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ORIGINAL ARTICLE

Cross-linking of collagen I by tissue transglutaminase provides a promising biomaterial for promoting bone healing

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Abstract Transglutaminases (TGs) stabilize proteins by the formation of ε(γ-glutamyl)lysine cross-links. Here, we demonstrate that the cross-linking of collagen I (COL I) by tissue transglutaminase (TG2) causes an alteration in the morphology and rheological properties of the collagen fibers. Human osteoblasts (HOB) attach, spread, proliferate, differentiate and mineralize more rapidly on this cross-linked matrix compared to native collagen. When seeded on cross-linked COL I, HOB are more resistant to the loss of cell spreading by incubation with RGD containing peptides and with α1, α2 and β1 integrin blocking antibodies. Following adhesion on cross-linked collagen, HOB show increased phosphorylation of the focal adhesion kinase, and increased expression of β1 and β3 integrins. Addition of human bone morphogenetic protein to HOB seeded on TG2 cross-linked COL I enhanced the expression of the differentiation marker bone alkaline phosphatase when compared to cross-linked collagen alone. In summary, the use of TG2-modified COL I provides a promising new scaffold for promoting bone healing.

Keywords Tissue transglutaminase · Cross-linking · Collagen I · HOB · Integrins

Abbreviations

TGs	Transglutaminases
TG2	Tissue transglutaminase
HOB	Human osteoblasts
Collagen I	COL I
FAK	Focal adhesion kinase
PKCα	Protein kinase α
hBMP7	Human bone morphogenetic protein 7
bALP	Bone alkaline phosphatase
ECM	Extra cellular matrix
MMPs	Matrix metalloproteinases
AFM	Atomic force microscopy
RGD	GRGDTP
RAD	GRADSP
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
OPN	Osteopontin
VEGF	Vascular endothelial growth factor
RT	Reverse transcription
TIMP	Tissue inhibitors of MMPs
VCAN	Versican
VTN	Vitronectin
HA	Hydroxyapaptite
Si-CaP	Silicate-substituted calcium phosphate
PE	Polyethylene

Introduction

During the last decade, there have been many significant advances within medicine and biotechnology, particularly in the field of tissue engineering. Collagens are one of the most popular natural materials among the biomaterials. Being the most abundant protein in mammals, collagens are widely distributed in bone, cartilage, blood vessels, skin, cornea, tendons and ligaments (Kruger et al. 2013) and frequently investigated. Among the 28 collagen family members, collagen I (COL I) is one of the major

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extracellular matrix (ECM) proteins in bone (Ferreira et al. 2012). Providing the binding sites, such as RGD motifs, to the cell surface integrin receptors, collagens are essential for adhesion, cell survival, migration and to maintain the physical stability of tissues and organs (Plant et al. 2009). Like other proteins, collagens are biologically degradable by different proteases in the body, such as matrix metalloproteinases (MMPs) (McKleroy et al. 2013). To enhance the resistance of collagens to protease degradation and to further explore their application as biomaterials, the structure of collagen fibrils has been altered using chemical agents (Chan et al. 2013). Improvement of these agents has been done, but the toxicity that they induce in cells still remains a big problem. Therefore, there is urgency to discover new reagents to mediate the cross-linking of collagens with lower or no toxicity.

Mammalian transglutaminases (TGs) are Ca^{2+}-dependent enzymes which mediate the posttranslational modification of both intra-and extracellular proteins by catalyzing the formation ε-(γ–glutamyl) lysine bonds (Griffin et al. 2002). As such they are a natural choice for the cross-linking of natural polymer-based biomaterials. Among the eight catalytically active transglutaminases, tissue transglutaminase (TG2) is the most ubiquitous, found predominantly as a cytosolic protein, but also at the cell surface and in the extracellular matrix (Bergamini et al. 2011). As a multifunctional protein, TG2 is involved in various physiological and pathological conditions, such as wound healing (Wang and Griffin 2012), tumor progression and metastasis (Kotsakis et al. 2011; Wang and Griffin 2013; Mehta et al. 2010) and fibrosis (Huang et al. 2009; Olsen et al. 2011). By an unknown secretory mechanism, TG2 is externalized in association with cell surface receptors, such as syndecan-4, and deposited into the ECM (Wang et al. 2010, 2012). TG2, once deposited into the ECM, can increase the resistance of proteins to protease degradation by its cross-linking activity. In addition, once bound to its high affinity partner fibronectin, TG2 can rescue the loss of cell adhesion leading to cell death (also known as anoikis) by activating the syndecan-4 and α5β1 integrin co-signaling pathway involving focal adhesion kinase (FAK), protein kinase α (PKCα) and ERK1/2 (Wang et al. 2010, 2011; Wang and Griffin 2012).

The importance of TG2 in bone formation has been investigated by different groups (Heath et al. 2001; Piercy-Kotb et al. 2012; Chau et al. 2005; Tarantino et al. 2013; Orlandi et al. 2009). Griffin and colleagues first demonstrated that TG2 can be applied as a cross-linker for COL I (Chau et al. 2005). Compared to UV irradiation, physical heating and chemical treatment, TG2 provides a milder but still efficient cross-linking capability. As a mammalian enzyme, the application of TG2 in the cross-linking reaction overcomes the disadvantage of introducing non-biological toxic reagents into a physiological system. Compared to native collagen, TG2 cross-linked COL I showed a greater efficiency in inducing cell adhesion, spreading and differentiation in human osteoblasts, with no signs of cell toxicity (Chau et al. 2005). More recently, another TG family member Factor XIIIa has also been shown to be involved in bone formation (Piercy-Kotb et al. 2012). In keeping with the importance of TG2 in matrix deposition, it has also been recently shown to be important in angiogenesis by promoting matrix-bound vascular endothelial growth factor (VEGF) signaling during angiogenesis (Wang et al. 2013). Here, we demonstrate the importance of TG2 cross-linked COL I as a novel biomaterial in bone healing. We propose a mechanism for its increased biocompatibility, whereby TG2-modified collagen triggers an enhanced cellular response mediated by integrin binding.

