NCO-sP(EO-*stat*-PO) surface coatings preserve biochemical properties of RGD peptides

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Abstract. We have previously reported that star shaped poly(ethylene oxide-*stat*-propylene oxide) macromers with 80% EO content and isocyanate functional groups at the distal ends [NCO-sP(EO-*stat*-PO)] can be used to generate coatings that are non-adhesive but easily functionalized for specific cell adhesion. In the present study, we investigated whether the NCO-sP(EO-*stat*-PO) surfaces maintain peptide configuration-specific cell-surface interactions or if differences between dissimilar binding molecules are concealed by the coating. To this end, we have covalently immobilized both linear-RGD peptides (gRGDsc) and cyclic-RGD (RGDfK) peptides in such coatings. Subsequently, SaOS-2 or human multipotent mesenchymal stromal cells (MSC) were seeded on these substrates. Cell adhesion, spreading and survival was observed for up to 30 days. The time span for cell adherence was not different on linear and cyclic RGD peptides, but was shorter in comparison to the unmodified glass surface. MSC proliferation on cyclic RGDfK modified coatings was 4 times higher than on films functionalized by linear gRGDsc sequences, underlining that the NCO-sP(EO-*stat*-PO) film preserves the configuration-specific biochemical peptide properties. Under basal conditions, MSC expressed osteogenic marker genes after 14 days on cyclic RGD peptides, but not on linear RGD peptides or the unmodified glass surfaces. Our results indicate specific effects of these adhesion peptides on MSC biology and show that this coating system is useful for selective testing of cellular interactions with adhesive ligands.

Introduction

The surface design of biomaterials is a key element controlling their integration into the surrounding tissue and cell adhesion (1). The biomaterial surface modulates cellular responses in terms of adhesion, proliferation, inflammatory reactions and survival.

Due to its surface properties an artificial implant usually induces the production of a fibrous tissue covering its surface after implantation. This fibrous layer reduces the tissue-implant contact, which may result in loosening of the implant or stimulation of further inflammatory processes. There are several strategies to enhance the ingrowth of non-biological materials by addressing non-specific cell-surface interactions. Alterations of the physical surface properties like hydrophobicity or surface topography are possibilities. For example, by creating nano-sized features on titanium-based surfaces the expression of bone sialoprotein (BSP) and osteopontin was stimulated by osteogenic cells (2). The enhancement of osteoblast adhesion and function on different nanostructured metal and ceramic materials was demonstrated in further studies (3,4).

A more biomimetic strategy is to control adhesion, proliferation and differentiation of osteogenic cells by biochemical modification of the implant surface. While coating with hydroxyapatite is often used in combination with an alteration of surface roughness to improve osteointegration of implants (5), this approach does not minimize non-specific interactions e.g. interactions with plasma fibronectin that do not necessarily contribute to an optimal bone ingrowth. They may instead lead to negative effects like foreign body reaction and fibrotic processes (5,6). Specific tuning of the material surface can be used to minimize these non-specific effects and may rely on the molecular mechanisms of cell adhesion mainly mediated by integrins which provide selective interactions with the proteins of the extracellular matrix (7). A number of integrin subtypes recognize the tripeptide sequence Arg-Gly-Asp

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(RGD) as their ligand, but show preferential interactions depending on the amino acids flanking the RGD motif as well as on the overall conformation of the peptide (8-11). Therefore, linking of adhesion peptides to biomaterials is a widely used approach to improve biocompatibility, biological activity and the interaction with cells (12-14).

We have previously described a system for surface coating by preparing ultra thin films (30±5 nm) of reactive star shaped NCO-sP(EO-*stat*-PO) macromers with 80% EO content and isocyanate functional groups at the distal ends (15). These films exhibit minimal interactions with immobilized proteins and allow the reversible folding of the immobilized proteins (16,17). Unmodified NCO-sP(EO-*stat*-PO) coatings prevent non-specific protein binding and the adhesion of cells under standard cell culture conditions over at least 4 weeks. This feature also reduces bacterial adhesion and creates additional antimicrobial properties (18). Because of the unique switch from the chemically reactive isocyanate groups to an inert polymer layer, during the NCO-sP(EO-*stat*-PO) layer preparation, an easy integration of cell-binding supporting RGD peptides was demonstrated (19).

