phenotypic maturation of vascular and bone-derived cells. Nanopatterned surfaces provided good support for the adhesion, spreading, growth and metabolic activity of these cells. All these surfaces could be useful for tissue engineering, construction of cell arrays and biosensors.

Acknowledgements

This study was supported by the Academy of Sciences of the Czech Republic (grants No. 1QS500110564, KAN400480701, KAN101120701, IAAX00100902) and the Grant Agency of the Czech Republic (grant No. 305/08/0108). Mr. Robin Healey (Czech Technical University, Prague) is gratefully acknowledged for his language revision of the manuscript. We also thank Mrs. Vera Lisa and Mrs. Ivana Zajanova (Inst. Physiol., Acad. Sci. CR, Prague) for their excellent technical assistance in cell culturing and immunocytochemical techniques.

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VASCULAR SMOOTH MUSCLE CELLS IN CULTURES ON BIOFUNCTIONALIZED CELLULOSE-BASED SCAFFOLDS

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Abstract

Viscose, dialdehyde cellulose and oxidized 6-carboxycellulose with 2.1 or 6.6wt.% of –COOH groups were prepared. The materials were subsequently functionalized with arginine or chitosan. Both unmodified and biofunctionalized materials were seeded with vascular smooth muscle cells. The morphology of the adhered cells indicated that oxidized 6-carboxycellulose with 2.1% content of –COOH groups was the most appropriate of all tested materials for potential use in tissue engineering. The shape of the cells on this material was elongated, which demonstrates adequate adhesion and viability of the cells, while the morphology of the cells on other tested materials was spherical. Moreover, the stability of 6-carboxycellulose with 2.1wt.% of –COOH groups in the cell culture environment was optimal, with a tendency to degrade slowly with time. The highest stability was found on the viscose samples, whereas there was very low stability on oxidized 6-carboxycellulose with 6.6 wt. % of –COOH groups, and also on dialdehyde cellulose. Functionalization with arginine or chitosan increased the number of adhered cells on the materials, but not markedly. We did not obtain a significant elevation of the cell population densities with time on the tested samples. These results suggest the possibility of using a cellulose-based material in such tissue engineering applications, where high proliferation activity of cells is not convenient, e.g. reconstruction of the smooth muscle cell layer in bioartificial vascular replacements.

Key words: *oxidized cellulose, tissue engineering, biofunctionalization, chitosan, arginine, vascular smooth muscle cells*

[Engineering of Biomaterials, 89-91, (2009), 21-24]

Introduction

Cellulose, composed of glucose monomers, is a polysaccharide commonly occurring in nature. Oxycellulose is cellulose oxidized by oxidizing agents, such as NO_2 or $NaClO_2$, which induce conversion of the glucose residues to glucuronic acid residues, i.e. compounds containing –COOH groups [1]. The concentration of these groups modulates the pH, swelling in a water environment, degradation time, drug loading efficiency and other behavior of the material [2]. In addition, –COOH groups, which are polar and negatively charged, can be used for functionalizing the oxidized cellulose with various biomolecules.

Oxidized cellulose has been widely used for many years as a wound-healing material due to its excellent properties, such as high absorbability, antibacterial and antiviral activity, non-toxicity and effects that prevent the formation of tissue adhesion after surgery [3-6]. Due to its ability to initiate or accelerate blood coagulation at the site where it is applied, oxidized cellulose has been used as a hemostatic material [7]. This material is also promising as a carrier for controlled drug delivery [2]. The possible use of various types and modifications of cellulose-based materials in tissue engineering is under extensive scientific research [8-10]. For example, cellulose fabrics [8] and injectable cellulose-based hydrogels [9] have been successfully tested as carriers for chondrocytes for cartilage regeneration in vitro and in vivo.

In this study, we have investigated the adhesion, growth and morphology of vascular smooth muscle cells on various types of cellulose materials for potential use in soft tissue engineering. Four basic groups of materials were prepared: primal viscose (VIS), oxidized 6-carboxycellulose with 2.1wt.% of –COOH groups (2.1), oxidized 6-carboxycellulose with 6.6% of –COOH groups (6.6) and dialdehyde cellulose from cotton knitting (DAC). The samples were further functionalized with arginine or chitosan. Positively-charged amine groups in these biomolecules were expected to support the adsorption of cell adhesion-mediating molecules and cell adhesion. At the same time, amino acids with the basic side chain (lysin, arginin), as well as chitosan, were expected to balance the relatively acid character of oxidized cellulose molecules [11].