Materials and methods

Reagents and antibodies

Reagents were purchased from Sigma-Aldrich (Dorset, UK), unless stated below. Human bone morphogenetic protein (hBMP-7) was purchased from R&D Systems (Abingdon, UK). Guinea pig transglutaminase 2 was extracted and purified according to a specific protocol (Leblanc et al. 1999). Enzyme activity, ranging between 4 and 5 U/mg, was evaluated using the hydroxamate assay (Folk and Chung 1973). Rat tail collagen was extracted following the method described by Chau et al. (2005). The irreversible active-site-directed TG2 inhibitor R283 (1,3-dimethyl-2-[(2-oxopropyl)thio] imidazolium chloride) (Freund et al. 1994) was synthesized at Aston University.

Cell culture

HOB cells were kindly supplied by Professor S. Downes and Dr. S. Anderson (School of Biomedical Sciences, University of Nottingham) and used at low-passage number. The cells were cultured in DMEM medium supplemented with 10 % (v/v) heat-inactivated FBS, 1 % (v/v) non-essential amino acids and 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin and maintained in a humidified atmosphere at 37 °C and with 5 % (v/v) CO_2.

Preparation of the native and cross-linked collagen

The extracted COL I, neutralized and adjusted to pH 7.4 using 1 M NaOH in the presence of 10× PBS in serum free DMEM), was used as native collagen. Part of the

neutralized COL I mixture was treated with TG2 (10 μg/ml unless otherwise stated) with addition of 5 mM DTT, 5 mM $CaCl_2$ in 10 mM Tris–HCl pH 7.4 to reach the desired concentrations. 96-well tissue culture plates were coated with either native or cross-linked COL I and allowed to gel at 37 °C overnight in a humidified atmosphere. Following polymerization, wells were washed once with sterile PBS, pH 7.4, and once with serum-free cell culture medium and used immediately.

Atomic force microscopy (AFM)

Neutralized and treated COL I samples were added directly on glass slides (BDH, Poole, UK) and allowed to polymerize at 37 °C for 2 h. After washing once with distilled water, the samples were left to air dry for a further 30 min. A Dimension 3100 Atomic Force Microscope (Veeco Instruments Ltd., Cambridge, UK) was used for sample imaging; the 'tapping mode' was set with a drive frequency close to the resonance frequency of the cantilevers. Scan size was set at 0.8–8 μm (scan rate 1 Hz). Measurements were made on three representative images with $n = 50$.

Rheological studies

The elastic (G') and the viscous (G'') moduli of native and TG2-treated collagen (250 μg/ml) were evaluated using a Bohlin C-VOR KTB 30 rheometer (Malvern Instruments, Worcestershire, UK), fitted with a cylindrical cuvette geometry and 2 cm diameter serrated plates. The plate-gap dislocation distance was set at 0.8 mm with sample conditions maintained at a constant temperature of 37 °C.

Cell proliferation assay

Viable cell number was measured using the XTT II assay kit (Roche, UK) as previously described (Kotsakis et al. 2011). Briefly, 96-well plates were coated with native or TG2 cross-linked COL I. 2×10^5/well of HOB cells in serum-free DMEM medium were seeded into the wells and incubated for 16 h. 30 μl of the XTT II reagent mixture was added directly into the medium and the absorbance (490 nm wavelength) was measured after a further 4-h incubation at 37 °C using a Microplate Reader (Biotek, Bedfordshire, UK).

Cell adhesion assay

The HOB cell adhesion assay was performed as reported by Wang et al. (2011). Briefly, HOB cells were seeded on native or TG2 cross-linked collagen (5 mg/ml COL I, 10 μg/ml TG2) at a density of 2×10^4 cells/well in serum-free DMEM medium. The cells were allowed to adhere for 40 min in the presence of the treatments as indicated below. After washing once with PBS, pH 7.4, the adherent cells were fixed, permeabilized and stained with May-Grünwald and Giemsa co-stains. The attached and spread cells were photographed with an Olympus DP10 digital camera using a Nikon CK2 microscope and quantified using Scion Image™ software (Scion Corporation, Maryland, USA). Spread cells were expressed as a percentage of the total attached cells. GRGDTP (RGD) and the control GRADSP (RAD) (Bachem, Nottingham, UK) peptides between the ranges of 50 and 500 μg/ml were used for the RGD effects on cell adhesion assay, while the functional blocking antibodies against β1, α1 or α2 integrin subunits (Santa Cruz, UK) were used between 1:500 and 1:10,000 dilutions.

Table 1 Antibodies used in this study

Antigen	Species source	Company
β1 Integrin	Rabbit, polyclonal	Santa Cruz (UK)
β3 Integrin	Rabbit, polyclonal	Santa Cruz (UK)
α1 Integrin	Rabbit, polyclonal	Santa Cruz (UK)
α2 Integrin	Rabbit, polyclonal	Santa Cruz (UK)
p-Tyr397 FAK	Rabbit, polyclonal	Millipore (UK)
FAK	Mouse, monoclonal	Santa Cruz (UK)
α-Tubulin	Mouse, monoclonal	Sigma-Aldrich (UK)

Western blotting and immunoprecipitation

Western blotting was performed to detect the presence of target protein. HOB cells were cultured on native and TG2 cross-linked COL I (5 mg/ml COL I, 10 μg/ml TG2) in serum-free DMEM medium for 1 or 12 h and prepared in cell lysis buffer. After pre-clearing at 300 × g for 10 min and protein concentration quantification, 50–200 μg of total protein was used for Western blotting analysis with the specific antibodies listed in Table 1, while α-tubulin antibody was used as the equal loading control. Immunoprecipitation was performed to detect β1 integrins, Briefly, the HOB cell samples were pre-cleared with rabbit IgG for 90 min, followed by Protein A beads incubation for 90 min. The supernatants were incubated with 0.5 μg of anti-β1 integrin antibody for 90 min at 4 °C, followed by Protein A beads incubation for 2 h. The protein samples were extracted from the beads by boiling for 5 min and used for Western blotting using anti-β1 integrin antibody (Santa Cruz, UK).

