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Abstract

Collagen, type I, is a highly abundant natural protein material which has been cross-linked by a variety of methods including chemical agents, physical heating and UV irradiation with the aim of enhancing its physical characteristics such as mechanical strength, thermal stability, resistance to proteolytic breakdown, thus increasing its overall biocompatibility. However, in view of the toxicity of residual cross-linking agents, or impracticability at large scales, it would be more useful if the collagen could be cross-linked by a milder, efficient and more practical means by using enzymes as biological catalysts.

We demonstrate that on treating native collagen type I (from bovine skin) with both tissue transglutaminase (TG2; tTG) and microbial transglutaminase (mTG; *Streptoverticillium mobaraense*) leads to an enhancement in cell attachment, spreading and proliferation of human osteoblasts (HOB) and human foreskin dermal fibroblasts (HFDF) when compared to culture on native collagen. The transglutaminase-treated collagen substrates also showed a greater resistance to cell-mediated endogenous protease degradation than the native collagen. In addition, the HOB cells were shown to differentiate at a faster rate than on native collagen when assessed by measurement of alkaline phosphatase activity and osteopontin expression.

Keywords: Bioactivity; Biocompatibility; Collagen; Fibroblasts; Osteoblasts; Tissue engineering

1. Introduction

Collagen is a very popular biomaterial due to its biocompatibility, i.e. the ability to support cell adhesion and proliferation. It is also biodegradable and only weakly antigenic—able to persist in the body without developing a foreign body response that could lead to its premature rejection [1]. The replacement of skin with artificial collagen–GAG matrices has been investigated since the early 1980s and is now in clinical use [2,3]. The primary reason for the usefulness of collagen in biomedical applications is that collagen can form fibres with extra strength and stability through its self-aggregation and in vivo cross-linking [4]. Unfortunately, collagen, like many natural polymers, once extracted from its original source and then reprocessed, suffers from weak mechanical properties, thermal instability and ease of proteolytic breakdown. To overcome these problems, collagen has been cross-linked by a variety of agents—a subject of much recent research to find methods of preventing rapid absorption by the body [4]. However, these methods suffer from the problem that the residual catalysts, initiators and unreacted or partially reacted cross-linking agents used can be toxic or cause inflammatory responses if not fully removed or, simply, not cost-effective or practical at the large scale [5–7]. As a consequence, research continues to find alternative methods to stabilise collagen which are *natural*, milder, efficient and more practical.

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Transglutaminases (EC 2.3.2.13) are a group of enzymes that can catalyse several types of post-translational modifications to proteins. The most important of these reactions results in the cross-linking of peptides or proteins to form multimers via a $\varepsilon(\gamma$-glutamyl)lysine linkage using the side chains of lysine and glutamine residues. Transglutaminases are also able to covalently attach primary amine containing compounds to peptide bound glutamine, facilitating modification of the physical, chemical and biological properties of proteins [8]. For these reasons, transglutaminases have been utilised by the commercial sector in many different processes and have attracted much attention from the research community [9]. Microbial transglutaminase has been used to cross-link gelatin matrices to further increase their strength [10] and, also, to incorporate cell adhesion factors within the gel matrix, resulting in an enhancement of cell proliferation [11].

Interestingly, a novel component of the cell/tissue response to cell damage and stress is tissue transglutaminase (tTG), a Ca^{2+}-dependent mammalian form of the enzyme, which modulates cell–matrix interactions, tissue stability and a variety of other cell functions [12,13]. The entire tissue repair process is regulated by the interaction of cells with the surrounding extracellular matrix (ECM), ensuring cell adhesion, survival and proliferation [14,15]. To date, the cross-linking function of tTG in the ECM leading to ECM stabilisation/remodelling has been identified in a number of biological processes important for tissue repair [12]: in addition, at least three of the nine genes so far characterised are thought to be naturally involved in the wound healing response process [see review, 16].

The aim of this study was to investigate the use of the two different transglutaminases; the mammalian (tTG; TG2; TG-2; isolated from guinea pig liver) and the microbial enzyme (mTG; isolated from *Streptoverticillium mobaraense*) in the modification of collagen type I with the view to investigate potential application as a biocompatible natural polymer for use in soft and hard tissue repair.

2. Materials and methods

All water used was deionised using an Elgastat System 2 water purifier (ELGA Ltd., UK) and a Milli-Q water purifier (Millipore Waters, UK). All chemicals were purchased from Sigma-Aldrich, Poole, UK, unless otherwise stated. Sterile preparation of stock solutions and chemicals were performed either by filtration through a 0.22 μm Whatmann sterile filter and/or autoclaving at 121 °C at 1 bar for 1 h.

2.1. Cell culture

Human osteoblast (HOB) cells, isolated from explants of trabecular bone dissected from femoral heads following orthopaedic surgery as previously described [17] were kindly supplied by Professor S. Downes and Dr. S. Anderson (School of Biomedical Sciences, University of Nottingham) and used during this investigation. Human foreskin dermal fibroblast (HFDF) cells isolated from human neonatal foreskin were also used. Both cell lines were used during their low-passage number, ranging from 11 to 15 passages. Cell lines were cultured and maintained, in vitro, as monolayers in T-flasks using DMEM, supplemented with 10% heat-inactivated (56 °C for 1 h) FCS, 1% non-essential amino acids and 2 mM L-glutamine. Periodic additions of 1% penicillin–streptomycin were used to avoid bacterial contamination. Flasks were kept in a humidified-atmosphere incubator at 37 °C and with 5% CO_2. Cells were routinely passaged and never allowed to reach greater than 90% confluency at any one time. For detachment, standard trypsinisation was performed using 0.25% (w/v) trypsin/2 mM EDTA solution in PBS solution.

2.2. Cell viability and proliferation

Cell counts and viability estimations were performed using the standard trypan blue exclusion technique by means of a 0.22 μm sterile filtered 0.5% (w/v) trypan blue solution and a haemocytometer. Non-viable cells stained blue due to the loss of their membrane integrity and, hence, allowed the passage of dye into the cell. Viable cells remained colourless.

Cell proliferation and viability were also measured using the CellTiter AQ One Solution Cell Proliferation™ assay kit (Promega, Southampton, UK. Cat no. G3580). Assays were performed, with reduced lighting, simply by the addition of 20 μl of CellTiter AQ reagent into the relevant samples in 100 μl of culture medium. These samples were then incubated in a humidified-atmosphere incubator at 37 °C and with 5% CO_2 for 90 min before the absorbance was read at 490 nm using a SpectraFluor® plate reader.

