Newly identified interfibrillar collagen crosslinking suppresses cell proliferation and remodelling

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ABSTRACT

Copper is becoming recognised as a key cation in a variety of biological processes. Copper chelation has been studied as a potential anti-angiogenic strategy for arresting tumour growth. Conversely, the delivery of copper ions and complexes in vivo can elicit a pro-angiogenic effect. Previously we unexpectedly found that copper-stimulated intraperitoneal angiogenesis was accompanied by collagen deposition. Here, in hard tissue, not only was healing accelerated by copper, but again enhanced deposition of collagen was detected at 2 weeks. Experiments with reconstituted collagen showed that addition of copper ions post-fibrillogenesis rendered plastically-compressed gels resistant to collagenases, enhanced their mechanical properties and increased the denaturation temperature of the protein. Unexpectedly, this apparently interfibrillar crosslinking was not affected by addition of glucose or ascorbic acid, which are required for crosslinking by advanced glycation end products (AGEs). Fibroblasts cultured on copper-crosslinked gels did not proliferate, whereas those cultured with an equivalent quantity of copper on either tissue culture plastic or collagen showed no effect compared with controls. Although non-proliferative, fibroblasts grown on copper-cross-linked collagen could migrate, remained metabolically active for at least 14 days and displayed a 6-fold increase in Mmps 1 and 3 mRNA expression compared with copper-free controls.

The ability of copper ions to crosslink collagen fibrils during densification and independently of AGEs or Fenton type reactions is previously unreported. The effect on MMP susceptibility of collagen and the dramatic change in cell behaviour on this crosslinked ECM may contribute to shedding some light on unexplained phenomena as the apparent benefit of copper complexation in fibrotic disorders or the enhanced collagen deposition in response to localised copper delivery.

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1. Introduction

Despite a considerable understanding of the intra- and intercellular role of copper, the role of this metal in the extracellular matrix (ECM) seems largely overlooked until now [1]. It is known that copper plays a structural role in many proteins and enzymes [2–7]. In addition, inorganic copper has been shown to exert pro-angiogenic response in vitro and vivo, to accelerate closure of wounds and to promote an immediate (within 24 h) synthesis of type I collagen in human fibroblasts [8–13]. Previously, we observed that copper unexpectedly induced substantial collagen deposition in vivo after 30 days [14]. In this study, we show in an orthotopic model that this collagen deposition appears to be a generalised response and is not restricted to soft tissues. The explanation for MMP susceptibility of collagen and the dramatic change in cell behaviour on this crosslinked ECM may contribute to shedding some light on unexplained phenomena as the apparent benefit of copper complexation in fibrotic disorders or the enhanced collagen deposition in response to localised copper delivery.

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could enhance collagen formation in an animal of otherwise normal nutritional status and diet [14]. In this study we found neither mRNA expression for type I collagen nor production of the protein itself occurred following exposure of primary human fibroblasts to copper in vitro.

With the aim of examining the physical interactions between copper ions and collagen, we exposed a tissue-equivalent reconstructed fibrillar collagen [18] to copper solution during mechanical compression. This resulted in what appeared to be a unique inter-fibrillar ionic crosslinking that rendered the collagen rigid and self-supporting. The effect was not repeatable with other metallic cations and was not affected by simultaneous exposure to glucose or ascorbic acid as it would be expected for an advanced glycation end products (AGEs) or Fenton type crosslink [19–22]. Curiously, in this study, human fibroblasts cultured on copper crosslinked collagen tissue equivalents did not proliferate yet they were able to migrate and remained metabolically active for 14 days. Matrix metalloproteinase (MMP) expression of human fibroblasts cultured on copper crosslinked collagen (CCC) resulted in a 6-fold increase in MMP-1 (collagenase-1) expression and a 3-fold decrease in MMP-13 (collagenase-3) expression, when compared with copper free collagen gels. The levels of copper that induced these striking changes in both physiological and biological properties were very low (70 ppm). An amount that is practically undetectable without complete collagen digestion and spectroscopy. The ramifications of the possibility that such low levels of copper may also accumulate in vivo are significant in light of the dramatic effects it induces. There is a growing body of literature indicating therapeutic action by copper level control mainly by chelation and our finding that collagenous ECM is so sensitive to this metal is of benefit in helping elucidate other biological functions of copper.