RNA isolation

Total RNA was isolated from 10^6 HOB cells cultured on native and TG2 cross-linked COL I (5 mg/ml COL I, 10 μg/ml TG2) for 12 h, using TRIzol® Reagent

Table 2 Primers used for qPCR study

Gene	Forward	Reverse
β-Actin	5′-GGCATCCTCACCCTGAAGTA-3′	5′-GGGTGTTGAAGGTCTCAAA-3′;
bALP	5′-CCACGTCTTCACATTTGGTG-3′	5′-GCAGTGAAGGGCTTCTTGTC-3′
OPN	5′-GACTCGTCTCAGGCCAGTTG-3′	5′-CTGGTATGGCACAGGTGATG-3′

(Invitrogen, Paisley, UK) and further purified using the RNeasy kit (Qiagen, West Sussex, UK) to remove genomic DNA and organic components. The RNA quality was checked using Bioanalyzer (Biotek, Bedfordshire, UK) and verified by agarose gel electrophoresis.

PCR array and real time-PCR study

cDNAs prepared by reverse transcription of 1 μg of RNA isolated from HOB cell seeded on native or cross-linked COL I were used for the PCR studies. PCR array was performed using the RT^2 Profiler PCR human extracellular matrix and adhesion molecules array (SABiosciences, Tebu-Bio Ltd., Peterborough, UK) on an Mx3000P QPCR System (Stratagene Technologies, UK) according to the manufacturer's protocol. Briefly, an initial denaturation at 95 °C for 10 min was followed by 15 s at 95 °C, 40 cycles of annealing at 60 °C for 1 min and a primer extension at 72 °C for 30 s. Data were analyzed using the Stratagene MxPro QPCR software. Five different housekeeping genes (β2-microglobulin, hypoxanthine phosphoribosyltransferase, ribosomal protein L13a, GAPDH and β-actin) were used to normalize the array. Additional positive controls and one negative control were also used. For the semi-quantitative reverse transcription (RT) PCR study, cDNA samples were used to detect the levels of bone alkaline phosphatase (bALP) and osteopontin (OPN) mRNAs. The human primers were designed to span introns to avoid amplification from contaminating genomic DNA (Table 2) and synthesized by Sigma Genosys (UK).

bALP activity assay

Differentiation of HOB cells was evaluated analyzing the bone alkaline phosphatase (bALP) activity using reagents and protocols from Sigma kit 104-LS (Sigma-Aldrich, Dorset, UK). Briefly, 2×10^5 HOB cells in complete medium were seeded onto native or TG2 cross-linked COL I (5 mg/ml COL I, 10 μg/ml TG2) for 2 weeks, and in some experiments, HOB cells were also treated with recombinant human bone morphogenetic protein 7 (hBMP7, 20 nM final concentration). The samples were fixed with 3.7 % paraformaldehyde in PBS, pH 7.4 for 30 min at room temperature. The presence of bALP was visualized using the Fast Violet salt and Naphthol phosphate according to the manufacturer's instructions. After washing with dH_2O and air dried, samples were photographed with an Olympus DP10 digital camera using a Nikon CK2 microscope.

Mineralization staining

The mineralized bone matrix by HOB cells was measured using Von Kossa staining (1901). Briefly, after culturing on native or cross-linked collagen for 12 days, the samples were was fixed with 3.7 % paraformaldehyde for 30 min and dehydrated with 70 % methanol for 1 h. After a quick wash with distilled H_2O, 2 % silver nitrate was used to stain the samples combined with a UV exposure for at least 20 min. After washing with distilled H_2O five times, the samples were fixed with 5 % sodium thiosulfate for 3 min. Following washing, the samples were imaged using a Nikon digital camera with a 20× objective.

Statistical analysis

All experiments were carried out three times in triplicate and the results expressed as mean ± SD. Independent data sets were analyzed with unpaired Student's *t* test or with a one-way ANOVA test. *, p value <0.05 was considered statistically significant.

Results

TG2 cross-linking alters the physical characteristics of COL I

Our previous data indicated that TG2 cross-linking led to alterations in COL I fibrillogenesis by increasing the rate of fibril formation. To further explore this finding, native and cross-linked COL I with 10 μg/ml of TG2 was analyzed using AFM. As shown in Fig. 1a, the untreated COL I shows a typical matted arrangement of straight fibrils, with diameter of 100–300 nm and the typical banding pattern (*D* period) of 67 ± 3.4 nm. The cross-linked COL I fibrils, although initially looking comparable, have a much closer banding pattern with a mean *D* period of 35 ± 11.8 nm (Fig. 1b). The variability in *D* period of cross-linked COL I fibrils was also greater than that of untreated COL I. When comparing the shear and viscous moduli of native (Fig. 1c) and cross-linked COL I gels (Fig. 1d), the shear modulus was increased to a maximum of 1.6 kPa in cross-linked