2.3. Attachment and spreading

Cells were seeded on the relevant substrate at a density of 625 cells/mm^2. After allowing cells to attach and spread, they were fixed in 3.7% (w/v) paraformaldehyde, permeabilised by the addition of 0.1% (v/v) Triton X-100 in PBS, before staining with May-Grunwald (0.25% (w/v) in methanol) and Giemsa stains (0.4% (w/v) in methanol, diluted 1:50 with water). Cells were then viewed at ×400 magnification using an Olympus CK2 microscope. Three separate fixed-size

random fields per sample were photographed with an Olympus DP10 digital camera. Pictures were analysed using Scion Image™ software (Scion Corporation, Maryland, USA). Spread cells were distinguished and characterised based upon the presence of a clear halo of cytoplasm surrounding their nucleus as previously described [18].

2.4. Alkaline phosphatase (ALP) activity

The ALP Optimized Alkaline Phosphatase EC 3.1.3.1 Colorimetric Test® kit (obtained from Sigma-Aldrich, Poole, UK. Cat no. DG1245-K) was used to quantify the ALP activity. Alkaline phosphatase hydrolyses *p*-nitrophenyl phosphate to *p*-nitrophenol and inorganic phosphate. The hydrolysis occurs at alkaline pH and the *p*-nitrophenol formed shows an absorbance maximum at 405 nm. The rate of increase in absorbance at 405 nm is directly proportional to ALP activity in the sample. Samples were treated according to the manufacturers' instructions and analysed using a Beckmann DU530 UV/Vis spectrophotometer.

2.5. Osteopontin (OPN) concentration

The OPN ELISA kit (obtained from CalBiochem, UK. Cat no. 499262) was used to quantify the concentration of OPN in the samples. The kit uses a polyclonal antibody to human OPN immobilised on a micro-titre plate to bind to the human OPN. The measured absorbance (450 nm) is directly proportional to the concentration of human OPN. Samples were treated according to the manufacturers' instructions and analysed using a SpectraFluor® plate reader.

2.6. Transglutaminase

tTG was isolated and purified from guinea pig livers using a combination of anion exchange, gel filtration and affinity chromatography as previously described [19]. Commercial samples of TG were also used during this investigation: tTG from guinea pig liver (Sigma-Aldrich, Poole, UK. Cat no. T5398) and microbial transglutaminase, mTG (Ajinomoto Corporation Inc., Japan), isolated from *Streptoverticillium mobaraense,* as the commercially available product, Activa™ WM. This required further purification steps to remove the maltodextrin ingredient: briefly, the Activa™ WM was dissolved in ice-cold 20 mM phosphate buffer, 2 mM EDTA pH 6.0 and filtered, before being loaded onto a 100 ml SP-Sepharose FF column overnight at a continuous flow rate of 5 ml/min. The column was then washed and proteins eluted, at the same flow rate, with a 0–1000 mM gradient of NaCl in 20 mM phosphate buffer, 2 mM EDTA pH 6.0. Fractions were assayed for protein using the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hertfordshire, UK. Cat no. 500-0120). Fractions containing mTG were pooled, aliquoted, freeze dried and stored at −70 °C. Before immediate use, tTG was pre-treated in 2 mM DTT in 50 mM Tris buffer (pH 7.4) for 10 min at room temperature to activate any oxidised enzyme, before addition to a final buffered solution containing 5 mM $CaCl_2$ and, a minimum of 1 mM DTT in Tris buffer. Typical activities for the transglutaminases used in this investigation were as follows: tTG: 11500–13000 U/mg and mTG: 16000–25000 U/mg.

2.7. Transglutaminase activity

The incorporation of [^{14}C]-putrescine into *N*,*N*′-dimethylcasein, as described previously [20], was used to assay for TG activity and monitor the effects of the inhibitors. Unit of transglutaminase activity is 1 nmol of putrescine incorporated per hour.

2.8. Collagen

Commercial calf skin collagen type I (Sigma-Aldrich, Poole, UK. Cat no. C9791) was used during this investigation. Native collagen samples were solubilised in 0.2 M acetic acid (Fisher Scientific, Loughborough, UK. Cat no. A/0400/PB17) at 4 °C with constant stirring for 24 h before use. Neutralisation of the collagen mixture was performed using a [5:3:2] ratio of [collagen: 2 × DMEM: 0.2 M NaOH buffer] respectively to a final of pH 7.2. Tissue culture plastic was then covered using this collagen mix (recommended at 6–10 μg/cm^2) before being placed into a humidified-atmosphere incubator for 12 h to allow gelation to occur. In general, 50 μl of the collagen mix was added to each well of a 96-well plate. Plates were used within 48 h of the collagen matrix formation.

2.9. Modified collagen by transglutaminase

Neutralised collagen mixture was subjected to treatment with both tTG and mTG. Samples of the neutralised collagen, as described above, were treated with 50–1000 μg/ml of tTG, in a reaction mix consisting of 2 mM DTT and 5 mM $CaCl_2$ in 10 mM Tris buffer (pH 7.4). Microbial enzyme was added in 10 mM Tris buffer (pH 7.4). Stock solutions of: 2 mg/ml tTG and mTG, 1 M DTT and 1 M $CaCl_2$ were used to minimise total volume changes. The enzymes were always added last to the collagen-reaction mix to minimise any self-imposed cross-linking. Controls using 10 mM EDTA (to block tTG activity) and an active-site directed inhibitor, R281 (a synthetic CBZ-glutaminyl-glycine analogue; 500 μM), were also included in each assay. For 96-well plates, 50 μl of the pre-treated collagen mixture was added to each well before being placed into a humidified-atmosphere incubator, at 37 °C and with 5% CO_2, for 8 h. On

removal, wells were washed twice with sterile distilled water and used immediately.