For specific functionalization of implant surfaces the preservation of sequence- and conformation-dependent biochemical properties of the peptides is necessary. Therefore, the primary aim of this work was to investigate if the micro-environment of the NCO-sP(EO-*stat*-PO) layer influences the functional properties of the covalently immobilized adhesion peptides. Secondly, we wanted to establish a substrate-specific experimental approach for testing different adhesion peptides without interference from non-specific protein binding, which occurs in most of the experimental setups used so far. To address these aims we compared linear RGD (gRGDsc) peptides with cyclic RGD (RGDfK) peptides that were integrated in an NCO-sP(EO-*stat*-PO)-coated surface, with respect to cell adhesion, proliferation and differentiation of bone marrow-derived multipotent mesenchymal stromal cells (MSC) and SaOS-2 cells.

Materials and methods

Preparation of NCO-sP(EO-stat-PO)-coated surfaces. A six-arm star-shaped polymer with a molecular weight of 12,000 g/mol was used in this study. The preparation of the star polymers has been described elsewhere (17). Glass substrates (d=18 mm) were coated as described earlier (19).

To obtain films that are modified with RGD peptides, the respective amount of linear (gRGDsc, 2 mg/ml THF) or cyclic RGD (RGDfK, 1 mg/ml THF) was dissolved in 9 ml of deionised water. This solution was mixed with the star polymer solution in 1 ml THF. The film formation was then done as described (19). The linear gRGDsc peptide was provided by Harm-Anton Klok (EPFL Lausanne) and the cyclic RGDfK peptide was prepared as previously described (20).

Cell culture. MSC were harvested from human bone marrow derived from routine surgical procedures with informed consent from six patients (age 19-30 years) in accordance with the terms of the ethics committee of the University of Ulm.
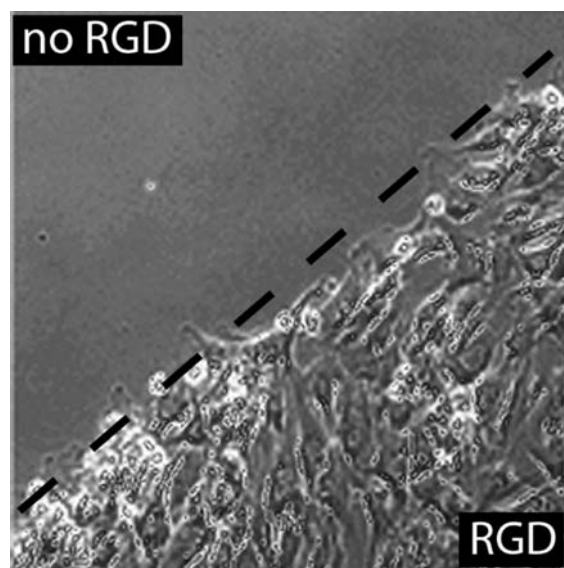

Figure 1. Cultivation of MSC on NCO-sP(EO-*stat*-PO)-RGD coated surfaces. After covering of the glass pane with NCO-sP(EO-*stat*-PO), half of the same substrate was dip-coated with NCO-sP(EO-*stat*-PO) containing cyclic RGD. A sharp borderline of attached cells to the non-adhesive part was detectable. The border is marked with a dotted line only on the picture and was not visible on the substrate (microscopic view, magnification x100).

Isolation and cultivation were done as described earlier (21). The preparation is specific for adherent mesenchymal progenitor cells and maintains the progenitor phenotype. After isolation, the cells were cultured in a basal medium (BM) consisting of DMEM with 10% FBS, 1% glutamine and 1% penicillin/streptomycin (Biochrom-Seromed, Berlin, Germany) at 37˚C, 5% CO_2 in 95% humidity. In order to differentiate the MSC into osteoblasts, the basal medium was supplemented with 10^{-7} M dexamethasone, 50 μg/ml ascorbic acid and 2.16 μg/ml ß-glycerophosphate (Sigma, Germany) for 14 days (differentiation medium, DM). The medium was changed twice a week. The osteogenic differentiation was assessed by RT-PCR of osteoblast-specific genes.