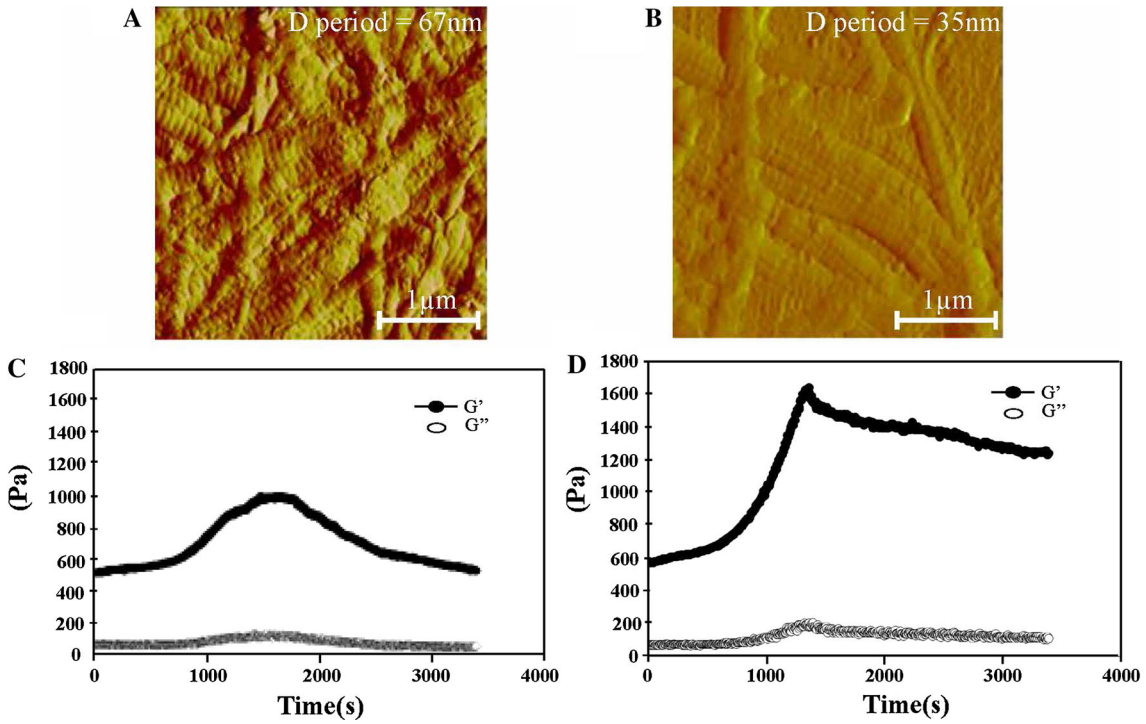

Fig. 1 **a** AFM analysis of TG2-cross-linked COL I and native COL I. **b** Rheological properties of native. **c**, **d** TG2-treated COL I

COL I, compared to a maximum of 1 kPa in untreated COL I, suggesting that cross-linking results in a stiffer gel.

TG2 cross-linked COL I promotes HOB cell proliferation

The proliferation rates of HOB cells cultured on 0.1–25 mg/ml native or cross-linked COL I (with 2–10 μg/ml TG2) were determined using the XTT II cell viability assay. Overall the proliferation of HOB cells was generally increased when seeded on TG2 cross-linked COL I, compared to the native collagen, but the proliferation became reduced as the concentration of COL I increased (Fig. 2). At the COL I concentration of 25 mg/ml, the proliferation rate of cells was comparable to that of the not-cross-linked collagen. Therefore, the concentrations of 5 mg/ml of COL I and 10 μg/ml of TG2 were chosen for the future experiments.

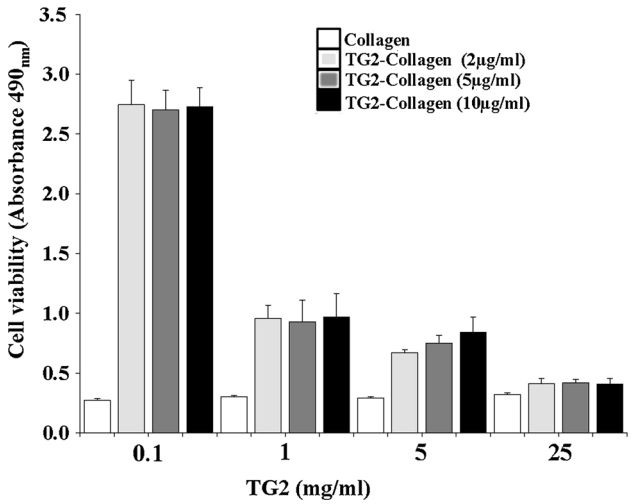

Fig. 2 Viable cell number of HOB cells cultured for 24 h on 0.1–25 mg/ml native or cross-linked collagen using 2–10 μg/ml TG2 and measured using the XTT II assay kit

Gene expression profile of HOB cells cultured on native and TG2 cross-linked COL I

cDNA extracted from HOB cells cultured on native and TG2 cross-linked COL I (5 mg/ml COL I, 10 μg/ml TG2) was used to analyze the gene profile in these cells. Table 3 shows the fold increase of genes normalizing the mRNA expression of the genes to GAPDH using the $2^{-\Delta\Delta CT}$ method. A significant overexpression of β1 and β3 integrin subunits from the cells seeded on cross-linked COL I was detected, as well as the inhibitors of the metalloproteinases TIMP-1 and TIMP-2, compared to the cells on native collagen. To verify the array data, protein expression of β1 and β3 integrins was further analyzed by Western blotting. As shown in Fig. 3a, increased levels of these integrins were found in the cells cultured on TG2 cross-linked COL I, compared to the control cells on native collagen, confirming the DNA array result.