Material and methods

Preparation of biofunctionalized scaffolds

Cellulose-based materials (listed above) in the form of woven fibrous scaffolds were exposed to solutions of arginine or chitosan (USA, Sigma-Aldrich) for 2 hours at laboratory temperature (20°C). The quantities of particular substances used for preparation of the solutions are summarized in TABLE 1. The samples were then washed twice for 2 hours in isopropyl alcohol, and were air-dried. Dialdehyde cellulose from cotton knit functionalized with arginine could not be created because of its high tendency to disintegrate.

Prepared bio-functiona-lized material	Primal textile material [g]	Arginine or chitosan [g]	Acetic acid [ml]	Distilled H_2O [ml]
VIS_arg	6 g	2 g Arg	0 ml	150 ml
VIS_chit	6 g	6 g Chit	2.5 ml	500 ml
2.1_arg	6 g	1.48 g Arg	0 ml	150 ml
2.1_chit	6 g	6 g Chit	2.5 ml	500 ml
6.6_arg	6 g	4.76 g Arg	0 ml	150 ml
6.6_chit	6 g	6 g Chit	2.5 ml	500 ml
DAC_chit	2.4 g	2.4 g Chit	1 ml	500 ml

TABLE 1. Quantity of ingredients in the arginine or chitosan solution used for functionalization of the primal materials.

Cells and culture conditions

The fibrous scaffolds were cut into square pieces (2x2 cm) and sterilized by UV light from both sides, 1 hour for each side. Earlier pre-experiments had shown that UV light sterilization is the most appropriate method for this kind of material, as sterilization in ethanol or by autoclaving have caused morphological, chemical and mechanical instability of samples. After sterilization, the samples were inserted into 12-well culture plates (well diameter 2.2cm; TPP, Switzerland) and fixed to the well bottoms with plastic rings to avoid flotation of the sample. Then they were seeded with vascular smooth muscle cells (VSMC), derived from the complex of the tunica intima and media of the rat thoracic aorta by an explantation method. The cells were used in passage 4 and in a density of 60,000 cells/well (i.e., about 21,000 cells/cm^2). The cells were cultured in Dulbecco's modified Eagle's Minimum Essential Medium (DMEM; Sigma, U.S.A., Cat. No D5648; 3 ml/well), supplemented with 10% of fetal bovine serum (FBS; Sebak GmbH, Aidenbach, Germany) and gentamicin (40µg/ml, LEK, Ljubljana, Slovenia), at 37o C in a humidified atmosphere containing 5% of CO_2 in the air. Materials with 6.6wt.% of –COOH groups, especially those non-modified with arginine and chitosan, caused an excessive decrease in the pH of the culture medium, as indicated by the phenol red pH indicator contained in the medium, which changed the colour of the medium from pink to yellow as early as 3 hours after seeding. The medium on these samples was therefore replaced by fresh medium, and after replacing it the same effect did not recur.

On the 2nd, 4th and 7th day after seeding, the cells were rinsed with phosphate-buffered saline (PBS; Sigma-Aldrich), detached from the materials by a trypsin-EDTA solution (Sigma, U.S.A., Cat. N^0 T4174) and counted using a Burker haemocytometer. For each time interval and experimental group, two independent samples were used, and another parallel group of samples was used for evaluating the cell morphology. For visualization, the cells were fixed with 70% cold ethanol (-20^{o}C, 5-10 min) and stained with a combination of the following fluorescence dyes: the cell membrane and cytoplasm was stained with Texas Red C_2-maleimide (Molecular Probes, Invitrogen, Cat. No. T6008; 20ng/ml PBS), and the cell nuclei were stained with Hoechst 33342 (Sigma, U.S.A.; 5µg/ml PBS) for 1 hour at room temperature. Digital pictures of the cells were taken using a conventional fluorescence microscope (Olympus IX 50, Japan) and a confocal microscope (DM 2500, Leica, Germany).

Statistical analysis

The quantitative data was presented as mean ± SEM (Standard Error of Mean) from 36 measurements. Multiple comparison procedures were performed by the One Way Analysis of Variance (ANOVA), Student-Newman-Keuls method, using SigmaStat software (Jandel Corp. U.S.A.). P values equal to or less than 0.05 were considered significant.