2.10. Determination of ε-(γ-glutamyl)lysine cross-link

Cross-linked and native samples of collagen were proteolytically digested as previously described [21] which included an initial digestion with microbial collagenase (*Clostridiopeptidase A.*; 1 mg/ml, Sigma-Aldrich, Poole, UK. Cat no. C9891) prior to the addition of further proteases. After digestion, samples were freeze dried and then resuspended in 0.1 M HCl and sonicated for 2 min to aid dispersion. An aliquot (90 μl) was mixed with 110 μl of loading buffer (0.2 M lithium citrate, 0.1% phenol pH 2.2) and loaded onto a Dionex DC-4A resin column 0.5 cm × 20 cm using a Pharmacia Alpha Plus amino acid analyser. Derivatisation was performed post-column using *o*-phthaldialdehyde (0.8 M boric acid, 0.78 M potassium hydroxide, 600 mg/ml *o*-phthaldialdehyde, 0.5% (v/v) methanol, 0.75% (v/v) 2-mercaptoethanol, 0.35% (v/v) Brij 30) and the absorbance was measured at 450 nm. Dipeptide was determined by addition of known amounts of ε(γ-glutamyl)lysine to the sample and comparing peak areas.

2.11. Coomassie blue staining assay of cell cultures

The capacity of both the HOB and HFDF cells to degrade type I collagen was assessed as previously described [22]. Briefly, native and TG pre-treated collagen samples gels were plated out at 50 μl per well of a 96-well plate. Hundred microlitres of 2×10^4 cells/ml, cultured in complete media, was then added to the wells in triplicates. Plates were then kept in a humidified-atmosphere incubator for the relevant time point(s). After incubation, cells were removed from the collagen matrix by addition of 0.5% (w/v) sodium deoxycholate in 10 mM Tris–HCl. A rinse with distilled water was performed before the collagen samples were stained with a 0.1% (w/v) Coomassie Brilliant blue stain solution (50% (v/v) methanol; 10% (v/v) acetic acid; 40% (v/v) dH_2O). Samples were allowed to stain for 5 min before a further rinse with distilled water. Unstained areas, which appeared lighter blue, gave an indication of collagen degradation by cells. Two separate fixed-size random fields per triplicate samples were photographed using an Olympus CK2 microscope and DP10 digital camera.

2.12. Protein concentration

The total protein content of the collagen samples was determined by the Lowry method [23] using the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hertfordshire, UK. Cat no. 500-0120).

2.13. Collagenase degradation of matrices following cell culture

Collagen substrates were subjected to digestive treatment with 100 μl of a 1 mg/ml microbial collagenase solution (*Clostridium histolyticum,* Sigma-Aldrich, Poole, UK. Cat no. C9891) followed by 100 μl 0.25% (w/v) trypsin/2 mM EDTA solution in PBS solution for 24 h at 37 °C. Samples were washed twice with PBS followed by a wash with distilled water before the enzymatic digestion treatment.

2.14. Zymography

Gelatin and collagen zymography were carried out as previously described [24] with the following adaptations: resolving gels were mixed with the following components, in order: 1 ml of 5 mg/ml of type I collagen solution (Sigma C9791) in 20 mM acetic acid (for collagen zymography)/1 ml of 5 mg/ml porcine gelatin (Sigma G2625) in H_2O (for gelatin zymography), 3.1 ml H_2O, 2.5 ml of 1.5 M Tris–HCl pH 8.8, 3.33 ml of 29% acrylamide/1% *N,N′*-methylene bisacrylamide, 50 μl of 10% ammonium persulphate, 10 μl of *N,N,N′,N′*-tetramethylethylenediamine (TEMED). SDS was found to cause precipitation of the collagen and so was not added to the resolving gel. Stacking gels were poured in the usual way, i.e. 0.65 ml of 29% acrylamide/1% *N,N′*-methylene bisacrylamide, 3 ml H_2O, 1.25 ml 0.5 M Tris–HCl pH 6.8, 50 μl of 10% SDS, 25 μl of 10% ammonium persulphate, 5 μl of TEMED.

Samples containing matrix metalloproteinases (MMPs) were diluted 1:1 with loading buffer (1 M Tris–HCl pH 6.8, 50% glycerol, 0.4% bromophenol blue) and electrophoresed at 100 V in standard Laemmli running buffer (24 mM Tris–HCl, 192 mM glycine, 3.47 mM SDS, pH 8.3), avoiding overheating (approx. 4–5 h). After electrophoresis, gels were washed twice, with shaking, for 30 min each in 200 ml of 2.5% Triton X-100, to remove SDS and recover MMP activity. The gels were then placed in digestion buffer (100 mM Tris–HCl, 5 mM $CaCl_2$, 0.005% Brij-35, 1 μM $ZnCl_2$, 0.001% NaN_3, pH 8) for 16–48 h at 37 °C. Gels were stained with 0.2% Coomassie Brilliant blue R-250 in 50% ethanol, 10% acetic acid for 2 h and destained by microwaving for 15 min (full power 850 W) in three changes of deionised H_2O.

2.15. Statistical analysis of data

Differences between datasets (shown as mean ± SD) were determined by the Student's *t*-test at a significance level of $p < 0.05$.

3. Results

3.1. Cross-linking of collagen by microbial and tissue transglutaminases

Native collagen (type I) was treated with both tTG and mTG, separately, in order to catalyse the formation of ε-(γ-glutamyl)lysine cross-linking. The extent of cross-linking for each of the TG treatments is shown in Table 1. Treatment of collagen with increasing concentrations of TG led to a corresponding increase in the amount of ε-(γ-glutamyl)lysine bonds present—with up to 1 mol of cross-link per mole of collagen monomer. Treatment with mTG gave a much greater increase (almost two-fold) in the amount of isopeptide formed for the equivalent protein concentration of transglutaminase used. However, the increased specific activity of the mTG probably accounts for the differences noted.

3.2. Resistance of native and cross-linked collagen to cell-mediated degradation

Collagen treated with 50 μg/ml TG showed a greater resistance to cell-mediated degradation as compared to the native collagen, when HOB cells and HFDF were seeded onto the collagen matrices and incubated for 72 h. Following removal of cells, visual comparison of the Coomassie blue stained matrices and measurement of the residual collagen indicated the mTG-treated collagen to be more resistant than tTG-treated collagen (Table 2).

3.3. Matrix metalloproteinases secreted by HFDF cells grown on transglutaminase collagen matrices

Following growth on type I collagen, fibroblasts showed an induction of a wide array of collagenases and gelatinases when compared with growth on tissue culture plastic-ware alone (Fig. 1). After growth on transglutaminase cross-linked type I collagen, the induction of active MMP1 (45 kDa), Fig. 1A, is much less pronounced compared to growth on native collagen, whereas the induction of active MMP2 (66 kDa) and MMP9 (86 kDa), Fig. 1B, was increased particularly when the cells were grown on collagen cross-linked by tTG.