Table 3 Gene expression by PCR array

Gene	Fold increase
ADAMTS1	+2.77
COL1A1	+3.29
COL2A1	+3.27
COL15A1	+2.97
FN	+2.89
α1 Integrin	+1.18
α2 Integrin	+1.76
α3 Integrin	+1.34
β1 Integrin	+8.45
β3 Integrin	+8.87
MMP1	+2.48
MMP8	+2.68
MMP13	+3.53
MMP2	+2.10
MMP9	+2.44
MMP3	+2.21
TIMP1	+6.32
TIMP2	+7.83
VCAN	+2.04
VTN	+0.90

Assessment of the RGD integrin-mediated cell binding using inhibitory synthetic peptides

We next looked at the ability of the RGD containing peptide GRGDTP (which mimics the ECM binding site for many cell surface integrins) to block the cell adhesion and spreading of HOB on TG2 cross-linked COL I and compare this to cells seeded on native collagen. HOB cells were first pre-incubated with different concentrations of the integrin binding RGD peptide between 50 and 500 μg/ml, prior to seeding on the native and TG2 cross-linked COL I (Fig. 3b). This initial dose–response curve suggested that the percentage of spread cells on cross-linked COL I was always greater in the presence of RGD peptides. At 250 μg/ml, the control RAD peptide GRADSP showed no effect on HOB cell spreading compared to the untreated control in both native and cross-linked COL I samples (Fig. 3c), suggesting that there was no toxicity for both peptides at 250 μg/ml and the inhibition of cell attachment and spreading in the RGD treatment were purely due to the blocking of the integrin binding sites in the cells. When used at this non-toxic concentration, the RGD peptide led to a clear difference in the percentage of the spread cells on the different collagen matrices when observed under the light microscope after 3-h incubation (Fig. 3d, e).

Inhibition of cell adhesion using anti α1, α2 and β1 integrins function blocking antibodies and their intracellular signaling

Cell spreading on native and TG2 cross-linked COL I matrices (5 mg/ml of COL I, 10 μg/ml of TG2) was also measured using function blocking antibodies against α1, α2 and β1 integrins (Fig. 4a–c). Figure 4a shows that a greater percentage of cells spread on the TG2-modified matrix, compared to the native collagen when incubated in the presence of these different integrin targeted antibodies. The rabbit IgG polyclonal control antibody had no significant effect on cell spreading when compared to the non-treated control cells (data not shown). To further confirm the role of integrin signaling in the HOB cells seeded on cross-linked or native collagen, FAK phosphorylation at Tyr^{397} residue, the autophosphorylation site, was analyzed. Increased level of $FAKTyr^{397}$ was detected in the cells seeded on the cross-linked COL I, compared to native collagen (Fig. 5a, b).

HOB differentiation on TG2-cross-linked COL I

We next studied the effect TG2-cross-linked COL I on HOB differentiation and mineralization to see if this was enhanced when HOB cells are seeded on cross-linked COL I. After a 12-h culture of HOB, both OPN and bALP expressions were found to be increased twofold at the mRNA levels when analyzed by semi-quantitative RT-PCR and compared to native collagen (Fig. 6a). Biochemical evaluation of bALP showed that, after 14 days of culture, addition of the recombinant hBMP7 to the cells, seeded on the TG2 cross-linked COL I matrix, enhanced the expression of the bALP in HOB cells. The effect could be reversed using the site-directed TG2 inhibitor R283 confirming the involvement of TG2 (Fig. 6b). Analysis of the mineralization of HOB on cross-linked COL I compared to native collagen also demonstrated an increased ability of the cross-linked collagen to induce mineralization (Fig. 6c) in agreement with the increased levels of bALP induced by growth on the cross-linked COL I (Fig. 6b).

Discussion

Evidence suggests that TG2 may have a key role in wound healing and matrix turnover both as a cross-linking enzyme and as a novel cell adhesion protein (Wang and Griffin 2012) and its importance in human bone diseases, such as osteoarthritis, has been widely reported (Tarantino et al. 2013; Orlandi et al. 2009). Moreover, TG2 cross-linking