2. Materials and methods

2.1. Brushite preparation

Brushite cement was prepared as described previously [23]. Briefly, β-tricalcium phosphate (β-TCP) was synthesized by heating a mixture of monetite (DCPA; Mallinkrodt Baker, Germany) and calcium carbonate (CC; Merck, Germany) to 1050 °C for 24 h, followed by queench at room temperature in a desiccator. The product consisted of a phase pure and highly crystalline β-TCP as verified by X-ray diffraction (XRD). The sintered cake was crushed using a pestle and mortar, then passed through a 355 mm sieve and milled for 1 h. Equimolar amounts of β-TCP and monocalcium phosphate hydrate (Mallinkrodt Baker, Germany) were mixed with 0.8 M citric acid solution with a powder/liquid ratio 3.5 g/ml. Impaled with a cylindrical shape and a central cavity closed at one end were made from brushite cement. The phase purity of cements was analysed by X-ray diffraction using a Siemens D5005 diffractometer (Siemens, Karlsruhe, Germany) with monochromated Cu Kα radiation. The set cement density was measured using a helium pycnometer (Accylc1300®, Micromeritics). The specific surface area of cements was determined by using the Brunauer–Emmett–Teller (BET) method with helium adsorption–desorption (Tristar3000®, Micromeritics). After the setting of the cements and 12 h prior implantation, each sample was loaded with 560 ng of CuSO4 solution prepared in PBS. In sterile conditions 12 ml of solution were introduced with a thin tip into the central cavity of the implant and then absorbed by the cement. Control implants were loaded with 12 ml of vehicle solution.

2.2. Implantation, histological processing and histomorphometry

The implantations were performed in 9 New Zealand rabbits (3.5–4.0 kg) after approval by our local Institutional Animal Care Committees and in agreement with the guidelines of the Canadian Council for Animal Care. The rabbits were anaesthetized, the leg above and below the knee was shaved and the cutaneous surface was disinfected prior to the operation. A two cm incision was made above and below both the knee joints through the skin to expose the lateral condyle of the femur and the lateral tibia. One hole was made in the distal femur area and one hole in the proximal tibia to accommodate cylindrical implants (3.0 mm wide × 4.0 mm long). The incision was closed with a silk 4-0 suture. After surgery, 9 and 4 h prior to sacrifice, fluorescence markers (tetracycline, 25 mg/kg) were administered by intra-muscular injection to the animals to label actively mineralizing bone. The animals were sacrificed after 1, 3 and 9 weeks. Histological examinations were performed on dehydrated and resin embedded sections. Briefly, explants were fixed in 2.5% glutaraldehyde solutions and dehydrated in ascending concentrations of ethanol. The samples were then preinfiltrated for 24 h and infiltrated with resin for another 24 h before embedding in polymization resin at −20 °C for 14 days (Technovit, Leica Microsystems GmbH, Wetzlar; Germany). Following embedding, histological sections were taken using a micro saw (Leica Microsystems GmbH, Wetzlar; Germany), and the samples were stained with methylene blue/basic fuchsin staining for bone formation and sirius red staining for collagen deposition. One section remained unstained for fluorescence microscopy of the fluorochrome marker. For each histological section, 5 sites of double fluorescent labeling were selected. On each site, five measurements were taken and averaged in order to limit experimental errors resulting from different cutting angles in accordance with the original Frost’s procedure. The average distances were then divided by the number of days (5 days) between the two injections. The bone growth rate was obtained in micrometers per day.

The optical histological observations were used to perform the histomorphometrical analysis of the implant area, to calculate the bone growth, the percentage of bone and remaining material within the augmented tissues. The measured histomorphometrical parameters were the percentage of bone area in total implant area, the percentage of contact between bone and available implant outline in total implant area, the bone growth, the percentage of collagen area in total implant area, the percentage of contact between the deposited collagen and available implant outline in total implant area.

2.3. Cell culture reagents

Cell culture plastic ware was purchased from Corning-Costar (Corning Life Sciences, USA). Fetal bovine serum was obtained from HyClone (Cat No SH 30396-03, Fisher Scientific, Canada). DMEM (Cat No 319-020-C), d-glutamine (Cat No 609-065-EL), penicillin/streptomycin antibiotics (Cat No 450-201-EL), trypsin/ethylenedi-amine tetraacetic acid (T/E, Cat No 325-042-EL) were obtained from Wisent (Wisent Inc., Canada). - Ascorbic acid (AA, Cat No A5960) were from Sigma® (Sigma–Aldrich Co, USA). All other chemicals were from standard laboratory suppliers and of the highest purity available.

2.4. Fibroblastic cell culture

Human fibroblasts were derived from foreskins, after written informed consent which was approved by the Centre Hospitalier Universitaire de Quebec (CHUQ’S) Ethics Committee. Cells were used at passages 10–25. They were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Sigma Chemical Co., St Louis, MO) supplemented with 5% foetal bovine serum (FBS) and containing antibiotics (100 μ/l penicillin and 100 μg/ml streptomycin) and antifungals (250 pg/ml fungizone). Ascorbic acid (10 μg/ml) was added daily. Cell cultures were supplemented with fresh medium every other day. Fibroblasts were plated at a concentration of 2–3 × 104 cells/cm² and grown in a tissue culture incubator.