The osteoblastic cells were cultivated under the same conditions in DMEM with 10% FBS, 1% penicillin/strepto-mycin and ascorbic acid 50 mg/l.

RT-PCR analysis. The phenotypic status of the MSC, the differentiated progenitor cells, and osteoblasts was affirmed by RT-PCR analysis of the following osteoblastic markers: alkaline phosphatase (AP), osteocalcin (OC), BSP, RUNX2 (RX2) and osteonectin (ON; SPARC). Collagen type I α 1 (COL1A1), and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used to verify the PCR results as internal standards. Therefore, total RNA was isolated from 10^5 cells with the RNeasy® system and reverse transcription was done with Omniscript™ RT kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. PCR-primers for GAPDH, COL1A1, AP, OC, RX2, and ON were described elsewhere (21). Amplicon size was set to 180-250 bp for GAPDH, COL1A1, AP, OC, RX2, and to 600 bp for ON and BSP. Temperature optimum was set to 60˚C. In all cases, the best primer pair was used. PCR reactions were performed with a Robocycler® (Stratagene, Amsterdam,
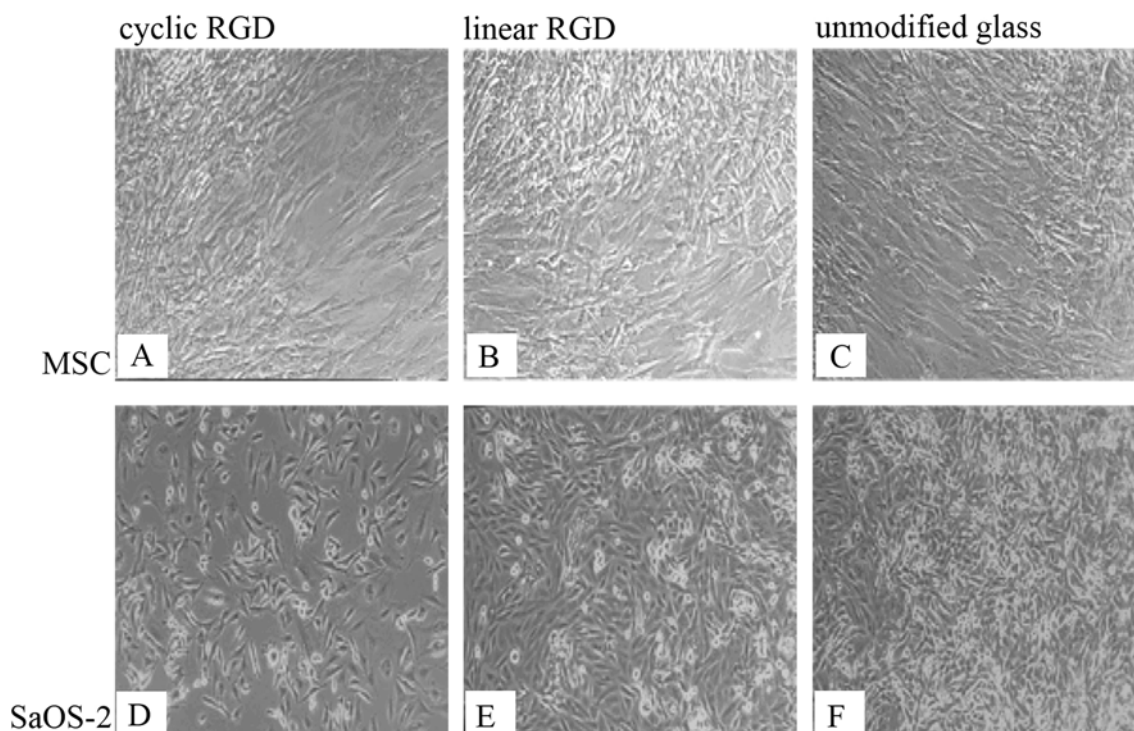
Figure 2. Cell attachment on different NCO-sP(EO-*stat*-PO)-RGD coatings and unmodified glass panes. No significant differences between linear and cyclic RGD coatings could be detected concerning cell attachment of MSC and SaOS-2 cells (microscopic view, magnification x100).

