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Osteoblastic potency of bone marrow cells cultivated on functionalized biometals with cyclic RGD-peptide

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Received 18 November 2012; accepted 3 January 2013
Published online 25 March 2013 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jbm.a.34590

Abstract: The fixation of cementless endoprostheses requires excellent fixation at the bone implant interface. Although the surface structures of these implants are designed to promote osteoblastic differentiation, poor bone quality may prevent or delay osseointegration. There is evidence that RGD peptides known as recognition motifs for various integrins, promote cellular adhesion, influence cellular proliferation, and differentiation of local cells. In this study, five different metal surfaces were analyzed: Sandblasted (TiSa) and polished (TiPol) Ti6Al4V, porocoated (CCPor) and polished (CCPol) cobalt chrome and polished stainless steel (SS) were coated by ethanol amine and poly(ethylene glycol) to attach covalently RGD peptides. Human mesenchymal stromal cells of healthy donors were cultivated onto prior functionalized metal surfaces for 14 days without osteogenic stimulation. Cell proliferation and differentiation were quantitatively evaluated for native (I), NaOH pre-activated (II), NaOH pre-activated, and PEG-coated (III) as well as for RGD (IV) coated surfaces. The RGD immobilization efficiency was analyzed by epi-fluorescence spectroscopy, cell morphology was documented by light and scanning electron microscopy. The RGD-binding efficiency was TiSa $>$ TiPol $>$ SS $>$ CCPor $>$ CCPol. RGD coated surfaces showed the highest average cell proliferation on CCPol $>$ SS $>$ CCPor $>$ TiSa $\geq$ TiPol, whereas cellular differentiation mostly correlated with the observed proliferation results, such as CCPol $>$ TiSa $>$ SS $>$ CCPor $>$ TiPol. Considering statistical analyses (significance level of $\alpha = 0.05$), the RGD-coating of all biometals in comparison and in respect of their particular controls showed no significant improvement in cellular proliferation and osteoblastic differentiation. © 2013 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 101A: 2905–2914, 2013.

Key Words: RGD peptide, bone marrow, mesenchymal stem cells, osteoblast, endoprosthesis

How to cite this article: Jäger M, Böge C, Janissen R, Rohrbeck D, Hülsen T, Lensing-Höhn S, Krauspe R, Herten M. 2013. Osteoblastic potency of bone marrow cells cultivated on functionalized biometals with cyclic RGD-peptide. J Biomed Mater Res Part A 2013:101A:2905–2914.

INTRODUCTION

Cementless endoprostheses are designed to support bony ingrowth, mineral formation and to effectively become "permanent" implants. Although the survivorship of cementless stems and cups in total hip replacements has shown excellent results, limited weight-bearing for several weeks is still recommended and limited loads are suggested in the early peri-operative period to prevent micro-motion and to promote direct bone/metal contact, which is essential for sound fixation and ingrowth. In addition, poor bone quality, such as osteoporosis, renal osteodystrophy, rheumatological disorders, or revision surgery may prevent or delay osseointegration of cementless orthopedic implants and hence promote aseptic loosening. Innovative biological treatment concepts "orthobiologics" are currently being evaluated in an effort to support bony ingrowth onto implants. The acceleration of bone ingrowth with biologically active engineered implant materials could potentially reduce recovery time and prevent some upcoming implant failures. The addition of growth hormones or modifications of the implant surfaces mimicking motifs of the extracellular matrix (ECM) are promising candidates to stimulate osteoblasts and induce biomineralization.

As typical amino-acid sequences of the cellular ECM, proteins such as fibronectin (FN), and vitronectin, RGD (a peptide containing arginine-glycine-aspartic acid) [Fig. 1(a)] act as recognition motifs for integrins, the cellular adhesion receptors. Integrins are a large family of heterodimeric transmembrane receptors mediating cell–matrix and cell–cell adhesion and they trigger signals regulating cell proliferation

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The implants were supplied by DePuy Orthopädie, GmbH, Kirkel, Germany.