3.4. Proliferation rates of HOB and HFDF cells on native and TG-treated collagen substrates

Proliferation rates of both HOB and HFDF cell lines were enhanced on TG-treated collagen substrates with a

Table 1
Measurement of ε-(γ-glutamyl)lysine in TG-cross-linked collagen

Sample	TG concentration (μg/ml)[a]	nmol of cross-link/mg protein sample	±Relative change to native collagen[b]	mol cross-link/mol of collagen[c]
Collagen	—	0.16	—	0.02
Coll-tTG	50	1.09	6.81	0.13
Coll-tTG	100	2.40	15.00	0.29
Coll-tTG	200	4.60	28.75	0.55
Coll-tTG	500	5.40	33.75	0.65
Coll-tTG	1000	8.90	55.63	1.07
Coll-mTG	10	0.90	5.63	0.11
Coll-mTG	50	2.00	12.5	0.24
Coll-mTG	200	4.90	30.63	0.59
Coll-mTG	500	8.40	52.50	1.00

Cross-linking of collagen type I by different amounts of tTG and mTG after 8 h at 37 °C was undertaken as described in the Methods.
[a]TG activity: tTG = 11500–13000 U/mg; mTG = 16000–25000 U/mg.
[b]Native collagen = 0.16 nmol cross-link.
[c]M_w collagen: 120 kDa.

Table 2
Degradation of native and TG-treated collagen type I by HOB and HFDF cells

Cell line	Collagen	Collagen-tTG (50 μg/ml)	Collagen-mTG (50 μg/ml)
HOB	24% ± 3.1	55% ± 1.9	59% ± 2.1
HFDF	14% ± 2.6	30% ± 2.3	38% ± 2.5

After 72 h culture, cells were removed with sodium deoxycholate, residual collagen was digested with microbial collagenase and trypsin, and solubilised protein was measured and expressed as a percentage of the initial level. Values are expressed as the mean ± SD from three independent experiments, each with triplicate samples.

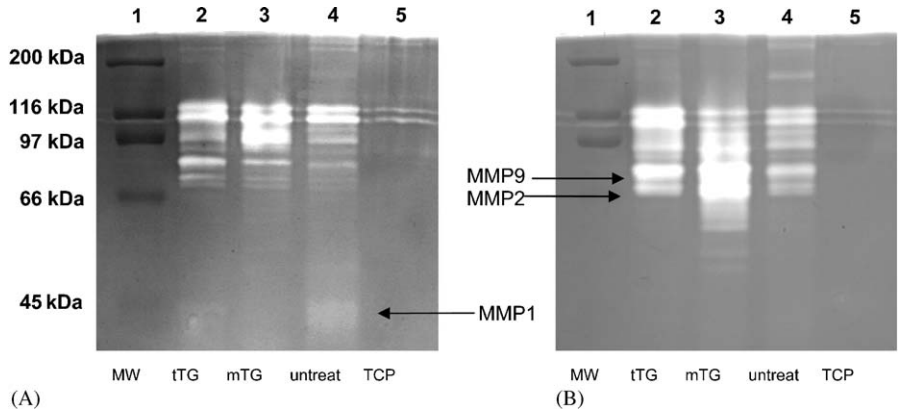

Fig. 1. Collagen (A) and gelatin (B) zymography of HFDF cell culture supernatants after 24 h growth on different media. Lane 1: molecular weight markers (BioRad 161-0317); lane 2: tTG-treated collagen; lane 3: mTG-treated collagen; lane 4: untreated collagen; lane 5: no collagen.