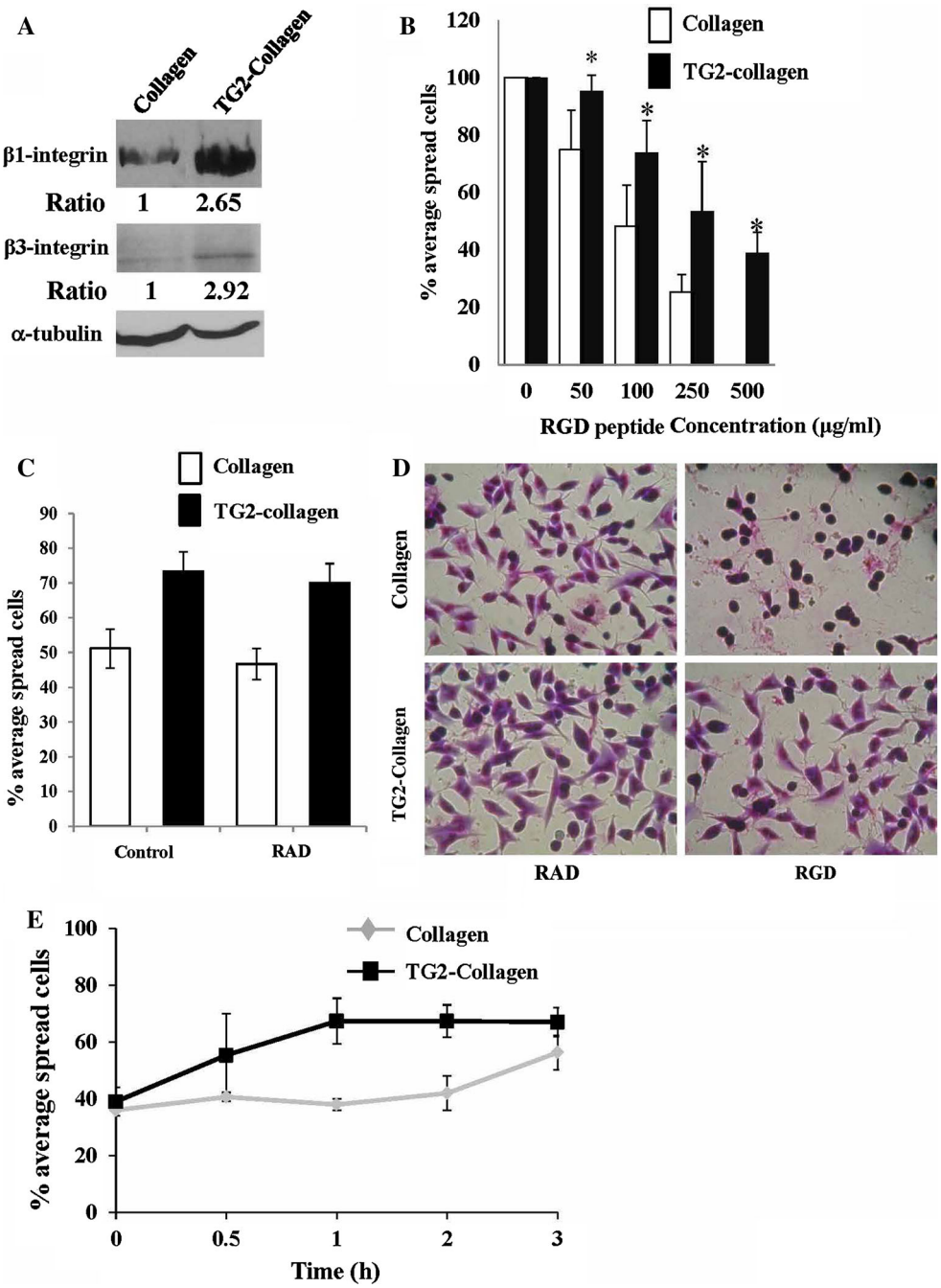

Fig. 3 **a** Representative Western blot for β1 and β3 integrins. The HOB cells, seeded on native or cross-linked COL I (5 mg/ml COL I by 10 μg/ml TG2), were lysed in cell lysis buffer and Western blotting performed to detect the presence of β3 integrin. For the β1 integrin, immunoprecipitation was carried on using anti-β1 integrin antibody and the presence of β1 integrin in the immunocomplex was detected via Western blotting as introduced in "Materials and methods". The mean ratios shown below are those between native collagen and cross-linked COL1 calculated from densitometry after normalization for protein loading ($n = 2$) using α-tubulin band. **b** Effect of different concentration (50–500 μg/ml) of the GRGDTP peptide on osteoblasts attachment on native and TG2 cross-linked matrices. The percentage of spread cells in the total adherent cells was calculated as described in "Materials and methods". **c** Cell response to 250 μg/ml RAD control peptide compared to control without peptide as described in **b**. **d** Light microscopy showing the effect of GRGDTP and GRADSP peptides (250 μg/ml) on cell spreading on native and TG2 cross-linked COL I over 40 min. **e** The percentage of the spread cells seeded on native or cross-linked COL I over a 3 h period. Cell spreading is expressed as percentage of total number of cells attached (using ~150 attached cells/field). *$p < 0.05$

activity has been shown to be important for matrix deposition and osteoblast differentiation (Chau et al. 2005; Heath et al. 2001). This study suggests a potential application for TG2 in bone healing when used to generate a novel biomaterial through the modification of COL I. Presently preferred methods for repairing bone defects involve the use of autographs whereby the patients own bone is used for the repair process. However, the limited amounts that can be taken and the prolonged pain at the harvest site are a major disadvantage of using autografts. As such many alternative methods have been sought and used in bone graft including the use of hydroxyapatite (HA),collagen, calcium sulfate, silicate-substituted calcium phosphate (Si-CaP) and polyethylene (PE) matrices modified with HA (Cameron et al. 2013). More recent methods have also involved the addition of osteoinductive growth factors to increase the osteoinductive properties of biomaterial used (Aravamudhan et al. 2013). Examples of these include the addition of recombinant human bone morphogenic proteins 7 (rhBMP-7) and 2 (rhBMP-2). However, there has been concern with regard to the side effects from the addition of these factors such as ectotopic

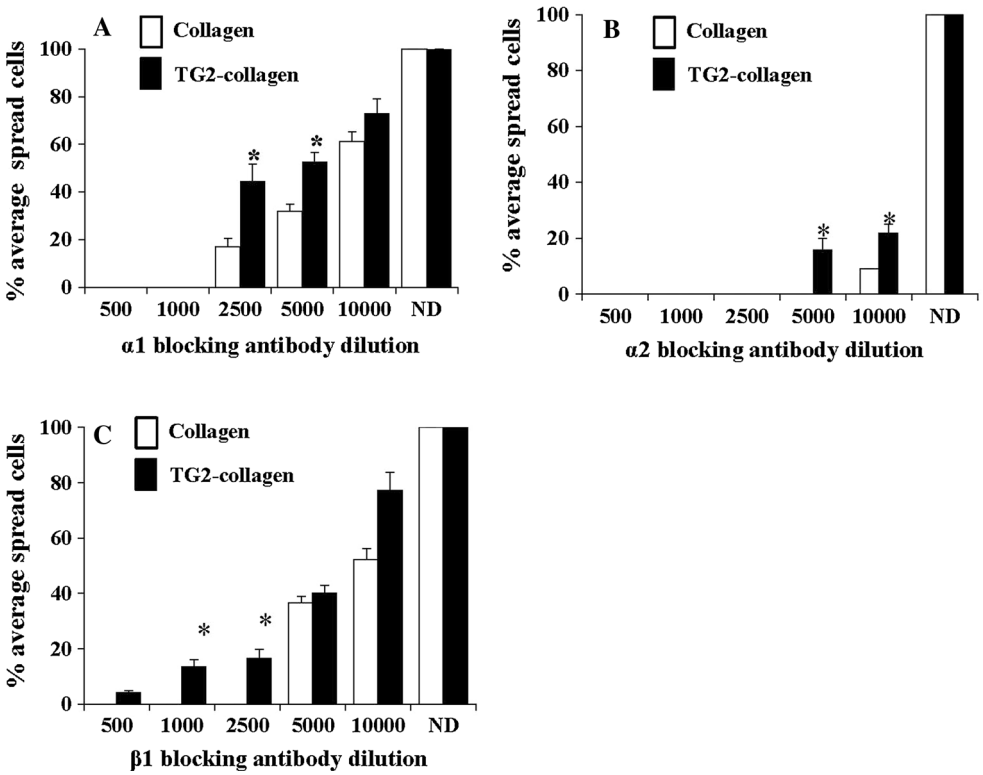

Fig. 4 Cell response to different dilutions (1:500–1:10,000) of **a** α1, **b** α2 and **c** β1 blocking antibodies on native and TG2 cross-linked matrices (5 mg/ml collagen, 10 μg/ml TG2). Cell spreading (using ~150 total attached cells/field) is expressed as percentage of total number of cells attached following 6 h of culture on the matrix (*$p < 0.05$) in the presence of antibody as introduced in "Materials and methods"