The Netherlands) using the HotStarTaq™ Master Mix kit (Qiagen). PCR was performed under linear conditions using the cycle profile: initial incubation for 15 min at 95˚C, followed by 30 cycles of annealing for 45 sec at 60˚C, extension for 45 sec at 72˚C and denaturation for 60 sec at 94°C and termination for 15 min at 72˚C.

PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide, visualized and a digital image obtained with the Gel Doc XR system (Bio-Rad, München, Germany).

Detection of cell adhesion. Cell attachment and adhesion on modified glass substrates was controlled by light microscopy using a Zeiss 'Axioskop mot plus' (Zeiss, Oberkochen, Germany) microscope with a Zeiss AxioCam MRc and the Zeiss AxioVision (v. 3.1) software.

Thiazolyl blue tetrazolium bromide (MTT) assay. MTT assays were carried out as follows: cells were trypsinized, counted, and seeded on NCO-sP(EO-*stat*-PO)-coated plates placed in 6-well plates. After 1 or 2 weeks, 3 ml of the culture medium were supplemented 1:40 with MTT solution (5 mg/ml) and the plates were returned to the 37˚C incubator for 2 h. Afterward, the supernatant was aspirated from each well, and replaced by 1 ml 0.04 M HCl/isopropanol. The plates were then incubated for 5 min at 37˚C. Subsequently, the optical density of 200 μl samples was measured at 590 nm using an ELISA reader (Dynatech Laboratories, USA).

Results

We were able to show in our present study that an NCO-sP(EO-*stat*-PO)-coated surface functionalized with linear or cyclic RGD peptides, preserving the different biochemical and biological properties of the bound peptides. Additionally, a specific, cell type-dependent, influence of the two tested peptides was shown.

The osteogenic differentiated MSC (dOB) and the SaOS-2 cells showed different responses in terms of cell adhesion, proliferation, and osteogenic differentiation on NCO-sP(EO-*stat*-PO)-RGD-coated surfaces.

Cell adhesion on linear and cyclic RGD peptide surfaces. MSC and SaOS-2 were seeded on NCO-sP(EO-*stat*-PO)-coated glass plates that were functionalized with different RGD peptides (Fig. 1). As shown the cells could not cover areas without RGD supplementation. Cell adhesion was detectable only in the presence of linear- or cyclic-RGDs. The principal cell adhesion properties of the cyclic RGDfK have been previously described (22,23). In the present study, cell adhesion on linear and cyclic RGD-modified surfaces showed no obvious differences (Fig. 2). Both MSC and SaOS-2 cells could adhere on the functionalized NCO-sP(EO-*stat*-PO) layers. Additionally, after 24 h of cultivation no differences concerning the cell shape, number of attached cells and percent coverage of the substrates were detectable on unmodified glass and NCO-sP(EO-*stat*-PO)-RGD surfaces. Obviously, the integrated amount of RGD peptides could substitute the non-specific adhesive protein binding on the unmodified glass surfaces.