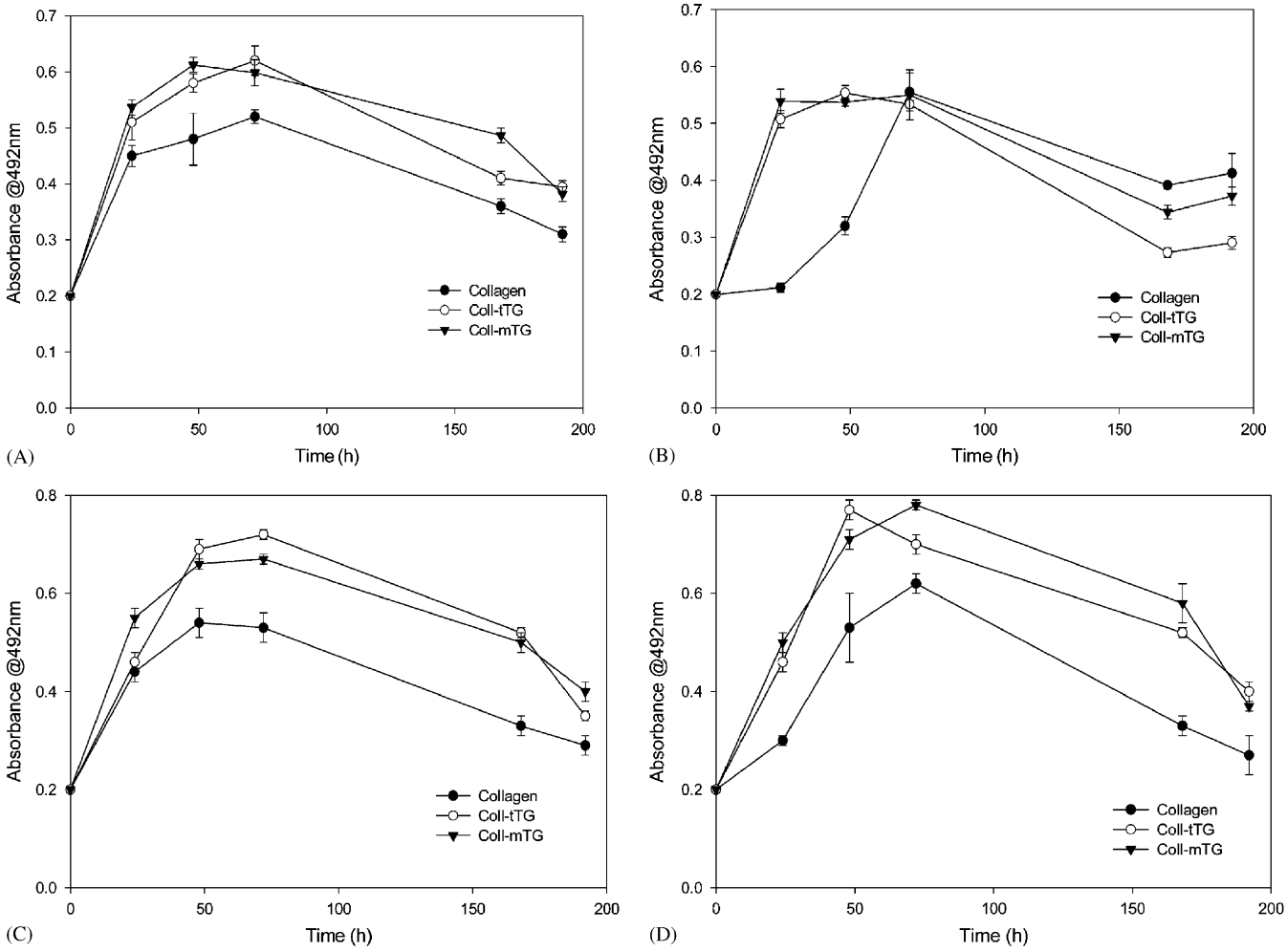

Fig. 2. Proliferation of HOB and HFDF cells cultured on native and TG-treated collagen substrates. HOB cells: (A) 50 μg/ml TG and (C) 100 μg/ml TG; HFDF cells: (B) 50 μg/ml TG and (D) 100 μg/ml. Proliferation rates were determined using CellTiter AQ solution. Results are the mean values ± SD from four independent experiments, each having triplicate samples.

greater level of viability achieved throughout the 196-h culture when compared with non-cross-linked collagen (Fig. 2). In the case of HOB cells, no significant difference in number of viable cells was observed with collagen treated with 100 μg/ml TG compared to 50 μg/ml TG. However, the HFDF cells showed an extended period of enhanced proliferation when cultured on collagen treated with 100 μg/ml TG (up to 48 h),

compared to 50 μg/ml TG (up to 24 h), and maintained an increase in number of viable cells until 168 h of culture.

3.5. Attachment characteristics of HOB and HFDF cells on native and TG-treated collagen substrates

Figs. 3 and 4 show the short-term cell-attachment characteristics of HOB and HFDF cells, when cultured on native and TG-treated collagen. Increased numbers of both HOB and HFDF cells attached when cultured on transglutaminase cross-linked collagen. For the HOB cells, comparable cell attachment was observed on both 50 and 100 μg/ml TG-treated collagens (Fig. 4A and 4C) giving a significant increase of around ~20% in attached cells for the corresponding time points over the non-cross-linked collagen ($p<0.05$). Comparable enhancements in cell attachment on the cross-linked collagens were also observed for the HFDF cells ($p<0.05$) (Fig. 4B and 4D).

3.6. Spreading characteristics of HOB and HFDF cells on native and TG-treated collagen substrates

Figs. 3 and 5 show the short-term cell-spreading characteristics of HOB and HFDF cells when cultured on native and TG-treated collagen. Increased numbers of cells spread when cultured on 50 μg/ml transglutaminase cross-linked collagen. In the case of the HOB cells, a comparable increase of 5% in the spreading of the HOB cells, at each time point, is seen on both of the TG-treated collagens (Fig. 5A). In contrast, the HFDF cells showed significant non-spread cells on the 50 μg/ml TG-treated collagen—with increases of at least 10% observed for both of the TG-treated variants (Fig. 5B) ($p<0.05$).

A further increase in the number of spread cells was also observed on cross-linked collagen using 100 μg/ml transglutaminase. In the case of HOB cells, an increase of approximately 5% can be observed in spread cells (Fig. 5C). This behaviour increased with increasing time of culture. In contrast for the HFDF cells, although there was still an increase in the spreading characteristics on the TG-treated collagen, a much more distinct and

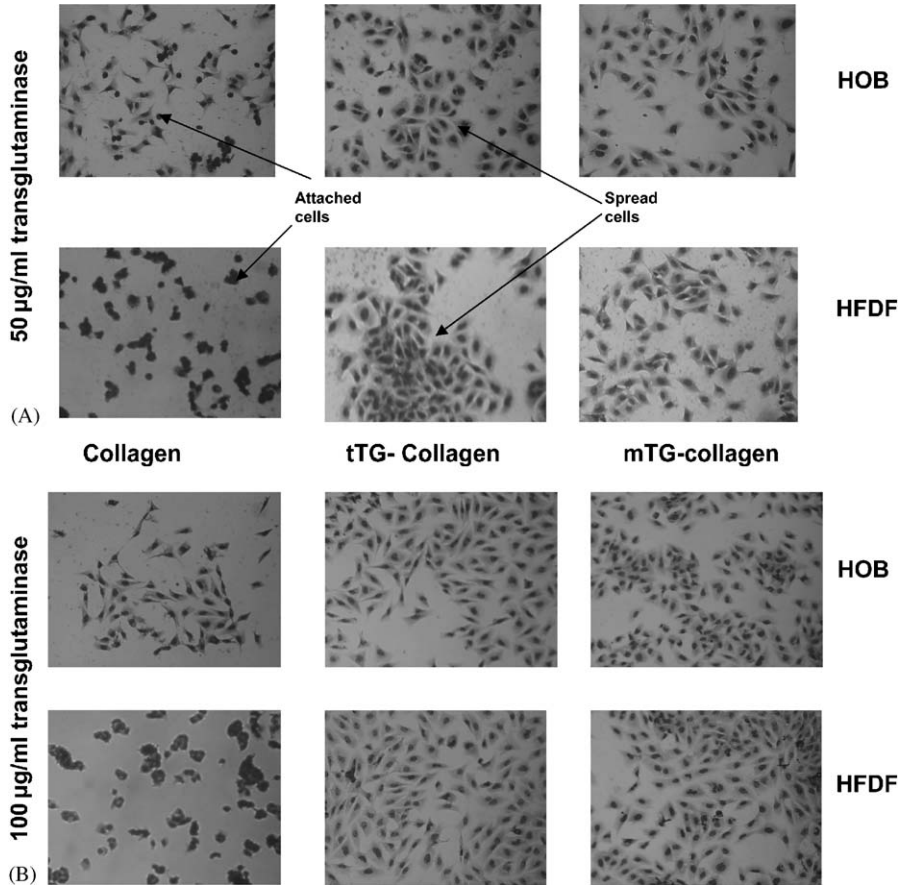

Fig. 3. Attachment and spreading of HOB and HFDF cells on native and TG-treated collagen type I. After 6 h incubation, cells were fixed using 3.7% (w/v) paraformaldehyde before being stained with May-Grunwald and Giemsa stains and then viewed at ×400 magnification.