Results and discussion

FIGURE 1 presents the number of cells on the 2nd, 4th and 7th day after seeding on various tested scaffolds. As can be seen, on day 2 after seeding, the highest number of cells was found on viscose modified with arginine. Functionalization with arginine also had a beneficial effect on cell colonization in oxidized 6-carboxycellulose with 2.1wt.% or 6.6wt.% of –COOH groups, where the highest number of cells was detected on day 4. Cell colonization was also improved by modifying the cellulosic materials with chitosan. On day 7,

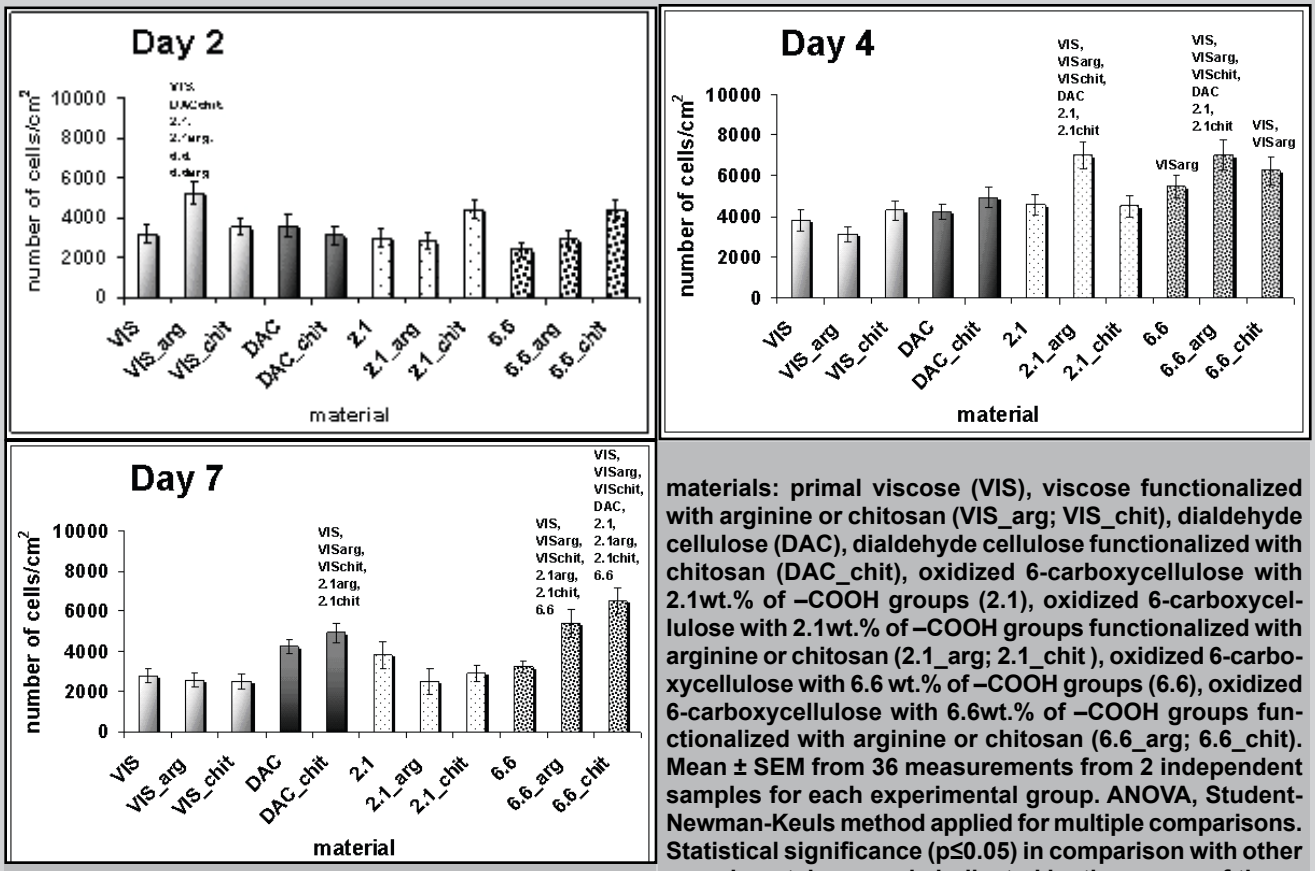

FIG.1. Number of rat aortic smooth muscle cells on days 2, 4 or 7 after seeding on various types of cellulose-based materials: primal viscose (VIS), viscose functionalized with arginine or chitosan (VIS_arg; VIS_chit), dialdehyde cellulose (DAC), dialdehyde cellulose functionalized with chitosan (DAC_chit), oxidized 6-carboxycellulose with 2.1wt.% of –COOH groups (2.1), oxidized 6-carboxycellulose with 2.1wt.% of –COOH groups functionalized with arginine or chitosan (2.1_arg; 2.1_chit), oxidized 6-carboxycellulose with 6.6 wt.% of –COOH groups (6.6), oxidized 6-carboxycellulose with 6.6wt.% of –COOH groups functionalized with arginine or chitosan (6.6_arg; 6.6_chit). Mean ± SEM from 36 measurements from 2 independent samples for each experimental group. ANOVA, Student-Newman-Keuls method applied for multiple comparisons. Statistical significance ($p \leq 0.05$) in comparison with other experimental groups is indicated by the names of these groups above the columns.