The time span between cell seeding and cell attachment, however, was faster on the NCO-sP(EO-*stat*-PO)-RGD surfaces compared to glass surfaces without modification. During the 28 days of cultivation, MSC, dOB and SaOS-2 proliferated and covered the complete area of the substrates (Fig. 3). Cell morphology did not change during cultivation.
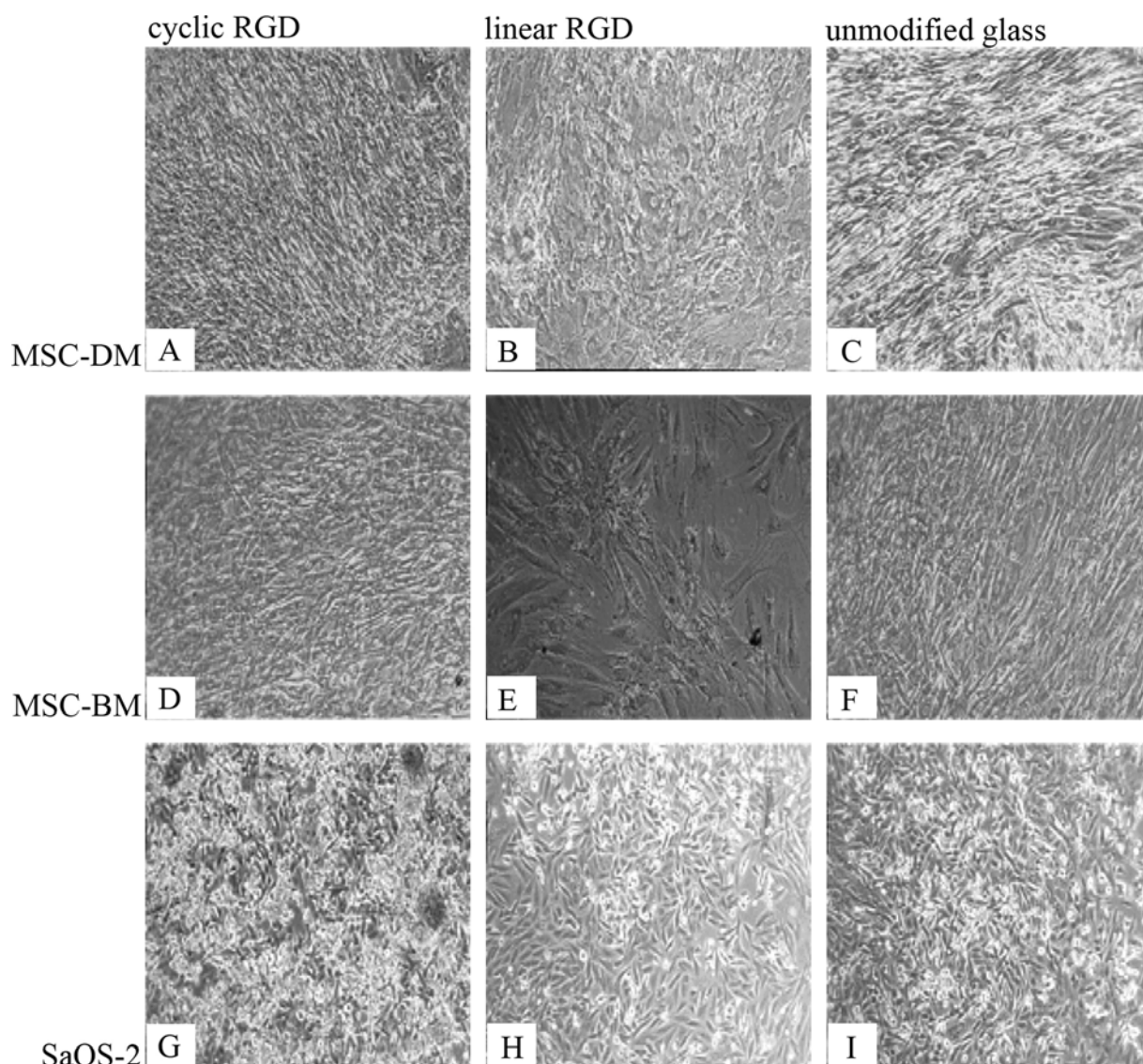
cyclic RGD　　　　　linear RGD　　　　unmodified glass

Figure 3. Differentiation and growth of MSC during 28 days of cultivation. DOB (differentiated osteoblasts) MSC (without differentiation conditions). (A) MSC with DM (differentiation medium) on cyclic-RGD-substrate [1 mg cyclic-RGD/ml NCO-sP(EO-*stat*-PO) = cyclic RGD]. (B) MSC with DM on linear-RGD-substrate [2 mg linear-RGD/ml NCO-sP(EO-*stat*-PO) = linear RGD]. (C) MSC with DM on glass without coating. (D) MSC with BM (basal medium) on cyclic RGD. (E) MSC with BM on linear RGD. (F) MSC with BM on glass. (G) SaOS-2 with BM on cyclic RGD. (H) SaOS-2 with BM on linear RGD. (I) SaOS-2 on glass.

No signs of enhanced cell death or cellular detachment were detectable.