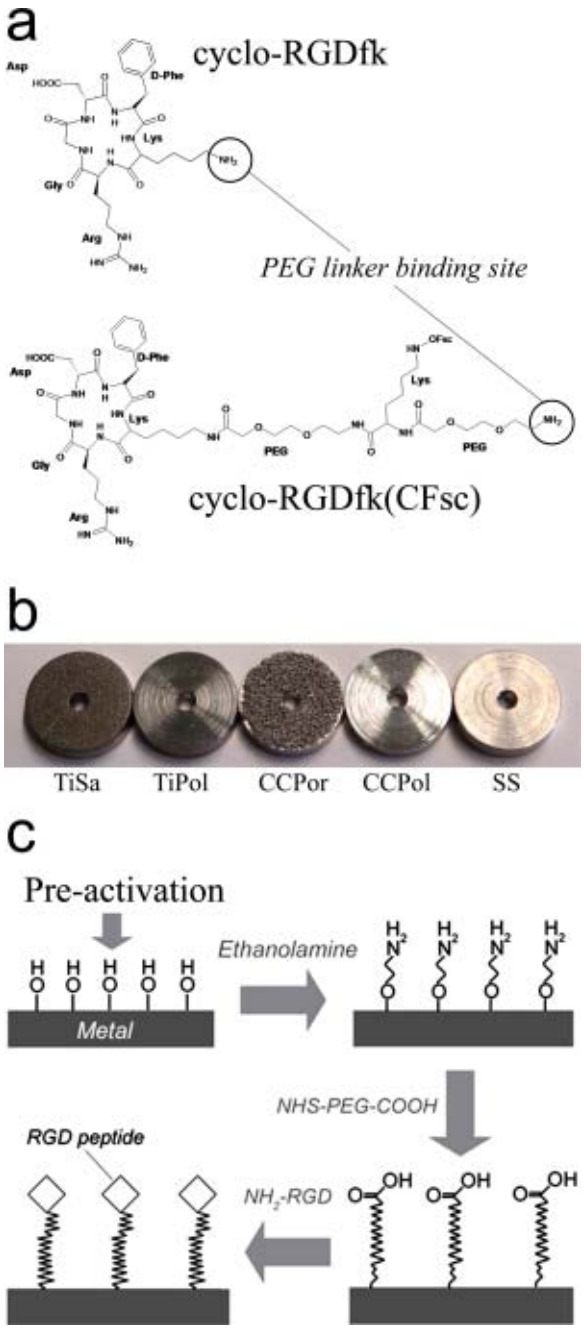

FIGURE 1. (a) Structure of used cRGD-peptides cyclo(RGDfk) and cyclo[RGDfK(PEGLys(CFsc)-PEG)]. (b) Overview of the cRGD-coated endoprosthesis biomaterials in the order TiSa, TiPol, CCPor, CCPol, and SS were used for covalent immobilization of the (c) different cRGD-peptides. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

and differentiation (e.g., focal contacts). Osteoprogenitor cells and osteoblasts express a wide panel of integrins which vary with their stage of development ($\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_8\beta_1$, $\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$, $\alpha_v\beta_6$-integrins) initializing chemotaxis, differentiation, and mineralization.[1–4] These integrins are expressed in relevant numbers by osteoblasts allowing cellular adherence onto different biometals such as titanium or cobalt-chromium-molybdenum alloys. In addition, mesenchymal stromal cells (MSC) promote bone regeneration *in-vivo* and may represent a new therapeutic tool for potential future applications in orthopedic surgery.[5–9]

Based on integrin–ECM interactions in osteoblasts, integrins represent an attractive option for the surface of biological implants. The large majority of work in this area has centered on presenting the short binding motif incorporating the RGD sequence from FN, bone sialoprotein and osteopontin.[10,11] In former studies, RGD peptides were used to enhance cell-adhesion and it could be shown that a critical minimum of RGD surface density is needed for cell response.[12] Density threshold values range hereby from 0.001 pmol/cm^2 to 1 pmol/cm^2.[13,14] To assess cell-adhesion strength centrifugal cell detachment assays were used[15,16] and it was assessed that cell-adhesion strength is influenced by spatial organization[17] and different flanking residues[18] of RGD peptides.

RGD-peptides attached onto metallic alloys by surface adsorption only, have shown neither an increase of cell-adhesion nor promotion of cellular spreading in bone marrow MSC (BM-MSC).[19] Moreover, osteoblastic differentiation was not stimulated and implant osseointegration was not promoted[20] by non-convalently bound RGDs. In contrast, biomineralization was induced in rat calvarial osteoblasts and also a higher cell-adhesion was observed in human bone cells[21,22] when the peptide was covalently bound to a biometal.[23]

Most studies have used titanium for the attachment of RGD, but many other alloys have been investigated in the quest for osseointegration *in vitro* and in the clinical setting. In a previously published study, we showed cellular adherence spreading and osteogenic differentiation of BM-MSC onto polished and sandblasted titanium (Ti6Al4V) as well as on polished/porous coated cobalt chrome biometals.[24] When the adhesion of osteoblasts and their progenitors onto these biometals is evaluated, there is evidence that covalently bound linear and cyclic RGDs[25] have advantages in contrast to soluble RGD.[26]

In this study, we have used an *in-vitro* model to evaluate the osteogenic potential of five different biomaterials with a typical surface topography frequently used in hip arthroplasty. Those biometals were covalently coated with cyclic RGD-peptide (cRGD). This *in-vitro* model has been examined to observe the proliferation and differentiation of human BM cells. The efficiency of cRGD binding was controlled by fluorescence spectroscopy techniques and the effects of cRGD coated biomaterials were evaluated for cellular morphology, proliferation, and osteogenic differentiation.

MATERIALS AND METHODS

Biomaterials

Five different biometals were used in this study. They were used as discoid shapes (size Ø 12 mm, with a central hole of 2 mm) with well defined surface structures. Sandblasted (TiSa) and polished (TiPol) Ti6Al14V, porocoated (CCPor) (Porocoat^{TM}), and polished (CCPol) cobalt chrome (Orthochrome^{TM}) as well as polished stainless steel (SS) (DePuy Systems, Kirkel, Germany) were used.[24] The materials were

chosen because they correspond to those used typically in orthopedic surgery for femoral stems and acetabular shells [Fig. 1(b)].

