Surface modification of cyclic olefin copolymers for osteochondral defect repair can increase pro-destructive potential of human chondrocytes in vitro

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SUMMARY

Introduction:

Materials on the basis of cycloolefin copolymers (COC) are suitable for subchondral defect repairs. The objective of this study was to evaluate the influence of surface modification of COC and COC/LLDPE blends on the viability and gene expression of chondrocytes.

Materials and methods:

Human chondrocytes were incubated on the surface of the studied materials. Half of the materials were plasmatically modified with subsequent type II collagen application. The gene expression of matrix metalloproteinases (MMP-1,-3,-13), pro-inflammatory cytokines (IL-1, TNF-alpha) and apoptotic molecules (BAX, Bcl-2) was evaluated using quantitative Taq-Man PCR after a 48-hour incubation. Chondrocyte viability was evaluated by the MTT test after 2, 4 and 8 days of incubation. The synthesis of MMPs was measured by ELISA assay in cell culture medium after 48 hours of incubation.

Results:

Chondrocytes incubated on plasmatically modified in contrast to unmodified materials demonstrated significantly increased gene expression of IL-1 ($p<0.05$), MMP-1 and MMP-3 ($p<0.05$ for both comparisons) as well as MMP-13 ($p<0.001$). Increased gene expression was confirmed by significantly increased production of active forms of particular MMPs into the cell culture medium. Unlike surface unmodified polymers, the modified materials showed time-dependent reduction of chondrocyte viability. The gene expression of TNF-α and apoptotic molecules by chondrocytes was not significantly changed by different materials.

Conclusion:

Cycloolefin copolymers and their blends may represent suitable materials for tissue engineering, however, their surface modification followed by collagen type II application may, at least under *in vitro* conditions, reduce the viability of chondrocytes and induce their pro-destructive behavior. The potential benefit or disadvantage of surface modifications of materials for osteochondral defect repairs needs to be further elucidated.

Key words: osteochondral defects, cycloolefin copolymer, chondrocytes, biocompatibility

Introduction

Osteoarthritis (OA) is one of the most frequent joint impairment with the underlying hyaline cartilage defect, gradual loss of proteoglycans and altered collagen structure (Goldring and Goldring 2007). The precondition for the development of these changes is a limited repair capacity of the extracellular matrix of hyaline cartilage. Mechanical forces play a major role in the development of OA. Age represents also an important risk factor. Nevertheless, osteochondral defects, which represent the predispositions of mechanical instability of the joint and subsequent development of the degenerative arthritic process, may occur also in the young age following a joint trauma (Kraan *et al*. 2002). Chondrocytes as well as subchondral bone are actively involved in the pathogenesis of OA by producing proinflammatory cytokines in an increased amount, e.g. tumour-necrosis factor (TNF)-alpha, interleukin (IL)-1, IL-6 or prostaglandins, nitric oxide (NO) and matrix metalloproteinases (MMPs) (Hedbom *et al*. 2002, Hulejová *et al*. 2007). Increased apoptosis of chondrocytes is expected to be significantly involved in the pathogenesis of advanced OA (Carlo and Loeser 2008).

Severe joint impairment by the osteoarthritic process or major cartilage defects often requires a total joint replacement. The matter of debate are smaller but symptomatic chondral and osteochondral defects that may be potentially treated by using methods of tissue-engineering, implantation of autologous chondrocytes, transplantation of osteochondral autografts, or an artificial osteochondral defect repair (Chajra *et al*. 2008). The purpose of such procedures is to reduce pain, induce regeneration of the affected articular cartilage and prevent the progression of OA. The research of biological materials for artificial replacement of skeletal elements is focused upon those materials whose biomechanical properties are as similar as possible to organic tissues and which are tolerated by the biological environment.