MTT-test. Cell proliferation testing was done by using the MTT-test comparing the proliferation rate of cells cultivated on commercial cell culture material with cells grown on NCO-sP(EO-*stat*-PO)-RGD surfaces. The influence of the NCO-sP(EO-*stat*-PO)-RGD coatings and cultivation conditions is apparent (Fig. 4). On cyclic RGD surfaces MSC proliferated under differentiation conditions (MSC-DM) 4 times faster as MSC-BM and SaOS-2 cells. In contrast, on linear RGD surfaces MSC proliferated 5 times faster under basal conditions (MSC-BM), which was also the case for cultivation on native glass surfaces.

Differentiation of cells on functionalized surfaces. In order to test if the NCO-sP(EO-*stat*-PO)-coated surfaces, which were
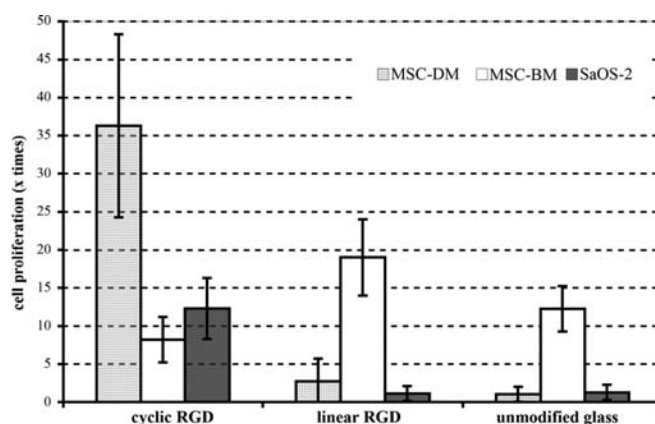

Figure 4. Cell vitality and proliferation on different surface coatings tested by the MTT assay. Cell proliferation was compared to the cell number when seeded (mean ± SD) (n=3).
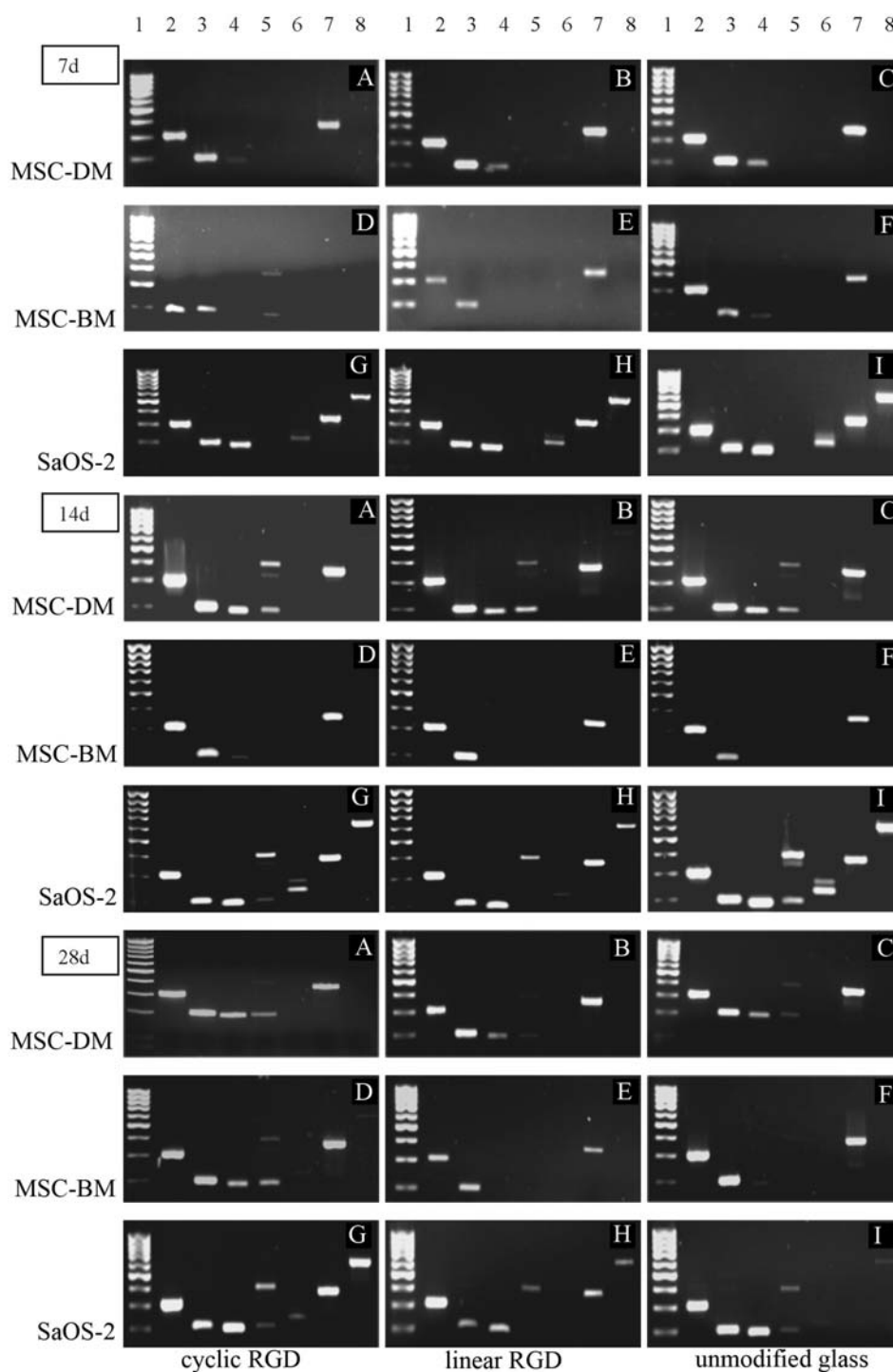
Figure 5. Osteogenic marker gene expression in MSC and SaOS-2. After 7, 14, and 21 days gene expression was assessed by RT-PCR. (A) MSC with differentiation medium (DM) on cyclic-RGD-substrate [1 mg cyclic-RGD/ml NCO-sP(EO-*stat*-PO) = cyclic RGD]. (B) MSC with DM on linear-RGD-substrate [2 mg linear-RGD/ml NCO-sP(EO-*stat*-PO) = linear RGD]. (C) MSC with DM on glass surface without NCO-sP(EO-*stat*-PO)-RGD. (D) MSC with BM (basal medium) on cyclic RGD. (E) MSC with BM on linear RGD. (F) MSC with BM on glass surface. (G) SaOS-2 with BM on cyclic RGD. (H) SaOS-2 with BM on linear RGD. (I) SaOS-2 with BM on glass. Lane 1, 100 bp DNA-ladder; 2, GAPDH; 3, collagen type I α 1; 4, alkaline phosphatase; 5, osteocalcin; 6, RUNX 2; 7, osteonectin; 8, bone sialoprotein.