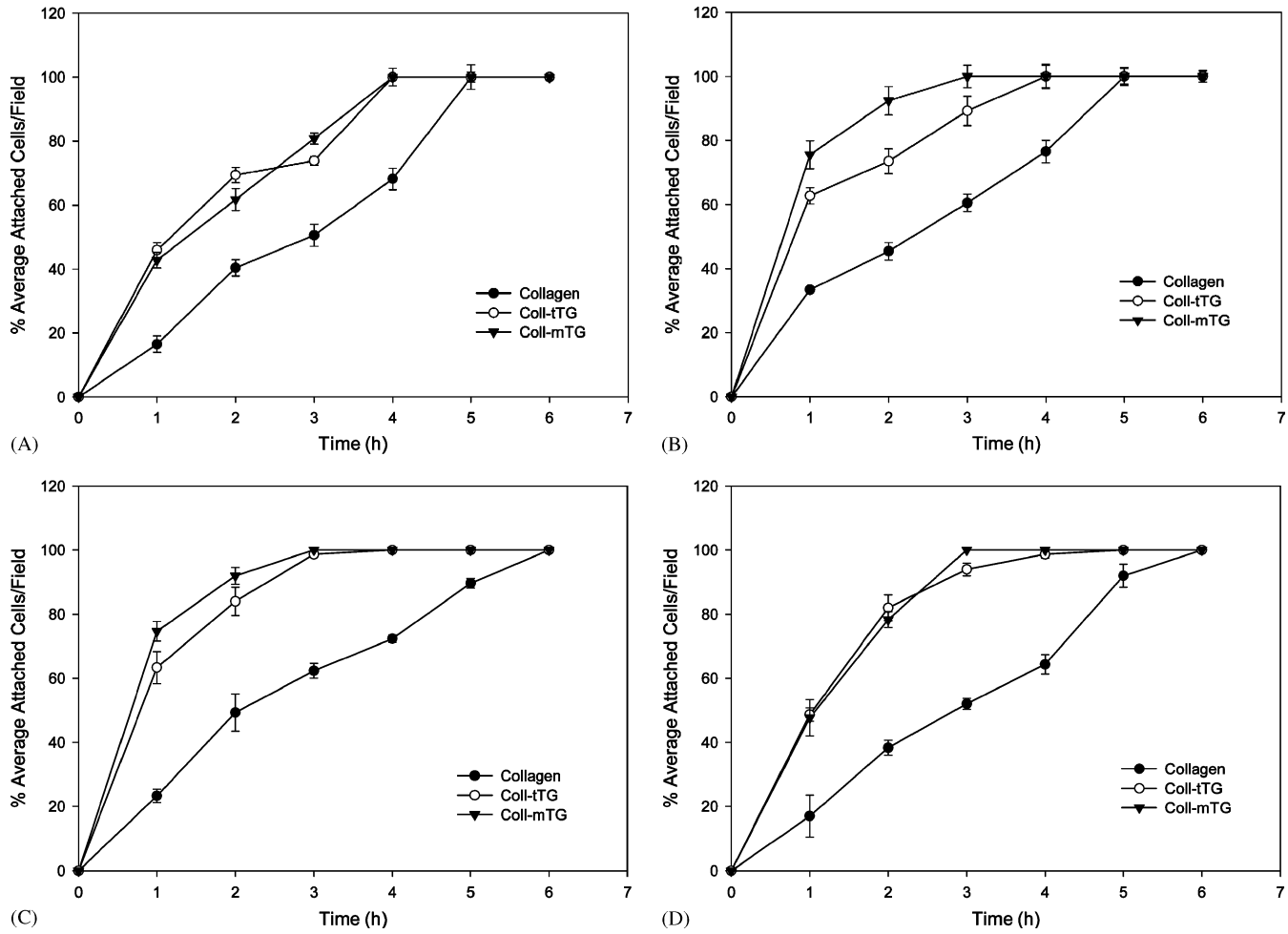

Fig. 4. Attachment of HOB cells and HFDF on native and TG-treated collagen substrates. HOB cells: (A) 50 μg/ml TG and (C) 100 μg/ml TG; HFDF cells: (B) 50 μg/ml TG and (D) 100 μg/ml TG. Attachment is expressed as a percentage of the total number of attached cells after 6 h. Results are the mean values ± SD from four independent experiments, each having triplicate samples.

significant behaviour was identified on the tTG-treated collagen with spread cells increasing by 15% for many of the time points. In contrast, the microbial-TG-treated collagen showed only a slight improvement in the spreading characteristics of cells (Fig. 5D) ($p < 0.05$).

3.7. Differentiation of HOB cells cultured on native and TG-treated collagen

Increases in ALP activity were observed in all the TG-cross-linked collagens, with the greatest increase seen with the tTG-treated collagen (Fig. 6A). A dose-dependent relationship between the concentration of TG and the increase in the ALP activity of the HOB cells ($p < 0.05$) was observed. However, with the highest concentration of mTG (250 μg/ml), there appeared to be a reduction in the corresponding amount of ALP activity when compared to tTG. In addition, higher OPN levels were observed in all the TG-cross-linked collagens—with the greatest increases seen with the 250 μg/ml concentration of transglutaminase (Fig. 6B). This gave rise to a two-fold increase of OPN expression over non-cross-linked collagen. In summary, a dose-dependent relationship between the concentration of TG and the increase in the OPN levels of the HOB cells ($p < 0.05$) was observed.

