cultures. The cocultures contained more cells of round shape which involved both dividing VSMC and non-spread macrophages. It was not possible to distinguish between the two cell types under the conventional light inverted microscope without specific VSMC or macrophage staining, i.e. anti α-actin or anti ED-1 staining (Figs 1 A-C).

Between days 2 and 5 of the experiment, the number of cells as well as their doubling times were similar in both pure and macrophage-enriched VSMC cultures (Fig. 2; doubling time 21.4±1.7 h and 20.6±1.3 h, respectively). However, from day 5, the cocultures of VSMC and macrophages proliferated two times more rapidly on average (doubling time 95±4 h vs 190±53 h in control pure VSMC cultures, $p<0.05$). On day 7, they reached a significantly higher population density by 40 %, (853 000±32 000 vs 608 000±25 000 cells/cm^2 in pure VSMC cultures, $p<0.001$) (Fig. 2).

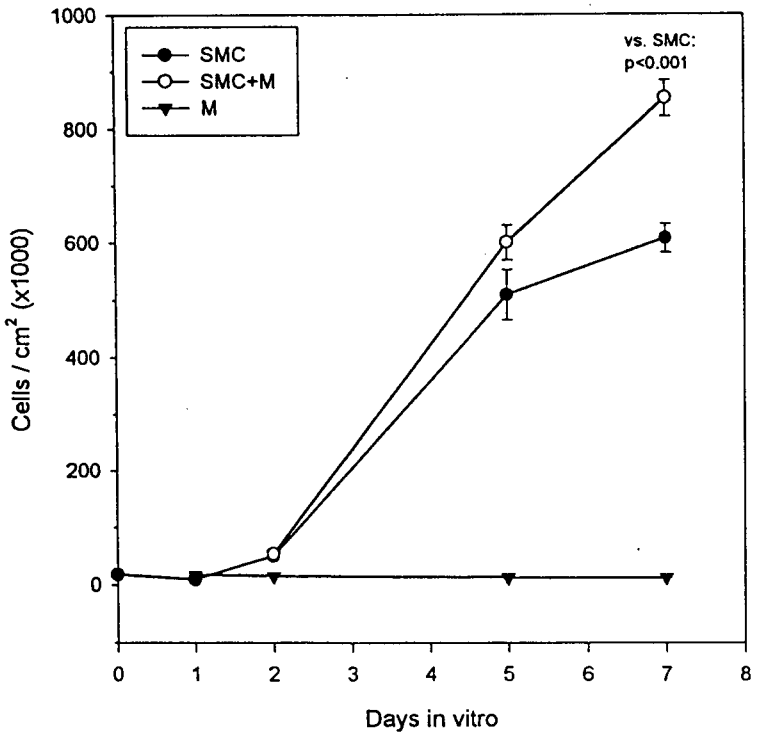

Fig. 2. *Growth curves of pure rat aortic smooth muscle cells (VSMC), pure rat alveolar macrophages (M) and cocultures of VSMC and macrophages (VSMC+M). Only adherent cells were included.*

Production of reactive oxygen species by macrophages activated by TiO_2 dust

In order to detect the production of reactive oxygen species, a method using luminol chemiluminescence was adopted according to our previous study (Vilím *et al.* 1987). Figure 3 shows the time-course of chemiluminescence production. It can be seen that the chemiluminescence intensity and therefore also the production of reactive oxygen species was maximal 9 min after addition of the dust and then slowly decreased. It was therefore assumed that after a 30 min incubation period collagen was exposed to all reactive oxygen species elicited by a single dose of TiO_2 dust.