functionalized with the linear (gRGDsc) or cyclic (RGDfK) peptides influence the osteogenic differentiation potential of MSC, different cultivation conditions were used. After 1, 2, and 3 weeks, the gene expression of osteogenic markers from MSC under basal conditions (MSC-BM), MSC under differentiation conditions (MSC-DM), and SaOS-2 cells under basal conditions was analyzed. In Fig. 5 the gene expression of

osteogenic marker genes in cells cultivated on NCO-sP(EO-*stat*-PO)-coated surfaces with linear or cyclic RGD peptides and on unmodified glass as a control, is shown. We observed that under basal conditions and after 28 days on cyclic RGD-coated surfaces MSC express the osteogenic markers AP and OC, while they did not express these markers on surfaces functionalized with linear RGD or cultivated on glass surfaces.

Nevertheless, under differentiation conditions osteogenic marker gene expression was noted in MSC on all tested surfaces. The expression of osteoblastic markers in SaOS-2 cells was preserved on surfaces coated with linear or cyclic RGD.

Discussion

Our studies with MSC, MSC differentiated into osteoblasts and SaOS-2 cells, showed that cell adhesion, spreading, and cell vitality on linear gRGDsc and cyclic RGDfK modified NCO-sP(EO-*stat*-PO) surfaces were significantly modulated by the presented peptides. The results confirmed that an effective covalent surface modification was achieved and we demonstrated that the coating conserves the biochemical properties of these different RGD peptides. The production process did not impair cell adhesion nor inhibit osteogenic differentiation. Using established cell culture protocols MSC could be differentiated into osteoblast-like cells in contact with the RGD-functionalized substrates. Negative effects from the NCO-sP(EO-*stat*-PO) coating on cell adhesion and differentiation were not observed.