RGD-coating

The cRGD-coating was performed with minor modifications according to the protocol of Janissen et al.[27,28] [Fig. 1(c)]. The metal surfaces were treated with air oxygen plasma (0.5 mbar, Power: 100 W, Model Femto, Diener, Ebhausen, Germany) for 20 min, as initial cleaning step and sonicated afterwards in a 5 % solution of alkaline detergent (Hellmanex II, Hellma, Mühlheim Germany) for 20 min at 37°C, washed for 5 min in ultra pure water (produced by Arium 611, Sartorius, Goettingen, Germany) and finally sonicated again in ultra pure water for 20 min. After an ultimate rinse in ultra pure water for 5 min, the surfaces were dried in a nitrogen flow. All the steps were carried out under a sterile laminar flow bench. The pre-activation of the surfaces which contains the generation of hydroxyl groups as surface grafting sites for later immobilization of the cRGD peptides was performed by incubating the metal samples in 2.5 *M* NaOH (VWR Int. GmbH, Darmstadt, Germany) for 24 h at room temperature (RT). After washing in ultra pure water and drying in a nitrogen flow, the metal discs were immersed in 5 *M* ethanolamine hydrochloride (Sigma-Aldrich, Taufkirchen, Germany) in water-free DMSO (Sigma, Taufkirchen, Germany) for 24 h at RT for homogeneous amination of the surfaces. Afterwards, the discs were washed five times with ultra pure water before they were dried again in a nitrogen flow. In the second step of the biometal functionalization, the amino-reactive hetero-bifunctional poly(ethylene glycol) NHS-PEG-COOH (MW 3400, custom-synthesised by Laysan-Bio, Arab, Alabama) was immobilized via peptide binding on the metal implants serving as a linker between the surface and the amino-labeled cRGD-peptide. To covalently couple this poly(ethylene glycol) (PEG) onto the aminated surfaces, 2 m*M* of the PEG linker was dissolved in water-free chloroform (VWR) with 0.5 % (v/v) triethylamine (Sigma) and the discs were incubated for 1 h at RT in the PEGylation solution. Afterwards, the functionalized biometals were washed five times in ultra pure water and dried in a nitrogen stream. 100 m*M* cyclic RGD-peptide (cyclo-(Arg-Gly-Asp-D-Phe-Lys), MW 603.7, Peptides Int., Louisville, KY) [Fig. 1(a)] was immobilized to the free accessible carboxylic groups of the PEG linker via peptide binding using 50 m*M* ethylenediaminecarbodiimide (EDC, Sigma) in acidic 100 m*M* 2-(N-morpholino)ethanesulfonic acid buffer (pH 4.75) as carboxyl activation substance.[29] After 1 h of incubation in a humid atmosphere at RT, the discs were washed twice for 15 min in 20 m*M* TRIS/HCl (with 150 m*M* NaCl at pH 7.4) (Sigma), then rinsed with ultra pure water for 5 min and finally each disc was incubated in 99.9 % ethanol (Uvasol, Merck, Darmstadt, Germany) until just before the cell experiments were started. To evaporate the ethanol, the discs were placed in petri dishes under a laminar air bench for 1 h to ensure sterile conditions before cell seeding. Each biometal was treated with the final concentration of 15×10^{-9} mol cRGD per disc surface of 1.1 cm^2.

Control of surface coating

The surface coating was investigated qualitatively and quantitatively by fluorescence microscopy to observe the overall binding efficiency and homogeneity with the additional use of carboxyfluorescein-labeled (CFsc), cyclic RGD peptides (cyclo [Arg-Gly-Asp-D-Phe-Lys(PEG-Lys(CFsc)-PEG)], MW 1379.59, Peptides Int., Louisville, KY). The same immobilization procedure described before was carried out on additional metal discs of the same type. Right after the immobilization procedure, the samples were measured using an inverted epi-fluorescence microscope (Olympus IX71, Hamburg, Germany) with a peltier-cooled photon-counting EMCCD camera (Andor IXON, 512 × 512 pixels, Belfast, Ireland). Fluorophore excitation was achieved by a 150 W Xenon-lamp with appropriate filter sets (Excitation: 475/35 nm, Emission: 535/40 nm; AHF, Tübingen, Germany). On each sample, the fluorescence intensity was measured by taking the average over five areas per image and metal disc of 10 × 10 pixels.[30]

Cell culture

Human BM was harvested from the iliac crest, generated, and expanded as described previously.[8] The use of human BM was approved by the Ethics Committee of the Heinrich-Heine University of Düsseldorf, Germany and informed consent was given by the patients. Aiming for a higher purification of the mesenchymal cell fraction the cultures were passaged twice to remove hematopoietic cells. Before the experiments, the mesenchymal stem cell character of the cells (MSC) was proven by flow cytometric analysis against defined antigens (CD105+, CD90+, CD73+, CD44+, CD14+, and CD 45−, CD34−) according to the criteria of the International Society for Cellular Therapy (ISCT).[31] The MSC of four healthy donors were cultivated onto the prepared biometal surfaces in 24-well plates with ultra low-attachment surfaces (Costar®, Corning, Corning, NY) to promote cellular adhesion and growth onto the biometals. Initially, a total volume of 50 µL cell suspension (corresponding 4×10^3 cells) was pipetted onto the biometal surfaces and incubated for 30 min allowing cellular attachment. Afterwards, the implant surfaces were totally covered with culture media and cultivated for 14 days without osteogenic stimulation [Dulbecco's modified Eagle medium DMEM, PAA Laboratories GmbH, Pasching, Austria with 1 g/L glucose, 20 % fetal bovine serum, PAA Laboratories supplemented with 100 units/mL penicillin, 2 m*M* L-glutamine and 100 µg/mL streptomycin (PAA)]. Culture conditions were 8.5 % CO_2 and 37°C. The culture medium was changed on every third day.

All experiments were carried out on (A) non activated surfaces (only treated with the washing procedure without further NaOH activation), (B) NaOH pre-activated surfaces, (C) NaOH pre-activated + PEG coated surfaces, and (D) NaOH pre-activated + PEG + RGD-coated surfaces for comparison (A–C served as control samples).

Cell proliferation measurements

Cell proliferation was quantitatively measured using lactate dehydrogenase (LDH) (CytoTox® 96, Promega, Mannheim, Germany), a stable cytosolic enzyme that is released upon cell lysis. Released LDH in culture supernatants was measured with a 30-min coupled enzymatic assay, which results in the conversion of a tetrazolium salt (INT) into a red formazan product. The strength of colour formed is proportional to the number of cells. Cell number was calculated using a standard curve based on the quantity of LDH for defined BM cells.