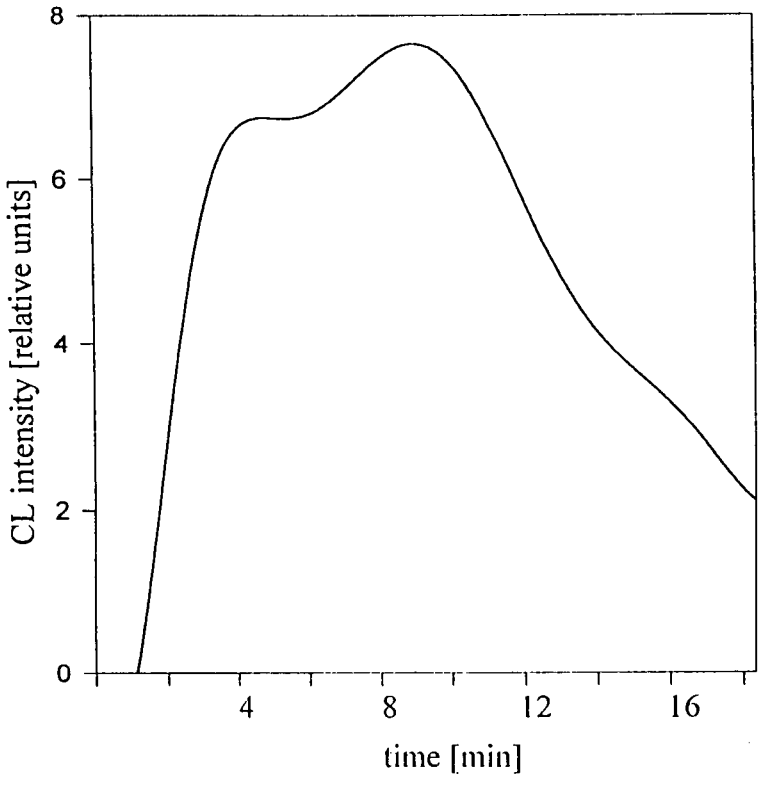

Fig. 3. *The time-course of chemiluminescence (CL) intensity elicited by single dose of TiO_2 dust (5 mg/ml) in alveolar macrophages. The dust was added at time 0.*