Out of all materials, synthetic polymers have a special position and are well tolerated by the biological environment (Martin *et al*. 2007). Biocompatibility of polymers in implants depends on interactions occurring between their surface and a biological environment. The first step of interaction of living body with a surface is protein adsorption. This interaction can be strongly influenced by modifying the surface chemistry, the surface charge and topography. Because of the inert nature of polymer surfaces they usually need to be functionalized. It was already reported that plasma treatment could modulate chondrocyte gene expression (Tsai *et al.* 2005). Collagen adsorption is known to promote cell adhesion and proliferation. For the purposes of this study, the authors selected cyclic olefin copolymers that demonstrated to be suitable for subchondral defect repairs. Surface modification by plasma and subsequent collagen application generally increases perspectives for the material application (Pešáková *et al*. 2005). In this paper, the authors evaluate the impact of surface modification of biological implants for osteochondral defect repair with plasma and subsequent collagen type II application on the viability and gene expression of human chondrocytes.

Materials and methods

Materials

Basic components:

COC - cycloolefin copolymer, chemically copolymer ethylen–norbornen (60-40), commercial product Topas 8007F- 400, Topas Advanced Polymers (Germany).

LLDPE –linear low density polyethylene, commercial product Exact 0102, Exxon, (USA)

Polymeric materials used for *in vitro* experiments:

a) COC

b) COC with surface plasmatic modification (N, O) and application of 0.3% type II collagen

c) COC/LLDPE (80/20) blend

d) COC/LLDPE (80/20) blend with surface plasmatic modification (N, O) and application of 0.3% type II collagen

COC and its blend were processed by mixing of components in the W 50 EH chamber of a Brabender Plasticorder at 190°C and 60 rpm for 8 min. All components were dosed into the mixer chamber together at the beginning of mixing. The molten material was taken out and pressed into plates from which the test samples were prepared. COC/LLDPE blend was irradiated by γ-rays (radiation dose 50 kGy) to obtain crosslinked structure in LLDPE particles dispersed in COC matrix.

One half of both the samples were modified by the nitrogen MW plasma using MW reactor equipped with SLAN I OV 425 magnetron (Plasma Consult) with power of 300 W, pressure of nitrogen 80 Pa, nitrogen flow 15 scm^3/min and exposure 15 min. It was deduced from photoelectron spectra that this treatment resulted in production of hydroxyl, carbonyl, carboxyl and ammine functional groups on the polymer surface. Before use, the samples were sterilized with ethylene oxide. Materials with plasmatically modified surface were sterile coated with type II collagen using 0.3% solution in PBS buffer and 2h incubation at room temperature. Collagen type II was prepared as describe previously (Pešáková *et al.* 1994).

Cell cultures

For *in vitro* experiments, two different cell lines of commercially available human chondrocytes (Normal Human Articular Chondrocytes, Knee cryopreserved – NHAC-kn13354 and NHAC-kn14757, DIAGENES sro., Czech Republic) were used. Chondrocytes were incubated in a specific differentiation medium (Chondrocyte Differentiation Medium CDM BulletKit, DIAGENES sro., Czech Republic) containing 5% FCS under 37°C in the atmosphere with 5% of CO_2. The fifth passage was used for the experiments. The testing of materials was conducted in 12-well plates with the materials of 21mm diameter inserted at the very bottom of each well.

Gene expression

Chondrocyte cell lines were seeded in the amount of 100. 000 cells per well and incubated for the period of 48 hours on individual materials, which were placed at the bottom of the relevant wells. All experiments were carried out in triplets. The negative control related to

modified materials was represented by the same number of chondrocytes incubated in the absence of materials. Following the time interval, cells were lysed using RLT Lysis Buffer (Qiagen). The total RNA was isolated from the cell lysate and by reverse transcription was transferred to complementary (c)DNA, which was used as the quantitative Taq-Man PCR reaction template. Gene expression of matrix metalloproteinases (MMP-1, -3 and -13), pro-inflammatory cytokines (IL-1, TNF-α) and apoptotic molecules (BAX, Bcl-2) (Sigma, Czech Republic) was evaluated. A probe for 18 S (Applied Biosystems) was used as the endogenous control.