After 14 days of cultivation, the supernatant was removed and the cells on the metal discs were washed three times with phosphate-buffered saline (PBS, PAA). For cell lysis, the 24-well-plates including cells onto the biometals were frozen at −80 °C. Three hundred microliter fresh PBS was added per dish and incubated on the shaker for 30 min at RT, centrifuged at 650 × g for 5 min. Afterwards 2 × 50 μL cell supernatant was transferred into a 96-well microplate. For the assay, 50 μL LDH substrate solution was added to each well and incubated for 30 min in the dark. After the addition of 50 μL 1 *M* acetic acid the reaction was stopped and the optical density was measured at 490 nm using a microplate reader (Cary 50 Bio, Varian, Darmstadt, Germany). The data were normalized to the corresponding cell number.

Cell differentiation measurements

Osseous alkaline phosphatase (ALP), a membrane-bound tetrameric enzyme attached to phosphatidyl-inositol moieties located on the outer cell surface, was assayed using the release of p-nitrophenol from p-nitrophenolphosphate (Sigma).[32] The culture medium was removed and the cells on the discs were washed three times with phosphate-buffered saline (PBS, PAA). After cell lysis at −80°C, 300 μL PBS was added and the wells were incubated on the shaker for 1 min at RT followed by centrifugation at 650 × g for 5 min. For the ALP assay, 2 × 50 μL cell supernatant was transferred into the wells of a 96-well microplate and 100 μL of the ALP-substrate was added. Immediately after the start and after 30, 40, 60, and 120 min the extinction was measured at 405 nm using a microplate reader (Cary, Varian). The reaction was finally stopped with 50 μL 3 *M* sodium hydroxide per well. The quantity of p-nitrophenol produced was calibrated with a standard curve of known ALP activity of bovine intestinal mucosa (Sigma). The specific ALP activity (pUnits ALP/cell × h) was determined by the quotient of ALP activity and cell number.

To quantify occurring cell-differentiation, the same amount of MSC which was used on the functionalized biometals was seeded onto borosilicate glass cover slides (Thermo Scientific, Waltham, MA) functionalized prior as followed: Pre-activated (pre-ac) = treated with NaOH, pre-ac + PEG = pre-activated with NaOH, and covalently bound PEG on ethanol amine, pre-ac + PEG + RGD = RGD covalently bound to the PEG surface and pre-ac + PEG + RGD-CFsc = RGD-CFsc covalently bound to the PEG surface, that were placed in sterile petri dishes (NUNC, Wiesbaden, Germany). After 7 days of cultivation in culture media without osteogenic stimulation, the glass cover slides were washed three times with PBS, fixed with 4 % paraformaldehyde (Histofix, Carl Roth GMBH, Karlsruhe, Germany) for 10 min at RT and washed with aqua dest three times. Afterwards, the glass cover slides were treated with AP-staining solution (Sigma), incubated for 1 h at RT, washed with aqua dest and finally covered with glycerine gelatine (Merck).

Cell morphology

Scanning electron microscopy (SEM) was used to document cellular morphology onto the biometal surfaces. After a cultivation period of 7 days, the culture medium was removed and the wells were rinsed three times with PBS and fixed with 2.5 % glutaraldehyde (Plano, Marburg, Germany) for 20 min at RT. After several washes in PBS, the samples were dehydrated stepwise twice for 15 min in acetone (50 % up to 100 %). The cells were dried in a critical point dryer (CPD 030, Bal-Tec, Witten, Germany) and subsequently gold sputtered (30-nm layer) in continuous argon influx using a sputter coater (108 auto, Cressington, Watford, England). The specimens were analyzed by a SEM (S-3000N, Hitachi, Krefeld, Germany).

RESULTS

Control of surface coating

The successful immobilization of the cRGD peptide was ascertained by attaching a fluorophore (CFsc) labeled cRGD-peptide to the metal surfaces with the described protocol. In qualitative aspect, the presence of bound CFsc-labeled cRGD biolayer could be detected on all tested biometals and the different surface topography of the used materials was clearly observable [Fig. 2(a–e)]. The CFsc-cRGD was homogenously distributed on TiPol, SS, and CCPol, while on the rough surfaces of TiSa and CCPor the varying levels resulted in a high standard deviation [Fig. 2(f)].

In direct quantitative comparison [Fig. 2(f)], as the fluorescence intensity is proportional to the amount of bound CFsc-cRGD molecules, the measured intensity of the covalently immobilized CFsc-cRGD peptide to the different biometals was comparably high on TiSa, TiPol, and non-significantly lower on SS considering the standard deviation. On CCPor and CCPol a significantly lower immobilization yield was observed in comparison with TiSa and TiPol of about 70 % and 50 %, respectively.

The ligand density measured via fluorescence microscopy and intensity comparison to a single molecule showed a minimal concentration of about 4 pmol/cm^2 (for CCPol) on the functionalized biometals with CFsc cRGD. Thus, it was guaranteed to get a higher density than the critical density of >0.01 pmol/cm^2 as described by Barber et al.[23]

Cell morphology

The evaluation of the cells attached onto the metal discs by SEM showed that cells could be seen on all metals within all treatments. The cells were spread onto the surfaces of TiSa, TiPol, CCPol, and SS while on CCPor the cells were connected to ball-like surface structures. The cellular