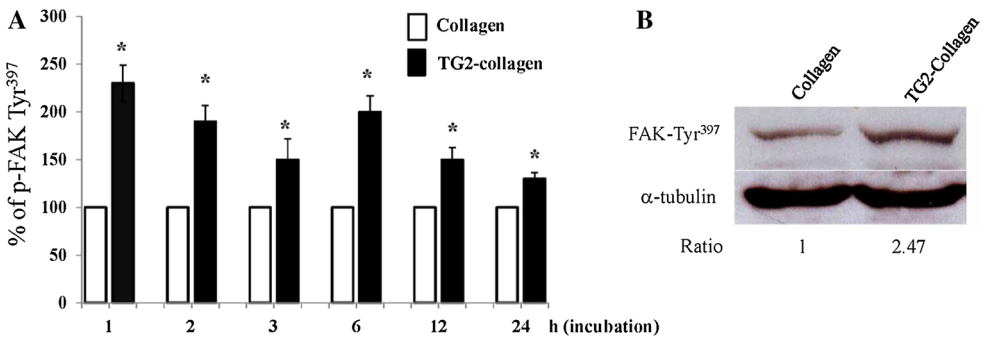

Fig. 5 **a** Shows the level FAK-Tyr^{397} phosphorylation in cells plated on TG2 cross-linked COL1 and native COL1 at different time points. The densitometry data taken from Western blots are shown as a percentage for the pFAK-Tyr^{397} in the HOB cells seeded on cross-linked collagen. Values were normalized to the pFAK-Tyr^{397} taken from cells plated on the native COL I at each time point (which was taken as 100 %). Data are from three separate experiments, *$p < 0.05$. **b** Representative Western blot showing the expression level of phosphorylated FAK in human osteoblasts after 1 h from the seeding on native and cross-linked COL I (5 mg/ml COL I, 10 μg/ml TG2). The mean ratio of native collagen to cross-linked COL1 shown below is calculated after densitometry of p-FAK after normalization of protein loading using α-tubulin ($n = 2$)

bone formation, depending on where the material is applied, e.g., spinal injuries can result in post-surgery complications (Rodgers et al. 2013). In this study, we show that the cross-linking of COL I by TG2 results in increased osteoconductive properties of the modified matrix, when compared to native collagen, and as such we provide possible reasons for this novel observation.

Our previous work demonstrated that the fibril growth phase of COL I treated with TG2 started at a much earlier time than in the native collagen (Chau et al. 2005). Here, we investigated the structure of the COL I fibrils in both native and TG2 cross-linked collagen. Using AFM, we demonstrate that the cross-linking reaction, mediated by TG2, induced a change in the banding pattern of the COL I fibers. An increase in the stiffness of the COL I gels treated with TG2 was also found. The shear modulus of cross-linked COL I was significantly higher than that of untreated collagen and that published elsewhere (Piechocka et al. 2011). Data showed that the gelification of COL I treated with TG2 occurred almost immediately compared to the