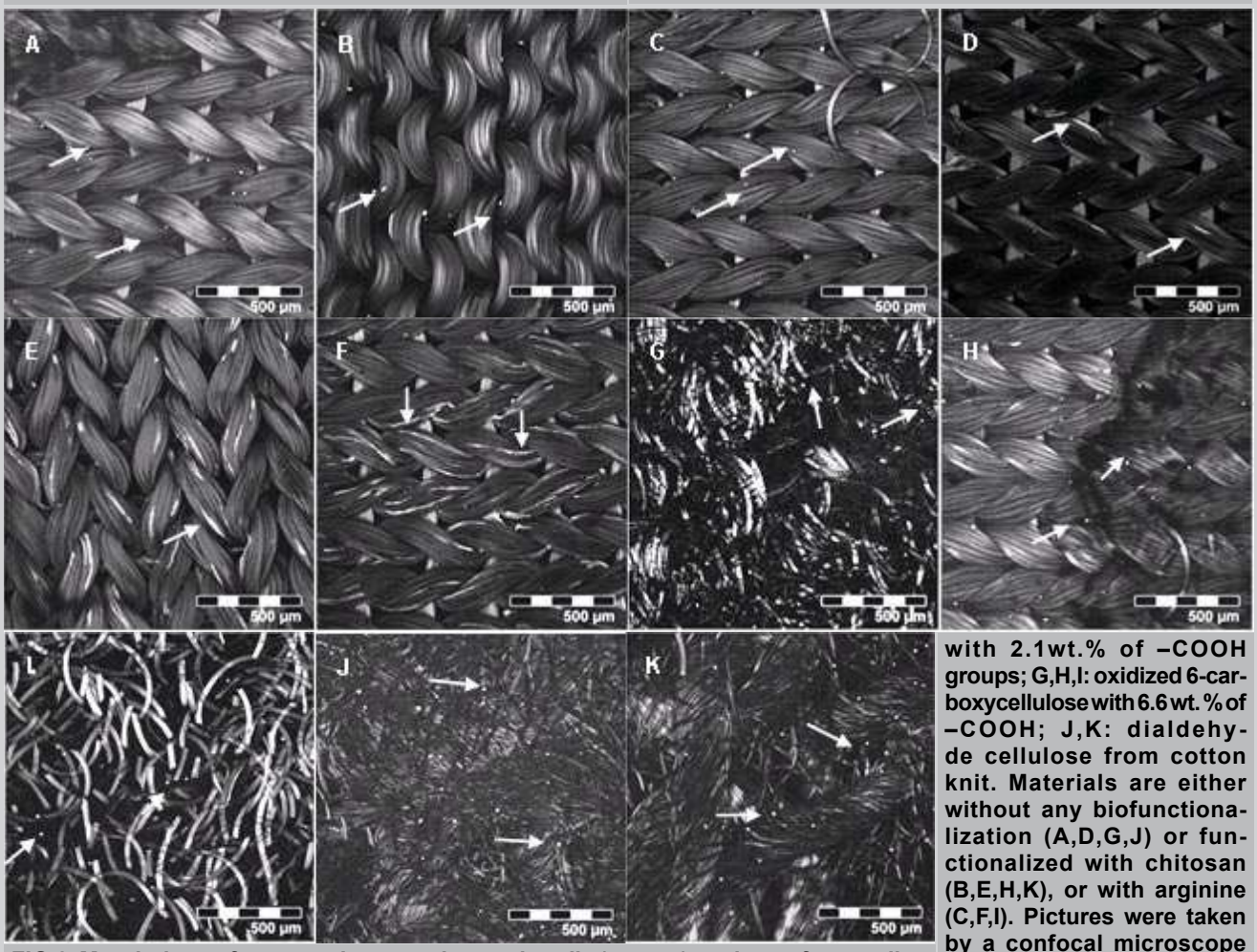

FIG.2. Morphology of rat vascular smooth muscle cells (arrows) on day 4 after seeding on cellulose-based materials. A,B,C: viscose; D,E,F: oxidized 6-carboxycellulose with 2.1wt.% of –COOH groups; G,H,I: oxidized 6-carboxycellulose with 6.6 wt.% of –COOH; J,K: dialdehyde cellulose from cotton knit. Materials are either without any biofunctionalization (A,D,G,J) or functionalized with chitosan (B,E,H,K), or with arginine (C,F,I). Pictures were taken by a confocal microscope (DM 2500, Leica, Germany).