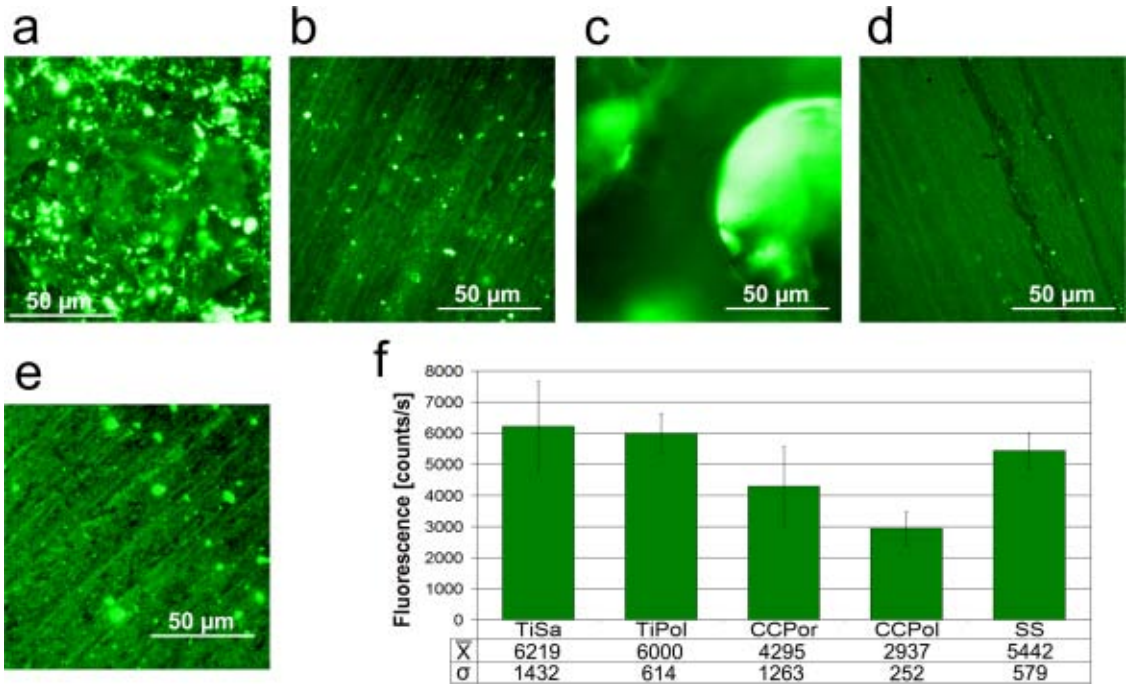

FIGURE 2. Fluorescent microscopy images of the five different biometal surfaces (a) TiSa, (b) TiPol, (c) CCPor, (d) CCPol, (e) SS after RGD-CFsc labeling. Measured fluorescence intensity of the CFsc-labeled cRGD covalently bound onto different biometals (data of five experiments) is shown in (f). The average fluorescence intensity and the standard deviation of each sample are indicated below the corresponding value bars. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

morphology did not differ between the different biometals (Fig. 3).

Cell proliferation and differentiation

The BM cells proliferated and differentiated on all five biometals over 14 days of cultivation as demonstrated in Figure 4(b,c) for four experimental repetitions. Native CCPol was taken as a reference because in average it has shown the highest proliferation and differentiation in comparison to the other biometals. For that reason, the final analyses of the assays were all normalized to the results of this metal type.

In average, the BM cell proliferation on RGD coated surfaces was highest on CCPol in comparison to the other used biometals followed by SS, CCPor, TiSa, and TiPol. The proliferation results of MSC on the biometals were independent from the cRGD coating and showed high variation. The applied statistical analyses (Kruskall-Wallis verified with Mann-Whitney, significance level of $\alpha = 0.05$) showed no significant differences between the cRGD-coated biometals and the respective controls (all biometals without cRGD-coating) for cell proliferation.

Over the cultivation period of 14 days, the cells differentiated into osteoblasts as shown by a positive staining for ALP of MSC on a NAOH + PEG + RGD-CFsc-treated glass surface [Fig. 4(a)]. The measured ALP activity for three experimental repetitions displayed by the cells grown on the differently treated biometal surfaces varied enormously [Fig. 4(c)] but correlated with cell proliferation [Fig. 4(b)].

The average ALP activity was highest on the CCPol biometal compared with the other RGD coated metals followed by TiSa, SS, CCPor, and TiPol, respectively. Similar to the proliferation results the statistical analyses of the ALP activity results (Kruskall-Wallis verified with Mann-Whitney, significance level of $\alpha = 0.05$) showed no significant differences between the cRGD-coated biometals. The respective controls of each biometal coating neither demonstrated a significant improvement of the cRGD-coating.

Summing up the results, no increase in biological compatibility could be detected by the applied coating method with cRGD compared with the controls. In general, the highest cell proliferation as well as cell differentiation was achieved in average on CCPol. In comparison with the other metals and in the context of this study design the best implant surface seems to be polished cobalt chrome (CCPol).

DISCUSSION

In this *in-vitro* study, the influence of RGD surface coating on the proliferation and differentiation of BM-MSCs was investigated for different biometals. The cells cultivated onto RGD-modified surfaces showed neither enhanced proliferation nor increased osteoblastic differentiation when compared with uncoated controls.