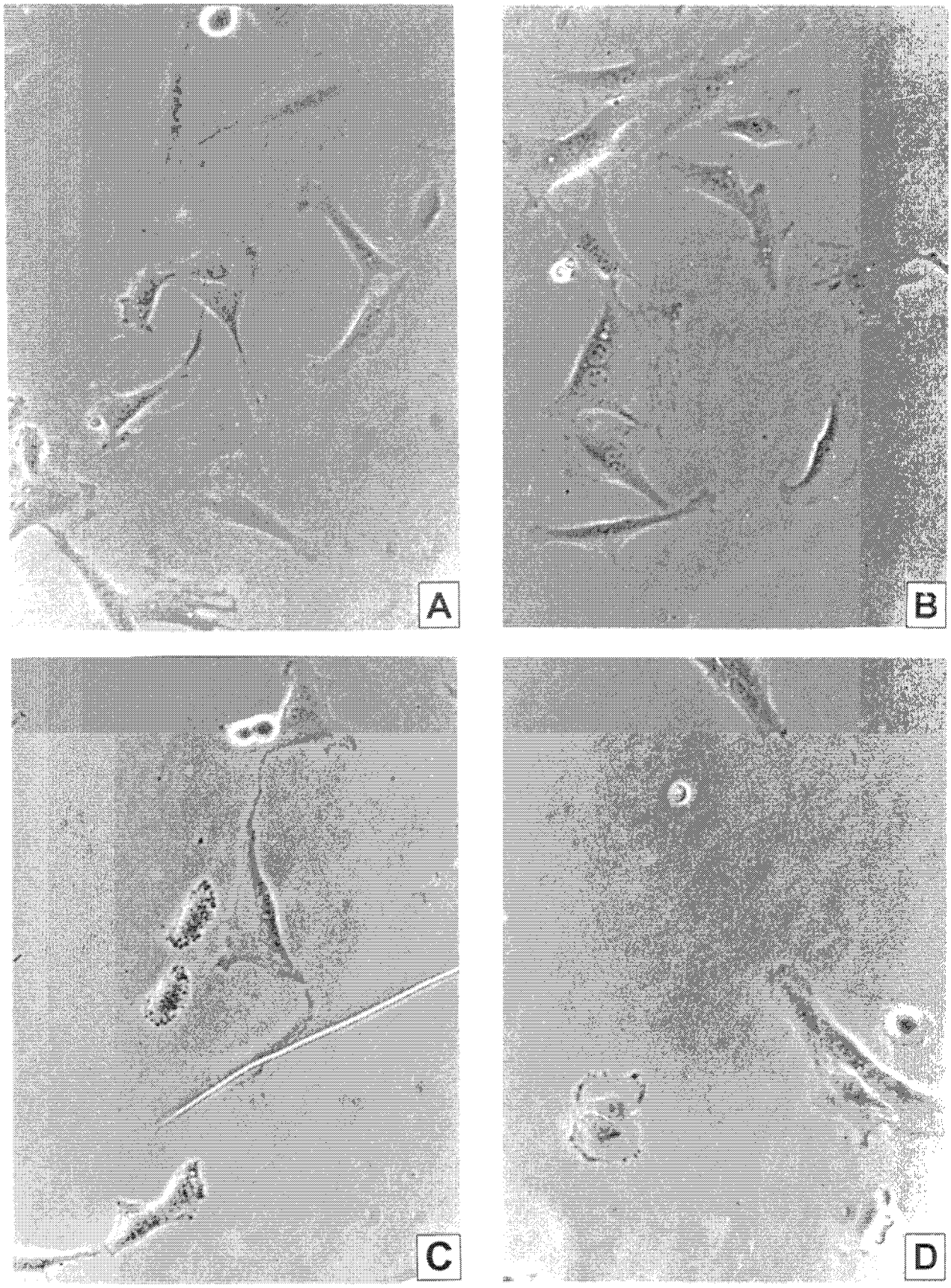

Fig. 4. *Morphology of rat aortic smooth muscle cells two days after seeding on normal unmodified collagen I (A), collagen I exposed to macrophages for 10 min (B), collagen I modified by macrophages for 120 min (C) and plastic GAMA dish (D). Phase contrast, obj. 20.*

During the period of macrophage activation, proteins were also released into the medium. This was documented by an increase in protein fluorescence measured as described in Materials and Methods. The incubation medium obtained after sedimentation of cells before the addition of dust had a fluorescence intensity of 15.9±2.3 RFU. This parameter increased to 113.2±5.6 RFU ($p<0.001$) after 10 min of incubation of the macrophages and stayed at this level for 60 min of the incubation. These measurements indicated that most of the protein was released from the macrophages during the first 10 min after activation with the dust. The total protein fluorescence intensity observed after 4 cycles of macrophage activation reached 237.3±9.2 RFU ($p<0.001$).

Adhesion and growth of VSMC on type I collagen modified by activated macrophages

One day after seeding, the absolute number of initially adhered cells was similar on the collagen modified by activated macrophages for 120 min, on both control collagens (i.e. exposed to macrophages only for 10 min and normal unmodified type I collagen) as well as on uncoated plastic dishes. However, the degree of cell spreading was much lower on 120-min-modified than on both control collagens. Many cells attached to this modified collagen remained round with a small adhesion area, while on both control collagens, significantly more cells were polygonal or spindle-shaped. The degree of cell spreading on 120-min-modified collagen was similar to that observed on uncoated plastic GAMA dishes (Figs 4 and 5). On day 2 and 3 after seeding, some of the cells spread on the 120-min-modified collagen became vacuolated, they regained their round shape and detached spontaneously from the growth support. All these findings were more apparent when the collagen was dried after the 24-hour-adsorption or adsorbed for 65 hours (data not shown).

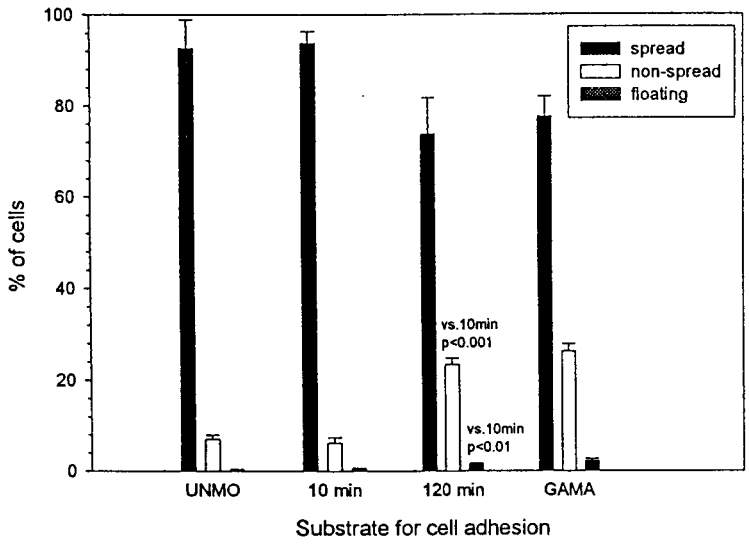

Fig. 5. *The percentage of attached spread, attached non-spread and floating cells in cultures of rat aortic smooth muscle cells one day after seeding on normal unmodified collagen I (UNMO), collagen I exposed to macrophages for 10 min (10 min), collagen I modified by macrophages for 120 min (120 min) and plastic GAMA dish (GAMA).*

Between days 1 and 5 after seeding, the doubling time of VSMC growing on 120-min-modified collagen was significantly longer than that on 10-min-exposed control sample (23.2±1.0 h vs 19.2±0.5 h, $p<0.05$). It did not differ significantly from those found on normal control collagen (20.9±0.8 h) and uncoated GAMA dishes (25.9 ±1.2 h).