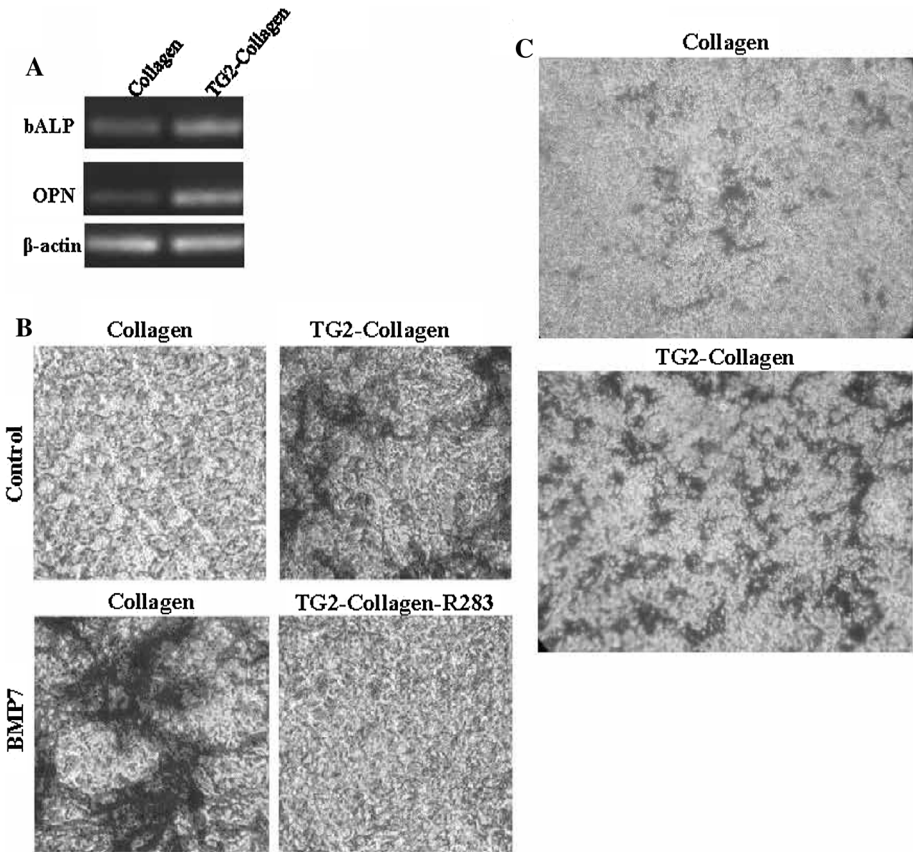

Fig. 6 **a** Bone alkaline phosphatase (bALP) and osteopontin (OPN) mRNA levels were measured by semi-quantitative RT PCR after culture of HOB on native and cross-linked collagen for 12 h. **b** Effect of TG2 cross-linked collagen and the addition of hBMP7 to the cell culture medium on HOB differentiation measured by the detection of bALP as described in "Materials and methods". The addition of the irreversible site directed TG2 inhibitor R283 (500 μM) was used as a control of the TG2-mediated effect. **c** HOB cell mineralization assay. The mineralized bone matrix formed by HOB after culture on native and TG2 cross-linked COL I was measured using Von Kossa staining (1901) as described in "Materials and methods". Briefly, after culturing on native or cross-linked collagen for 12 days, the samples were fixed and dehydrated as described in "Materials and methods". 2 % silver nitrate was used to stain the samples with UV exposure for at least 20 min. After washing with dH_2O for five times, the samples were further fixed with 5 % sodium thiosulfate for 3 min. Following washing, the samples were imaged using Nikon digital camera with a 20× objective

native collagen. It is well known that the cross-linking reaction mediated by TG2 protects proteins from enzymatic digestion by collagenases, gelatinases and stromelysins as demonstrated by Chau et al. (2005). Interestingly, the PCR DNA array also showed high expression of the tissue inhibitors of MMPs (TIMPs), including TIMP1 and TIMP2, in the HOB cells seeded onto TG2-cross-linked COL I, compared to native one further facilitating the increased stability of the cross-linked matrix although we have no explanation for this observation.

Given the changes in the fibril structure and shear modulus of cross-linked COL I, we next investigated the cell response to the TG2 cross-linked COL I. Different combinations of COL I and TG2 were used in the initial experiment, including 2, 5, and 10 μg/ml of TG2 and 0.1, 1, 5 and 25 μg/ml. A significant increase in HOB proliferation occurred in every cross-linked COL I matrix at the different concentrations, apart from 25 mg/ml and especially in soft scaffold because of the low concentration of COL I. We found that cell proliferation was reduced with the progressive increase in the stiffness of the matrix as the COL I concentration increased (from 1 to 5 mg/ml), becoming comparable to that of the native COL I when the COL I concentration reached 25 mg/ml. We therefore hypothesized that the cross-linking is able to increase the rigidity of the scaffold when the concentration of COL I ranges in certain limits. This will favor the proliferation of the osteoblasts that prefer a stiff matrix rather than a soft scaffold. However, as the COL I concentration increases, the enzyme is unable to increase the rigidity any further due to the already dense nature of the fibrils.

A further interesting observation was the increased expression levels of cell surface integrins found in the HOB when plated on cross-linked COL I, suggesting that the TG2 cross-linked COL I affects HOB behavior by altering the cell surface integrin receptor profile. It has

been reported that cell surface β integrins are required for the externalization of TG2 and its role in regulating cell adhesion and migration (Akimov and Belkin 2001). Moreover, extracellular TG2 can rescue the RGD-independent cell adhesion via syndecan-4 and its co-receptor β1 integrin (Telci et al. 2008). Previous studies (Chau et al. 2005) indicated that the MMP profiles of fibroblasts, when cultured on cross-linked COL1 changed to that seen with native COL1 with a reduction of active MMP-1 and a corresponding increase in active MMP-2, suggesting that the cells may recognize the modified collagen as more like its denatured form gelatin. In view of these different findings, the effect of TG2-cross-linked COL I on HOB cell adhesion was studied using both the synthetic RGD peptides which mimic the integrin binding site for many ECM proteins and also the functional blocking antibodies against the α1, α2 and β1 integrins to which COL I can bind. Both increased amounts of RGD peptides and the functional blocking antibodies were required to block the spreading of HOB cells seeded on cross-linked COL I, compared to HOB cells seeded on native collagen. This suggests that more ligand/or binding antibodies are needed to saturate the increased amounts of integrins in the HOB cells on TG2-cross-linked COL I. This was confirmed by Western blotting and PCR array which demonstrated increased expression levels of α1, α2, β1 and β3 integrins in the HOB seeded on cross-linked COL I. However, it cannot be ruled out that increased binding sites for these integrins also become available on the modified COL I after its TG2-mediated cross-linking. In addition, higher levels of phosphorylated FAK at Tyr 397 residue, a crucial downstream signaling molecule in integrin signaling transduction (Wang et al. 2011; Parsons 2003), were detected in the HOB cells seeded on cross-linked COL I, further confirming the involvement of integrins in the cell reaction to cross-linked COL I. To further analyze the effect of TG2-cross-linked COL I and of HOB differentiation, and the effects of hBMP7 on this process, we investigated bALP deposition after a 14-day culture on the different COL I matrices. Increased bALP was found in the cells seeded on cross-linked COL I, compared to the native collagen, and even more in the presence of BMP7, confirming the involvement of TG2 in HOB differentiation. These data were confirmed at the earlier time period of 12 h via RT-PCR for both OPN and bALP. Importantly, the increased presence of bALP in the cells cultured on the cross-linked COL1 was paralleled by a comparable increase mineralization.

To summarize, the cross-linking of COL I by TG2 promotes the formation of a stronger and more stable collagen structure inducing the increase of integrin receptor expression, i.e., α1, α2, β1 and also β3 in HOB, and the activation of their downstream signaling via FAK. This, in turn, increases the ability of the osteoblast to adhere to the modified COL I promoting increased differentiation and mineralization.

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