Special emphasis in this study was placed on covalent binding of the RGD-peptide to the five different metal surfaces. To compare the functionalized surfaces, coating efficiency was verified by quantitative and qualitative fluorescence microscopy. Thus, a correlation could be drawn

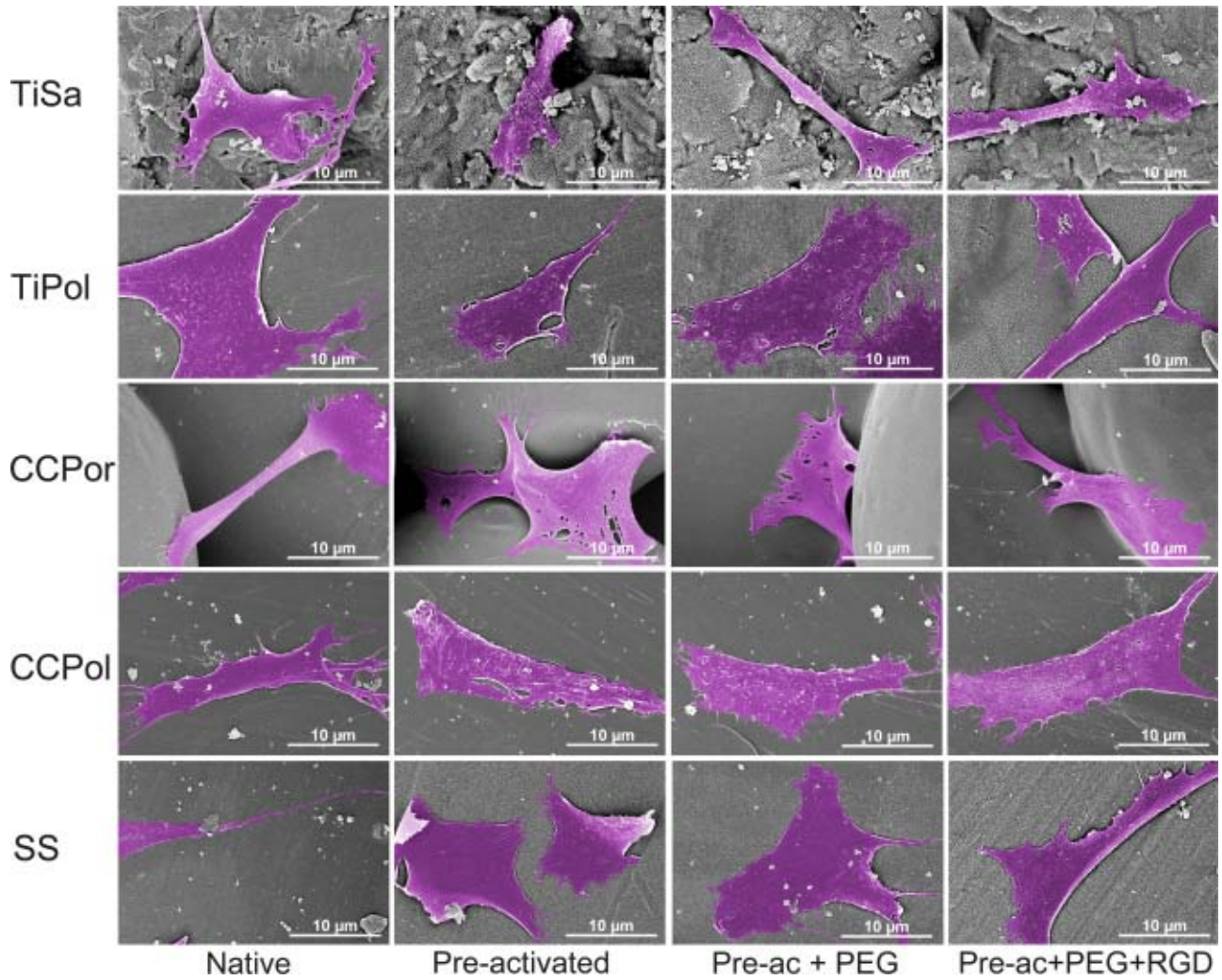

FIGURE 3. Colour enhanced SEM (20 kV, 700×) analysis of the cells adhering to the differently treated biometals after 7 days of incubation: Native surface, pre-activated (pre-ac) = treated with NaOH, pre-ac + PEG = pre-activated with NaOH and labeled with PEG, pre-ac + PEG + RGD = RGD covalently bound to the surface. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

between coating efficiency and cell culture results. To ensure an exact correlation between proliferation and differentiation, supernatants for assays were always taken from the same microplate wells. The highest average cell proliferation and differentiation was detected on CCPol, which was the metal surface with the lowest amount of RGD-coating. Thus, according to our data, BM-MSCs lead to higher proliferation and osteoblastic differentiation rates when the least RGD was attached to the surface that they are cultured onto. However, SEM showed adherent cells on all biometals and surfaces, and cellular morphology did not differ among the different biometals. In our experiments, we used a cyclic RGD-sequence which is also feasible for *in-vivo* application because it has superior solution stability compared with linear RGD-peptide over a pH range of 3–7.[26]

Anyhow, RGD is not the only candidate for improving cellular adherence onto biomaterials. Other *in-vitro* studies on human bone derived cells[21] or human BM-MSCs have also focused on the improvement of cell-adhesion by functionalising the metal surface with either collagen I, vitronectin or by hydroxyapatite coating.[25,33,34] In contrast to our study, the cell culture periods were much shorter in these studies, such as 15–60 min,[21] 1–3 h,[33] and 1–24 h,[25,34] respectively, leading to a positive effect of RGD-coating on cell-adhesion. Although cell-adhesion onto a biomaterial may be a prerequisite for initial local proliferation in mesenchymal cells, the positive influence over a culture period of 14 days remains doubtful, as illustrated by our data.