Within the first hour after cell seeding the majority of the cells attached to both RGD peptides correlated with a strong cell spreading, while the cell attachment on unmodified glass surfaces was slower and corresponded to a round cell shape. This probably reflects the different amount of presented binding sites. In order to attach to biomaterial surfaces a cell has to flatten and spread its plasma membrane over the substratum, which is indicative of a high number of binding sites on the material surface (24,25). Coating with NCO-sP(EO-*stat*-PO)-RGD reduced the time for cell adhesion, because of the immediate availability of sufficient cell binding motifs described by several studies (26,27). This is important, because NCO-sP(EO-*stat*-PO) without functionalization, inhibits cell adhesion by preventing any non-specific protein binding to coated surfaces. Therefore, the addition of cell binding molecules is required in order to use such coatings for biomaterials that have to be integrated into tissues (16). The unmodified glass surfaces first have to be passively coated by a sufficient amount of adhesive proteins, derived from the supplemented serum (e.g. fibronectin) or secreted by the cells themselves, to allow cell adhesion. This may be the major reason for the longer time period necessary to attach and spread on the uncoated glass surface.

The linear and cyclic RGD peptide integration differentially modified the proliferation rate of the different cell types as shown by the MTT assay. While it has been previously reported, that both the osteogenic differentiation of MSC (28,29) and the interaction with different biomaterials affect cell proliferation (30), it has not been described that the physio-chemical properties of linear and cyclic RGD peptides also influence the proliferation rate of human MSC depending on the presence or absence of conditions inducing osteogenic differentiation. This may partly be explained by the fact that the cyclic RGD pentapeptides have higher binding affinity and selectivity for $\alpha v\beta 3$, $\alpha v\beta 5$ and to a much lesser extent for $\alpha IIb\beta 3$ integrin receptors (31-33). Human MSC express $\alpha v\beta 3$ and $\alpha v\beta 5$ integrin receptors (34) and these receptors have been implicated in the functional regulation of osteogenic cells (35).

In the present study, the NCO-sP(EO-*stat*-PO) surface functionalized with linear RGD peptides did not induce or inhibit osteogenic differentiation of MSC and maintained the differentiation status of SaOS-2 cells. This is in accordance with previously published results (19). The cyclic-RGD-functionalized surfaces supported osteogenic differentiation of human MSC even under basal conditions. This may at least partly be explained by the more rigid binding through $\alpha v\beta 3$ and $\alpha v\beta 5$ integrin receptors since associated effects on the cytoskeleton might contribute to the fate determination (36). Our results are in agreement with a recent study on the osteogenic differentiation of MSC in a 3-dimensional alginate matrix in which cyclic and linear RGD peptides were incorporated (37). In contrast, Yang *et al* reported, that incorporation of linear RGD adhesive peptides in a polyethylene glycol diacrylate hydrogel stimulates osteogenesis of bone marrow stromal cells (38). Possibly, the 3-dimensional cultivation conditions used by Yang *et al* contributed to this opposite result since we used a 2-dimensional system. The interaction of adhesive ligands and their spatial presentation clearly deserves further investigation.

Taken together, our studies on cell adhesion and cell shape showed that cell adhesion on linear or cyclic RGD-functionalized NCO-sP(EO-*stat*-PO) coatings is mediated by specific interactions between the RGD peptides, which were covalently linked to the polymer and the corresponding integrin receptors on the cell surface. The NCO-sP(EO-*stat*-PO) coating did not affect the biological and chemical properties of the integrated linear or cyclic RGD peptides. This is supported by the higher MSC proliferation under differentiating conditions and the induction of osteogenic differentiation under basal conditions on cyclic RGD modified coatings. Therefore, ultrathin coatings with the NCO-sP(EO-*stat*-PO) system and specific functionalization with adhesive peptides represent a promising strategy to improve cell-biomaterial interactions at the bio-interface (39). The NCO-sP(EO-*stat*-PO) system proved to be a suitable tool to investigate the effect of integrated adhesion peptides with minimization of the non-specific interactions with secondary adsorbed proteins on surfaces.

Acknowledgements

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