4. Discussion

We have confirmed previous work [25], in demonstrating that treatment of collagen type I matrices with transglutaminases results in the incorporation of ε(γ-glutamyl)lysine, with both mTG and tTG introducing similar amounts of cross-link per unit activity. It has been previously demonstrated that collagen type I shows greater resistance to proteolytic degradation by matrix metalloproteinase 1 (MMP-1) in vitro after cross-linking by tTG [26]. Importantly, we have shown that collagen modified with tTG and mTG demonstrated greater resistance to the total complement of cell-secreted proteases and, as a consequence, improved resistance

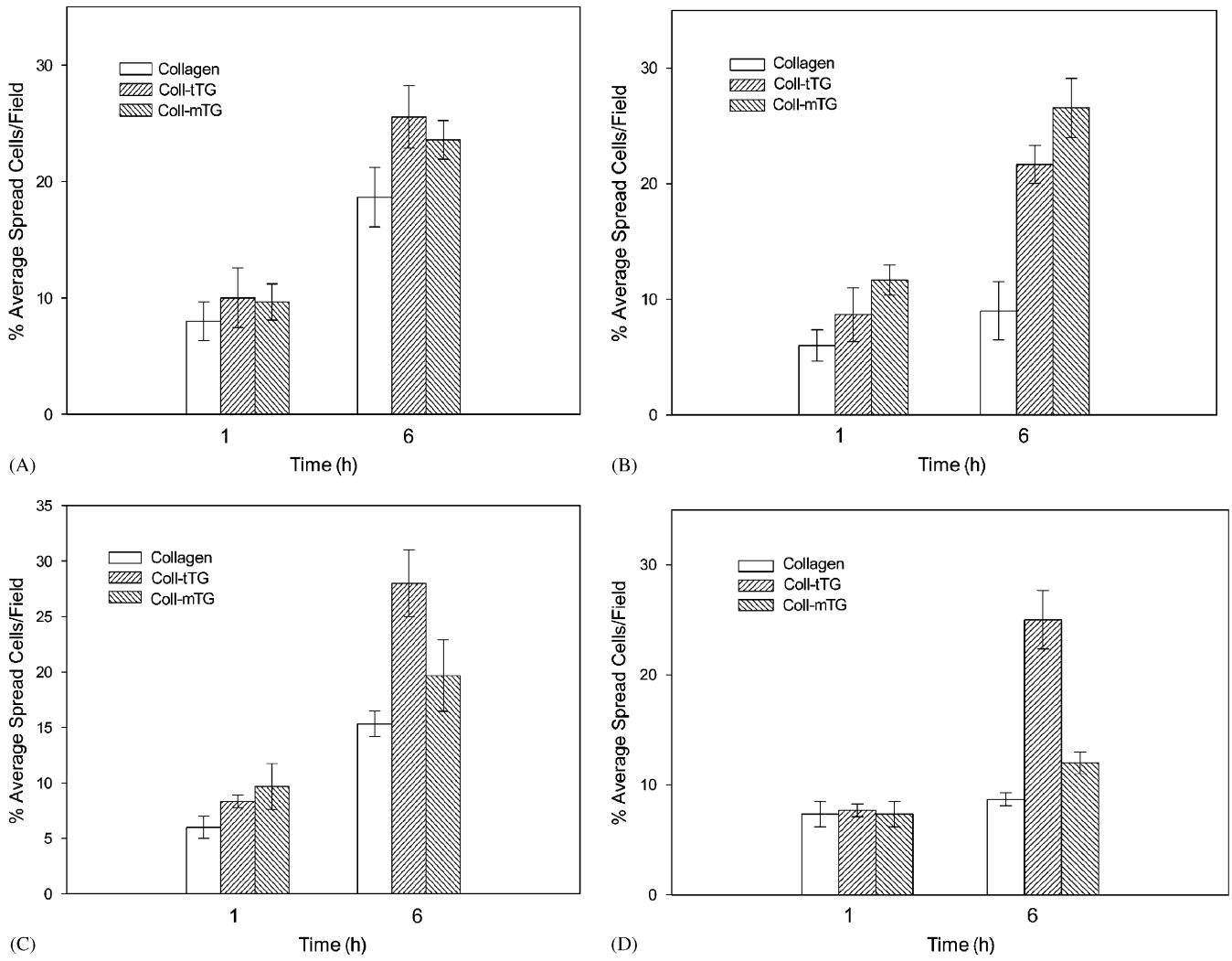

Fig. 5. Spreading of HOB cells on native and TG-treated collagen substrates. Cells were cultured for 1 and 6 h as described in the Methods. HOB cells: (A) 50 μg/ml TG and (C) 100 μg/ml TG; HFDF cells: (B) 50 μg/ml TG and (D) 100 μg/ml TG. Spreading is expressed as a percentage of the total number of cells in the field of view. Results are the mean values ± SD from four independent experiments, each having triplicate samples.

to cell-mediated degradation from cultured HOB and HFDF cells. The MMP expression profile of HFDF cells was found to alter as a result of growth on cross-linked collagen, with a reduction of active MMP-1 and a corresponding increase in active MMP-2, when compared to growth on untreated collagen. It is possible that this alteration in active MMP-1 accounts for the increased resistance of cross-linked collagen to cellular degradation. However, there is recent evidence to indicate that growth of either cultured hepatic stellate cells [27], rabbit periosteal fibroblasts [28] or human dermal fibroblasts [29] on collagen type I results in an increase in active MMP-2, which is associated with increased degradation of collagen in the matrix, independently of the collagenases [30]. In addition, changes in the mechanical characteristics of the collagen, which could be brought about by the introduction of ε(γ-glutamyl)lysine cross-link, can elicit mechano-chemical signalling via an integrin-dependent mechanism, resulting in alterations in gene expression, thus accounting for the changes in active MMP expression observed [28,28,31]. The importance of the mechanical nature of the matrix in MMP production is exemplified by the finding that bovine aortic or human umbilical endothelial cells repress their secretion of MMP-2 entirely when subjected to shear stress for as little as 30 min [32]. Transglutaminase-cross-linked collagen may be more efficient than native collagen at inducing this response to the ECM, either through the presence of cross-links which may disturb the native conformation, or via disruption of the native fibrillar form during fibrillogenesis. Alternatively, due to the increased resistance of the cross-linked collagen to MMP degradation, fibroblasts may elicit an enhanced MMP response in a futile attempt to increase the rate of collagen breakdown.