the highest cell population densities were obtained on DAC and 6-carboxycellulose with 6.6wt% of –COOH groups, both combined with chitosan. On the latter material with chitosan, the cell number was about twice higher than on non-modified 6-carboxycellulose with 6.6wt.% of –COOH groups.

Thus, as expected, the number of adhering cells and their subsequent growth were improved after functionalization of the materials with arginine or chitosan, although relatively slightly. This phenomenon can be attributed to the presence of positively-charged amine groups in these biomolecules. Positively-charged groups have been reported to support the adsorption of cell adhesion-mediating molecules (e.g., vitronectin, fibronectin) from the serum of the culture medium in appropriate geometrical conformations, which increase the exposure and accessibility of the active sites on these molecules, e.g. specific amino acid sequences like RGD, to cell adhesion receptors, e.g. integrins [12]. In addition, the basic character of arginine and chitosan molecules at least partly compensated the acidity of 6-carboxycellulose with 6.6wt.% of –COOH groups, which is a common problem of oxidized cellulose [11].

In addition, a significant contrast was found in the cell morphology, where oxidized 6-carboxycellulose with 2.1wt.% of –COOH groups (unmodified and functionalized with Arg or chitosan) appeared to be the most convenient material for cell adhesion, as the shape of the cells was elongated here. The morphology of the cells on other samples was spherical, which suggests weak spreading and adhesion of cells, and thus lower viability (FIG.2). The most appropriate shape that vascular smooth muscle cells can assume for proliferation is polygonal. For example, in cultures of VSMC obtained from the aorta of neonatal rats, the intensively proliferating cells were large, polygonal in shape and well spread, while the slowly growing cells were generally spindle-shaped and not well spread [13]. In our experiments, the VSMC on the tested materials could not acquire a polygonal morphology due to the knitted design of the materials, where the cells were guided to be arranged along the fibers in the scaffolds. This was probably a reason for their weak proliferation and for the only small increase in the cell population densities with time (i.e. from about 2000-5000 cells/cm^2 on day 2 to 2000-7000 cells/cm^2 on days 4 and 6, FIG.1), together with the acidic character of the cellulose materials and also their tendency to swell and degrade.

Higher oxidation of cellulose led not only to higher acidity, but also to lower stability of this material in the cell culture environment. The content of 6.6wt.% of –COOH groups appeared to be too high from this point of view, as these samples disintegrated in the culture medium after simple handling (e.g. washing in PBS, replacing them in another dish, staining, etc.) In addition, the stability of dialdehyde cellulose proved to be very low, probably because of the specific arrangement of the fibers in its fabric, which resembled a loose network, while in oxidized cellulose or viscose the fibers were densely packed in thick rope-like bundles. The highest stability was observed in the viscose materials, which allowed almost no tendency to degrade. The stability of oxidized cellulose with 2.1wt.% of –COOH was high enough, as it did not disintegrate during sample handling, and its degradation in the cell culture system was relatively slow in comparison with oxycellulose with 6.6wt.% of –COOH and dialdehyde cellulose.

Conclusion

The results obtained in this study indicate that oxidized cellulose with 2.1wt.% of –COOH groups could be an appropriate material for tissue engineering, due to its relatively high stability during handling and exposure to the cell culture environment, and particularly its biocompatibility, which can be further improved by modification with biomolecules, e.g. arginine. As this material induces spreading but no considerable proliferation of cells, it could be used in constructing bioartificial tissues or organs where high proliferation activity of cells is not desired, for example in replacements for blood vessels, where high growth of cells on the material or total bioinertness of the material may cause stenosis or other non-physiological behavior of the vascular prosthesis [14,15].

Acknowledgements

This study was supported by the Ministry of Industry and Trade of the CR (grant No. 2A-1TP1/073) and the Ministry of Education, Youth and Sports of the CR (Grant No. 2B06173). Mr. Robin Healey (Czech Technical University, Prague) is gratefully acknowledged for his language revision of the manuscript.

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