Between days 5 and 7 after seeding, however, the doubling time of VSMC growing on 120-min-modified collagen was significantly shorter than that on normal unmodified collagen (26.6±2.2 h vs 36.3±2.0 h, $p<0.05$) and also had a tendency to be shorter than that on 10-min-exposed collagen (26.6±2.2 h vs 31.8±2.9 h). On days 7 and 9 after seeding, the cells growing on all three collagens reached similar population densities. These densities were significantly higher than those on uncoated GAMA dishes ($p<0.001$) (Fig. 6).

Discussion

We have found that the cocultures of rat aortic VSMC and activated rat alveolar macrophages have a higher growth capacity in comparison to separate cultures of pure VSMC or macrophages. Between days 5 to 7 after

seeding, the cocultures of VSMC and macrophages exhibited a significantly shorter doubling time and they reached a significantly higher population density on day 7. Two possible explanations will be discussed here. First, stimulation of VSMC growth results from increased production of ROS (and/or products of NO-ROS interaction) and peptide growth factors released from activated macrophages. Second, the increase of cell number is the result of the proliferation of macrophages, which do not exert any proliferative activity in separate cultures without vascular smooth muscle cells.

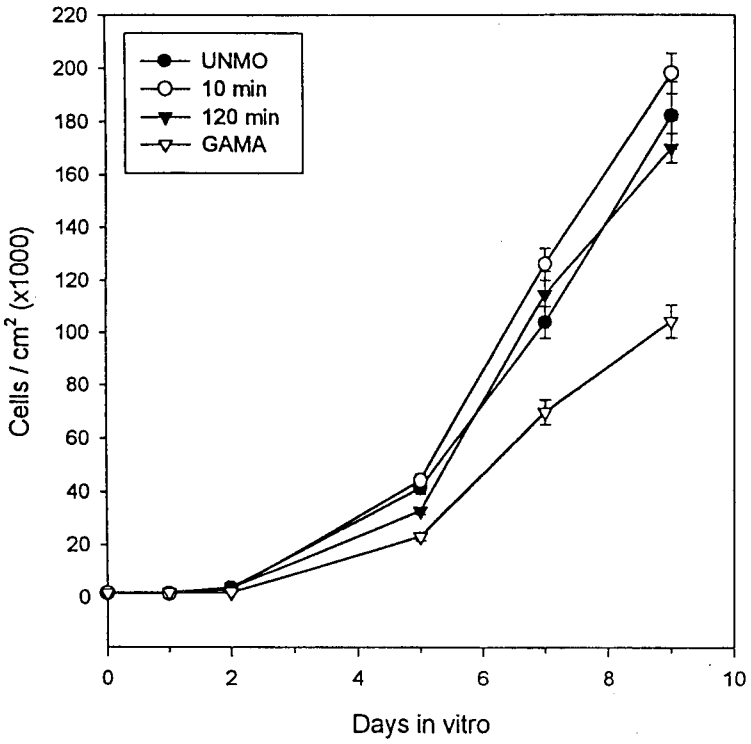

Fig. 6. *Growth curves of rat aortic smooth muscle cells on normal unmodified collagen I (UNMO), collagen I exposed to macrophages for 10 min (10 min), collagen I modified by macrophages for 120 min (120 min) and plastic GAMA dish (GAMA).*

At lower concentrations, the ROS function as physiological mediators of cellular responses, i.e. similarly as the growth factors. In VSMC they can activate receptor and non-receptor tyrosine kinases, mitogen-activated protein kinases, transcription factor NF-kB, the expression of Bcl-2 gene and protooncogenes c-fos and c-jun and autocrine production of growth factors (Baas and Berk 1995, Ozer *et al.* 1995, Bae *et al.* 1997, Delafontaine and Ku 1997, Gebicki 1997, Rao 1997). Burdon (1996) hypothesized that normal production of reactive oxygen species is necessary for normal transduction of cellular signals regulating cell growth. The possibility that increased oxygen radical species production is consistent with vascular remodeling in pulmonary hypertension, is supported by the fact that exposure of animals to chronic hyperoxia stimulates vascular cell growth and fibroproduction. Animals kept in a hyperoxic atmosphere (more than 80 % O_2 in inspired air) develop pulmonary vascular remodeling similar to that described after exposure to a hypoxic environment (Jones *et al.* 1984, Reid 1986). In hyperoxia, the production of radical oxygen species in the lung tissue is definitely increased (Freeman and Crapo 1981). At higher concentration, ROS can cause growth arrest, damage and death of cells (Fanburg and Lee 1996). For example, they inhibit protein kinase C, activate proteases and endonucleases, increase the expression of heat-shock proteins (Nishigaki *et al.* 1991, Ozer *et al.* 1995, Pirillo *et al.* 1997). However, the dying cells and their surviving neighbors can release growth factors (e.g. the fibroblast growth factor) which can finally stimulate the proliferation of a cell population originally damaged by the oxidative stress (for review see Ozer *et al.* 1995).