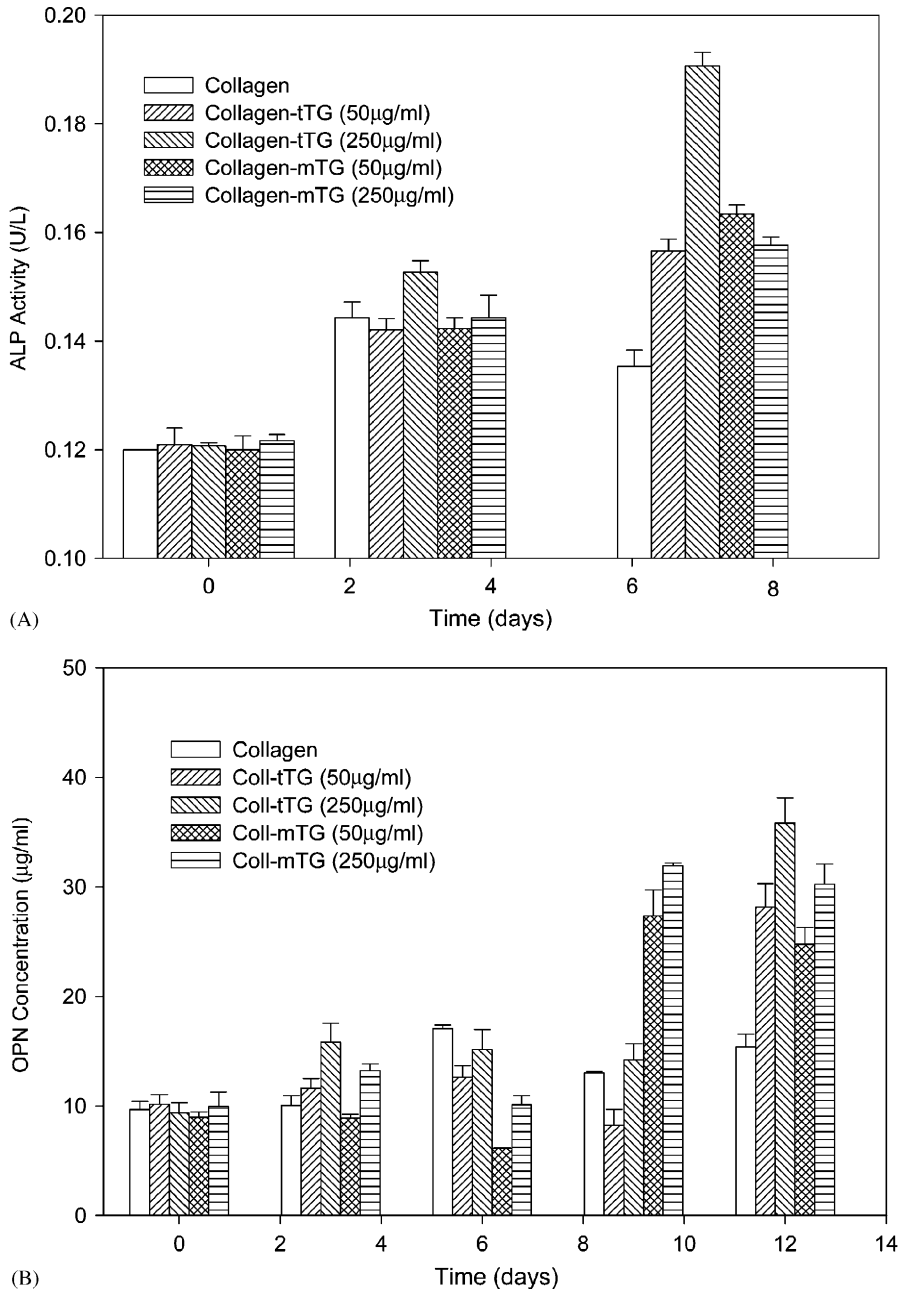

Fig. 6. Differentiation of HOB cells cultured on native and TG-treated collagen type I. Alkaline phosphatase (A) and osteopontin levels (B) were measured as standard biochemical markers of osteoblast differentiation. Results are the mean values ± SD from three independent experiments.

The proliferation rates and the attachment and spreading characteristics of HFDF and HOB cells were increased after growth on TG-cross-linked collagen when compared to culture on native collagen; furthermore suggesting alteration of the natural collagen confirmation resulting in the exposure of cryptic cell binding sites within the cross-linked collagen [16]. Importantly, long-term growth and survival rates were also maintained. These data confirm that the cross-linked collagen is eliciting a different cellular response compared to untreated collagen. HOB cells were also found to differentiate more rapidly after culture on TG-modified collagens as demonstrated by the corresponding increases in ALP activities and the earlier appearance of OPN. A possible explanation for this is that the TG-treated collagen matrix is in contact with the cells longer due to its increased proteolytic resistance thus providing the required integrin-mediated signal to the HOB cells necessary for differentiation [33].

The ability of tTG to act as a cell adhesion protein is well documented, promoting increased cell proliferation, spreading and attachment [34,35]. In fact, tTG, as well as Factor XIII, are critical components in the wound healing process and also in bone modelling and bone

ossification [36], where up-regulation of TG activity and enhancement of ECM cross-link formation is observed [37–41]. The cell adhesion characteristics of tTG have already been exploited to enhance the biocompatibility of medical devices [42]. Treatment of collagen type I with tTG may therefore lead to a modified collagen that is mimicking the natural remodelling/healing processes experienced in vivo. However, the finding that both the mammalian and microbial enzymes alter the biological characteristics of collagen type I in the same manner, indicates that cross-linking is essential since it is unlikely that the microbial enzyme can act as a cell adhesion protein given the large biochemical and physical differences between the enzymes [43].

5. Conclusion

In conclusion, the cellular response of HFDF and HOB cells grown on transglutaminase-cross-linked collagen is altered in such a manner that they show enhanced attachment, spreading and proliferation. Another important finding was that HOB cells differentiated faster on the cross-linked collagen. The modified collagen was also degraded at a much slower rate than native collagen further enhancing its in vivo efficacy as a biomaterial. Transglutaminases, therefore, show considerable potential as alternative cross-linking treatments for the production of novel biomaterials that do not suffer from the same drawbacks as other chemical or physical methods.

Acknowledgements

The above work has been filed as a GB patent application and supported by grant number GR/521755/01 from the EPSRC and BLC Leather Technology Centre Ltd., Northampton, UK.

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