Evidence of cell viability and cytotoxicity of tested materials using MTT test

Cell viability was carried out by the MTT test (Sigma, Czech Republic). MTT is a colorimetric method, the distinction between viable and dead cells is based on the ability of viable cells to reduce yellow, soluble MTT tetrazolium salt (3-4,5-dimethylazol-2-yl)-2,5-diphenyl tetrazolium) by the succinate-dehydrogenase mitochondrial enzyme to insoluble blue formasan. Formasan is then dissolved by dimethyl sulfoxide (DMSO), or by another organic solvent, if applicable. The absorbance of the colour product is measured by photometry at 570 nm (ELISA reader SUNRISE). One chondrocyte cell line was incubated on tested materials for the period of 2, 4 and 8 days. After the particular intervals, MTT staining was carried out in one experiment to check for cell viability.

ELISA

Using the ELISA assay, levels of active MMP-1, -3, -13 (Ray Biotech, Inc., Norcross GA) were established in cell culture supernatant after 48 hours of incubation as described previously (Senolt *et al.* 2006). The analysis was performed using ELISA reader SUNRISE (Tecan, Salzburg, Austria) in the wavelength of 450 nm. The measured absorbance was related to the number of cells in the particular experiment. Testing was carried out in two independent experiments.

Statistical analysis

The statistical analysis was performed on GraphPad Prism 4 software. Paired t-test was used for comparison between two groups as appropriate. A p value <0.05 was considered statistically significant.

Results

Surface modification of materials induces matrix metalloproteinases expression

Figure 1 shows modulated gene expression of individual MMPs by human chondrocytes after 48-hour incubation with individual polymeric materials corrected to the gene expression of chondrocytes incubated without these materials. Plasmatic modification in contrast to unmodified materials significantly induced expression of MMP-1 and MMP-3 (fold increase: 8.14 vs. 0.78 and 3.77 vs. 0.55, respectively; $p<0.05$ for both comparisons) as well as MMP-13 (fold increase: 7.77 vs. 0.26; $p<0.001$). On the contrary, unmodified materials lead to reduced expression of particular MMPs by chondrocytes (Fig. 1). Increased gene expression of the MMPs was confirmed by measuring theirs active forms secreted by chondrocytes to the cell culture supernatants (Table 1). Modified in contrast to unmodified materials significantly induced production of MMP-1 ($p<0.05$), MMP-3 and MMP-13 ($p<0.001$ for both comparisons).

Proinflammatory cytokines and apoptotic molecules upon surface modification

Incubation of human chondrocytes with plasmatically modified compared to unmodified materials significantly increased expression of IL-1 mRNA (fold increase: 2.48 vs. 0.92; $p<0.05$) after 48 hours (Fig. 2a). The expression of TNF-α mRNA was not significantly changed upon the presence of tested materials (Fig. 2b). The gene expression of apoptotic molecules BAX and Bcl2 was also not significantly changed upon incubation of chondrocytes with plasmatically modified or unmodified materials (Fig. 3).

Chondrocyte viability upon surface modification

Cell viability and cytotoxicity of COC materials examined by the MTT test on Day 2, 4 and 8 is illustrated in figure 4. The viability/proliferation of chondrocytes on materials without plasmatic modification was gradually increasing. From Day 4 on, however, there were apparently less viable chondrocytes growing on the COC and COC blend modified materials compared to materials without modified surfaces. The greatest difference in chondrocyte viability was observed between modified and unmodified COC and COC blend materials on day 8.

Discussion

In the present study we examined the effect of modified and unmodified polymeric materials for osteochondral defect repair on human chondrocytes. We demonstrated that under *in vitro* conditions modified in contrast to unmodified surface of polymers increased expression of IL-1, induced production of several matrix metalloproteinases and reduced viability of human chondrocytes that may altogether potentially harm the cartilage homeostasis.