The significant increase in growth of the VSMC/macrophage mixed population was not observed before day 5 after adding macrophages. This delay suggests that the accelerated cell proliferation can be attributed to autocrine production of growth factors in macrophages and VSMC rather than to an acute effect of short-living radicals released from macrophages immediately after they had been added to VSMC cultures. In coculture with VSMC as well as in damaged

vessel wall *in situ*, the macrophages can be stimulated, e.g. by chemokines, cytokines and extracellular matrix molecules released from VSMC, to synthesize peptide growth factors mitogenic for both VSMC and macrophages. These factors include platelet-derived growth factor-like molecules, transforming growth factors, macrophage-derived growth factor, activin A and colony-stimulating factors (Kaufmann *et al.* 1990, Rosenfeld *et al.* 1992, Inoue *et al.* 1993, Ozer *et al.* 1995, Herembert *et al.* 1997, Plenz *et al.* 1997). On the other hand, short-living radicals can oxidize cellular and extracellular proteins and lipids, which are more stable, can persist, cumulate in the cultures and exert a long-term delayed effect on cell proliferation (Gebicki 1997).

In our study no special staining was applied to distinguish between VSMC and macrophages, e.g. anti alpha-actin and anti ED-1 staining (Skalli *et al.* 1986, Wiener *et al.* 1996). It is therefore possible that not only the VSMC but also macrophages can proliferate in VSMC/macrophage cocultures. Although in the present study, as well as in the study of Kaufmann *et al.* (1990), the macrophages seeded on plastic did not proliferate, they could do so in the presence of VSMC. The VSMC of damaged vessels as well as those in culture are able to produce the macrophage-colony stimulating factor, the main growth factor for macrophages (Rosenfeld *et al.* 1992, Herembert *et al.* 1997, Plenz *et al.* 1997).

The second result of our study is that the activated macrophages can influence VSMC adhesion by modifications of type I collagen. During the first five days in culture, the effects of 120-min-modified collagen on VSMC was slightly cytotoxic. This is documented by the round shape of many initially adhered cells, small cell adhesion area, vacuolization and spontaneous detachment of some cells. Furthermore, the resistance of these cells to the detachment by trypsin was somewhat lower. This lower adhesion ability and damage of VSMC can be explained by oxidative modification of collagen by the macrophages. The oxygen can be introduced in the amino acids of certain important sequences, such as RGD and DGEA, which bind to the ß1 integrin adhesion molecules on VSMC (Mattana *et al.* 1997). In our previous study we also observed a lower resistance to trypsin detachment in VSMC grown on collagen I oxidized by ultraviolet radiation (Bačáková *et al.* 1997c).

The proliferation of VSMC was also influenced by modifications of type I collagen by macrophages in this study. Between days 1 and 5, the doubling time of VSMC on 120-min-modified collagen was prolonged, which can be explained by the above mentioned damage and death of some cells. However, between days 5 and 9, the growth rate and population densities of VSMC on the collagen modified for 120 min were similar or even a little higher compared to cells grown on both control collagens. This could be due to the release of mitogens from damaged as well as from surviving VSMC (Ozer *et al.* 1995) and/or by the degradation of collagen molecules into smaller fragments which are mitogenic for mesenchymal cells (Gardi *et al.* 1990, 1994).

The proteins released by activated macrophages during collagen modification (see the Materials and Methods) can be excluded as the cause of different behavior of VSMC on collagens exposed to macrophages for 120 or 10 min, as their concentrations were similar in both collagen samples. The differences in the VSMC adhesion and growth was more probably due to some direct modification of collagen by the macrophages, such as oxidative damage and/or degradation.

Thus, the present study together with our previous reports (Wilhelm *et al.* 1996, Bačáková *et al.* 1997c), indicates that the activation of alveolar macrophages may participate in the process triggering vascular smooth muscle cell proliferation in hypoxic pulmonary hypertension. The mechanism may be related to a paracrine production of growth factors for VSMC by stimulated macrophages and/or to the oxidative modification of matrix proteins of the walls of peripheral pulmonary arteries.

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Abbreviations

DMEM	Dulbecco-modified Eagle minimum essential medium
FCS	Fetal calf serum
DT	Doubling time
M	Macrophages
PBS	Phosphate-buffered saline
RFU	Relative fluorescence units
ROS	Reactive oxygen species
SEVAC	Institute of Sera and Vaccines
SPF	Specific pathogen-free
UNMO	Unmodified collagen
VSMC	Vascular smooth muscle cells

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