In contrast to the large number of published experimental work focusing on ceramics, only a few *in-vitro* studies have investigated the proliferation and differentiation of human BM-MSCs onto biometals. Tosatti et al.[35] showed increased proliferation of mouse osteoblasts (MG 63) on RGD-coated surfaces (titanium, glass, and plastic), whereas cellular differentiation was not promoted by RGD after 7 days. In contrast to Tosatti et al.,[35] our biometal specimens were placed in ultra-low binding-well plates prohibiting cellular attachment to the plastic and forcing the BM–MSCs to attach to the metal discs. Thus, we ensured that only the

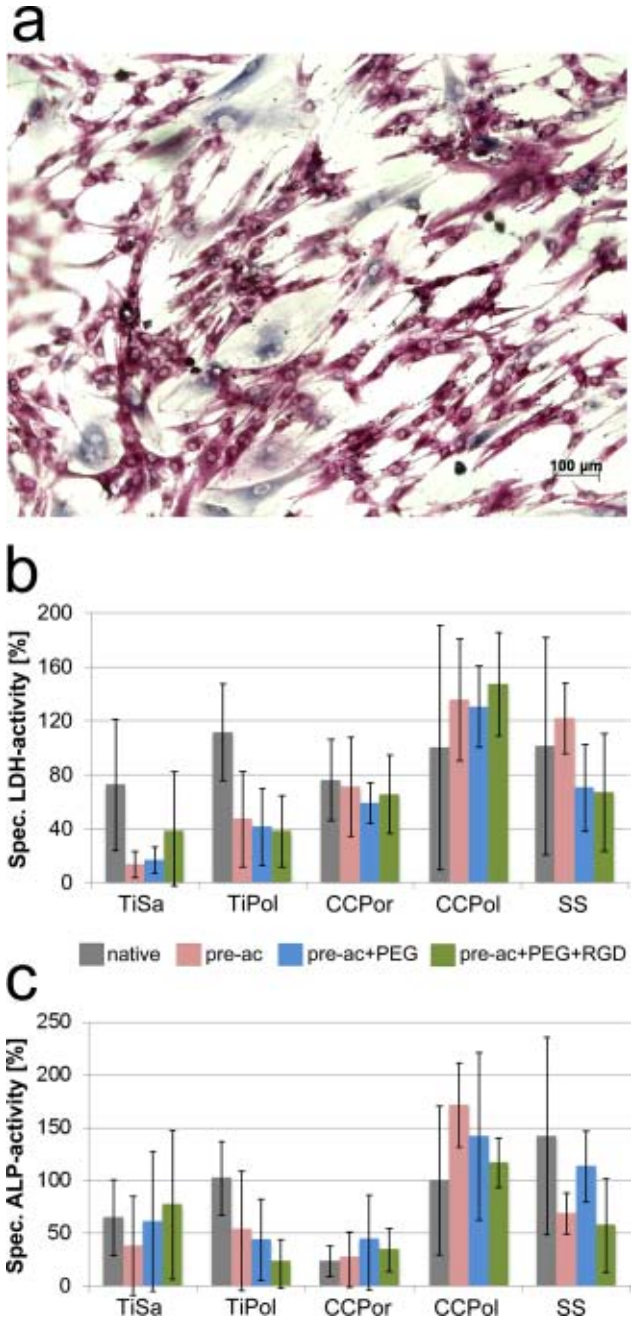

FIGURE 4. (a) Positive staining of the BM cells for ALP on the glass surface with pre-ac + PEG + RGD-CFsc = RGD-CFsc covalently bound to the surface after 7 days of *in vitro* cultivation. (b) Cell proliferation measured by specific LDH activity assay and (c) Cell differentiation measured by specific ALP activity (data of three experiments). The standard deviations of each sample are indicated by the corresponding error bars. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

cells attached directly to the metal surfaces were detected by the proliferation and differentiation assays.

An improvement of bony ingrowth on orthopedic endoprostheses is not only dependent on local cellular proliferation but also on osteoblastic differentiation and biomineralization. In relation to differentiation, a weakly positive effect was observed by Zreiqat et al.[22] using human bone derived cells showing increased expression of bone matrix mRNAs (osteocalcin, pro-collagen I) after 7 days, and increased expression of ALP-mRNA after 14 days as typical markers for an osteoblastic differentiation, if the cells were cultured on linear RGD-functionalized titanium surfaces. However, the protein level of pro-collagen was only higher on RGD-functionalized titanium on day 7, and was higher on bare titanium on day 14.

In contrast, Pallu et al.[34] did not show any superiority of RGD-coated titanium compared to pure titanium on the osteoblastic differentiation of human BM-MSCs after 10 days of cultivation on quantitative RT-PCR (Runx-2 and ALP).

In the study of Senyah et al.,[36] the adhesion of MC3T3-E1 cells on RGD-coupled borosilicate and titanium surfaces was non-specific and not necessarily dependent on a particular RGD sequence and therefore comparable with DGR, the retro-sequence of RGD.

The RGD-coupling method used in our study was a surface functionalization according to the protocols of Janissen et al.[27,28]—a proven method for covalent binding for different kinds of surfaces and biomolecules. Higher peptide concentrations than 14.5×10^{-12} mol cRGD per disc surface ($\sim$13 pmol/cm^2) were not feasible as in former experiments functionalized surfaces were already saturated at this amount of ligand density.

It is evident that *in-vitro* data cannot be translated directly to the *in-vivo* situation. Most *in-vivo* studies where titanium implants were treated with cyclic[10,37–40] or linear RGD[41] have shown positive effects on bone formation. Ferris et al. demonstrated a significant increase in new bone thickness around linear RGD-modified surfaces of polished titanium rods in rat femurs 2 and 4 weeks after implantation[41] Elmengaard et al.[10] showed that a cyclic RGD coating on unloaded press-fit titanium implants has an osteoconductive effect only directly at the interface 4 weeks post implantation in the dog femur. Kroese-Deutman et al.[38] demonstrated that bone formation was enhanced in rabbits on cyclic RGD-treated titanium after 4 and 8 weeks in contrast to native titanium fiber mesh only.