Due to limited repair capacity of the articular cartilage, most osteochondral defects caused by traumas are burdening adult and paediatric traumatology as well as orthopedic surgery. Pivotal research into the treatment of osteochondral defects shifts towards the search for a suitable material or "scaffold" with eligible biological properties (Zwingmann *et al*. 2007). Currently, several different materials are known such as metals, ceramics or organic polymers being used also for joint prosthesis. In the current study, the authors selected cyclic-olefin based polymers (COC and COC blend) for in vitro experiments. These materials allow active control of the properties such as the modulus of elasticity and tensile strength being suitable for medical application (Ramakrishna *et al*. 2001). Besides the physico-chemical parameters for the particular material, biocompatibility represents one of the greatest concerns. Toxic substances may cause impaired metabolic activity, reduced cell proliferation or viability, early chondrocyte apoptosis or an increased production of stress proteins and cytokines (Cornelis *et al*. 1991, Tanzi *et al*. 1992). Cell proliferation and viability, one of the key parameters for the determination of cytotoxicity of studied materials, are being often determined by MTT test (Willumeit *et al.* 2007, Shaw 1994). In the current study, we have not observed significant differences between the expressions of apoptotic molecules by chondrocytes exposed to modified or unmodified materials. However, using MTT test, we could demonstrated time-dependent decrease of viability/proliferation of human chondrocytes being exposed to modified polymers compared to unmodified polymers. Contrary, unmodified materials seem to be well tolerated. This finding is consistent with the observation that for example higher amounts of hydrophilic groups on polymer chains may reduce adhesion, proliferation and viability of chondrocytes (Pérez Olmedilla *et al*. 2006).

Furthermore, we demonstrated that the modified in contrast to unmodified materials could induce expression of proinflammatory cytokine IL-1 by human chondrocytes. Consistently with the evidence of IL-1 being a key regulator of articular cartilage degradation (Hedbom *et al*. 2002), we found an increased production of several MMPs by chondrocytes exposed to modified, but not to unmodified materials. Since collagen fragments were describe to up-regulate production of some MMPs, mostly MMP-13 (Sunk *et al*. 2007, Tchetina *et al*. 2007), it can be speculated that increased expression of MMPs can be the consequence of collagen application to modified surface of materials. Thereby, based on these data, one could not support the idea of benefits for plasmatic modification and subsequent collagen application of osteochondral defects implants. Contrary, it has been demonstrated that type II collagen-based matrices contribute to the invasion of cells that are directed into a chondrocyte phenotype and may be the choice for cartilage regenaration of the superficial layer (Buma *et al.* 2003). In addition, our data cannot definitely declare final assessment for the *in vivo* condition, as our preliminary results show a similar biological tolerance of both modified and unmodified polymers implanted into the artificially created osteochondral defects of porcine femoral condyles (unpublished observations). Accordingly, recent work has indicated a similar

proliferation and expression of type I, II and X collagens, including catalytic enzymes by bovine chondrocytes where modified collagen-based scaffolds were concerned (Chajra *et al*. 2008). Thereby, it seems that positive results of surface modification prevail over the negative results. However, further studies should confirm benefits or disadvantages of surface material modification used for osteochondral defect repairs.

In conclusion, cyclic olefin copolymers may represent suitable materials for tissue engineering; however, the results of this study suggest that, at least in vitro, plasmatic modification with subsequent collagen application may have an adverse effect on hyaline cartilage repair. More data should be brought by further experiments and *in vivo* studies of osteochondral defect implants.

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Table 1. Increased levels of active-MMPs secreted by chondrocytes to the cell culture supernatants after 48-hour incubation with modified in contrast to unmodified polymeric materials.

	Materials without surface plasmatic modification	Materials with surface plasmatic modification	*p*
MMP-1	120.15+/-83.64	237.22+/-135.43	<0.05
MMP-3	15.50+/-9.95	41.27+/-12.80	<0.001
MMP-13	2.32+/-1.65	24.61+/-10.07	<0.001

Legend to figures:

Figure 1. Increased gene expression of several matrix degrading enzymes (MMPs) by human chondrocytes after 48-hour incubation with modified in contrast to unmodified polymeric materials. Relative gene expression is corrected to the gene expression of control human chondrocytes incubated without these materials. White boxes represent unmodified materials, grey boxes represent modified materials.

Figure 2. Gene expression of proinflammatory cytokines IL-1 and TNF-α by human chondrocytes after 48-hour incubation with modified and unmodified polymeric materials. Relative gene expression is corrected to the gene expression of control human chondrocytes incubated without these materials. White boxes represent unmodified materials, grey boxes represent modified materials.