Another application of RGD for plastic and reconstructive surgery was shown by subcutaneous implantation of the linear RGD-peptide covalently integrated in PEG-hydrogels displaying a high biocompatibility and tissue integration without any foreign body reaction in rats,[42] and in sheep using RGD-alginate.[43]

In contrast to the aforementioned and other studies, only few RGD-applications on titanium implants showed decreased bone formation.[44,45] The utilization of cyclic RGD-peptide for bone regeneration in sheep displayed that PMMA-spacers covalently linked to RGD-peptide in a diaphyseal tibia defect, did not enhance torsional strength compared with PMMA-spacers alone.[46] While Schliephake et al.[45] described weakly positive effects of RGD-coated implants in dog mandible in earlier studies,[39,40] they did not find a significant increase in periimplant bone regeneration after 1 and 3 months using cyclic RGD-peptide anchored with phosphonate onto a dual acid-etched (DAE)

titanium surface. The authors attributed this to the well designed titanium surface superseding any additional covering with receptors for adhesion molecules.[45]

To our knowledge this work is the first study focussing on proliferation and differentiation of BM–MSCs cultivated onto covalently coated cRGD surfaces of five different biometals in direct comparison over 14 days. Using the cyclic RGD-peptide covalently linked to the metal surfaces, it did not result in any improvement of BM–MSC differentiation and proliferation and did not seem to be suitable for enhancement of bone apposition on implant surfaces and improvement in clinical outcome.

Based on the data of all these studies and our own data, the crucial question is why do RGD-coated biometals prevent cellular proliferation and do not promote osteoblastic differentiation *in-vitro*?

RGD sequences are not only required for integrin-mediated cell anchoring[2] but are also a stimulus for integrin signalling pathways which can modulate cellular morphology, motility, and differentiation. Based on these signalling effects, the statement that RGD-coated biomaterials promote integrin-mediated cell-adherence is not a general principle and more sophisticated as demonstrated for osteoblast progenitor cells. In the study of Taubenberger et al.,[47] the mechanism of integrin-binding to RGD-motifs that regulates cell-adhesion, proliferation and differentiation was addressed by analysing pre-osteoblast and MSC interactions with structurally well-characterized native (Col) and partially denatured (pdCol) collagen I matrices. They showed that pdCol presents RGD-peptides that trigger the binding of $\alpha_5\beta_1$- as well as αv-integrins and initiate cell processes such as osteoblast adhesion, -proliferation, -motility and -differentiation. Depending on cell type, there are different RGD-dependent pathways known that determine ion channel regulations. Arteriolar smooth muscle cells for example interact via RGD-ligands that connect to $\alpha_5\beta_1$-integrins in order to modulate the activity of large conductance [$Ca^{(2+)}$-activated $K^{(+)}$ (BK)] channels.[48,49] As a novel pathway to enhance and to mediate cell-adhesion and survival, tissue transglutaminase (TG2), an extracellular crosslinking enzyme involved in matrix turnover and in bone differentiation, represents a novel cell-adhesion and survival mechanism in human osteoblasts in association with FN.[50] Wang et al.[50] investigated the involvement of cell surface receptors and their intracellular signalling molecules to further explore the pathway mediated by this novel TG-FN heterocomplex. It was demonstrated that the presence of RGD peptides is crucial in regulating the compensatory effect of TG-FN on osteoblast cell-adhesion and actin cytoskeletal formation. Vonwil et al.[51] showed that a RGD-restricted substrate interface is sufficient for the adhesion, growth and cartilage forming capacity of human chondrocytes. Chondrocytes that were cultivated on RGD-modified and protein adsorption resistant tissue culture polystyrene (TCPS), could only bind to the surface through RGD-peptides. Moreover, two other studies reported contradicting findings about the influence of RGD-peptide modified materials on chondrocyte proliferation and matrix production.[52,53] RGD-initiated apoptosis was also reported for chondroblast precursors by Garciadiego-Cázares et al.[54] In this study $\alpha_5\beta_1$-integrins were blocked by specific antibodies or RGD-peptides to induce inhibition of prehypertrophic chondrocyte differentiation and ectopic joint formation between proliferating chondrocytes and hypertrophic chondrocytes.[54] In general these antagonistic effects of non-immobilized RGD-peptides can induce apoptosis of non-adherent cells. This principle is successfully used in therapeutic interventions for osteoporosis, renal failure, tumor therapy and angiogenesis.[55–57] However, the exact mechanisms of RGD-dependent integrin-binding for the regulation of cell-adhesion, -proliferation, and -differentiation are still not elucidated. Despite the fact that a common RGD-motif is present in proteins used for biomaterial functionalization *in-vivo*, a great variation of integrin-selectivity can be found within this group of proteins. Other adhesion-sequences of ECM-proteins or amino-acid-sidechains next to the RGD-sequence can influence specificity, selectivity, and affinity toward integrins and therefore interfere with the effect of the RGD-motif.[12,58,59]

CONCLUSIONS

In this study, only polished cobalt chrome with covalently bound RGD could show a slightly positive effect in cellular proliferation and differentiation. In result the RGD-coating showed no significant improvement of the biometals sand-blasted (TiSa) and polished (TiPol) Ti6Al14V, porocoated cobalt chrome (CCPor), and polished SS functionalized with cRGD compared with their native surfaces. Therefore, we believe that RGD-coating does not offer a significant improvement in cellular proliferation and osteoblastic differentiation.

ACKNOWLEDGMENTS

The authors thank Fares Haddad, Department of Orthopedic Surgery, University College Hospital, London, United Kingdom, for his support.

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