Figure 3. Gene expression of apoptotic molecules Bcl-2 and BAX by human chondrocytes after 48-hour incubation with modified and unmodified polymeric materials. Relative gene expression is corrected to the gene expression of control human chondrocytes incubated without polymeric materials. White boxes represent unmodified materials, grey boxes represent modified materials.

Figure 4. Plasmatic modification of materials reduced chondrocyte viability/proliferation as evaluated by MTT colorimetric method. White columns represent unmodified materials, grey columns represent modified materials.

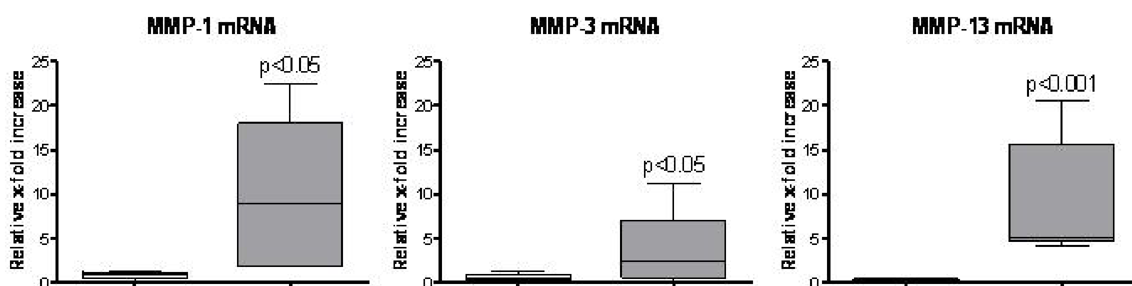

Fig. 1.

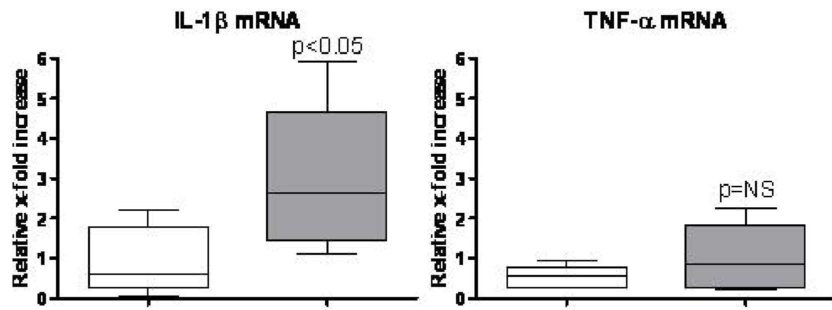

Fig. 2.

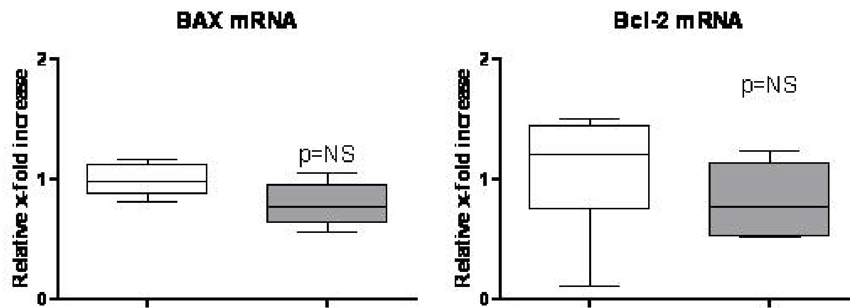

Fig. 3.

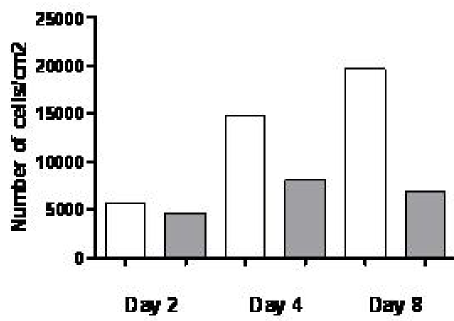

Fig. 4.