2.5. Collagen synthesis measurement

At day 7 of culture, at each medium change human fibroblast cell culture media were pooled (medium pool) corresponding to the most soluble collagen that has been released from cells. At day 15, media were pooled with the previous respective pools, and the cell-matrix layers were scrapped from wells with a rubber policeman and collected to be pooled separately (cell-matrix pool). The latter is composed mainly of insoluble collagen that has been deposited by the cells forming the extracellular matrix around the fibroblasts, and of collagen still present in cells prior to its release. Medium pools were then promptly frozen and then freeze-dried in a freeze-drying apparatus (BenchTop K, VirTis, Canada). Lyophilized pools were hy- drolyzed with 6 N HCl during 16 h at 110 °C and then neutralized with 10 N NaOH. Hydroxyproline contain were determined with chloramineT assay. Hydroxyproline standards were prepared in parallel with the samples. Briefly, samples were incubated with chloramineT for 25 min. After incubation 3.15 M perchloric acid is added and samples are vortex and incubated during 5 min at RT. After incubation, 20% p-dimethylaminobenzaldehyde is added. Samples are vortexed and incubated during 20 min at 60 °C and optical density was measured at 561 nm using a microplate reader (Tecan’s Infinite® F200, Tecan US Inc, USA). Prior to collect the cell-matrix pool, the numbers of cells present in wells were counted using the assay with trypan-blue staining.

2.6. Live/Dead assay

Confocal laser scanning microscopy (CLSM 5.1, Carl Zeiss, Germany) was used to monitor cell viability. Cell-seeded gels were incubated for 60 min in complete MEM with 1 μM calcine-AM and 2 μM ethidium homodimer-1 (Live/Dead® assay, Invitrogen, USA). Images were acquired using argon (488 nm) and HeNe633 (543 nm) laser excitations with 20X objective. Z-stacks were obtained through the entire thickness of the gels using 15 μm slices in 5 different regions, and assembled into maximum intensity projection images using the “Stacks-MultiID” plug-in from ImageJ software (Rasband, W.S., ImageJ, U.S. National Institutes of Health, USA, http://rsb.info.nih.gov/ij/, 1997–2010).
2.7. Alamar Blue® assay

For metabolic activity, samples were incubated in complete culture medium with 10% AlamarBlue® agent (Molecular ProbesTM, Invitrogen, USA) at 37 °C for 4 h. Post incubation, 100 μl aliquots of medium were collected in triplicate from triplicate samples and the fluorescence detection, indicative of cellular reduction of resazurin agent, was measured at 585 nm using 590 nm excitation with an EL800 Multi-mode Microplate Reader (Bertold Technologies, Bad Wildbad, Germany). Acellular scaffolds were used as background reference.

2.8. Migration assay

h-Fibroblasts were seeded (cell density of 3 × 10^5) cells/cm² and cultured on 15.4 wt% collagen gels assembled with and without copper. Gels were submersed in 800 μl of TRIZol (Invitrogen) reagent and total ribonucleic acid (RNA) species were extracted, as per the manufacture’s instructions. Messenger RNA were hybridized by deoxythymine oligomers (50 mM MLV reverse transcriptase) at 37 °C for 1 h as per manufacturer’s instructions with 10 nM (each) deoxynucleobacids (Invitrogen), 0.1 M dithiothreitol (Sigma), 40 U/μl rRNasin (Promega), First Strand Buffer (Invitrogen) and adjusted with RNAse/DNase Free H2O (Gibco). Enzymes were inactivated by incubation for 15 min at 75 °C and resulting cDNA templates generated were diluted 20-. Optimized primers synthesized by IDT technologies at 0.1 μM amplified pooled specimen templates verifying primer efficiency. Housekeeping gene primer was amplified with each sample assessing RNA extraction efficacy prior to qPCR commencement. Cycling conditions on a BioRad iCycler thermocycler were an initial 95 °C denaturation for 6 min, followed by 35 repeats of 95 °C denaturation for 30 s, 58 °C annealing for 45 s and 72 °C extension for 1 min and 30 s, concluding with an infinite loop of refrigeration. Results were analysed on 0.8% Aгарose gels electrophoresed at 75 V for 1 h stained with 5 μl 10 mg/mL ethidium bromide and fluororesced under UV. Applied Biosystems 7900HT Real Time PCR system conducted sample amplification in triplicates using manufacture pre-set cycling conditions in SDS v2.3 software with Power Sybergreen PCR master mix (Applied Biosystems).

2.10. Gels preparation

3.2 ml of acidic collagen solution were neutralized with 0.8 ml of 10% DMEM or PBS and 37 μl of 5 M NaOH and incubated for 30 min at 37 °C in a rectangular-shaped mould (40 × 18 mm). The so obtained prismatic gel was conditioned for 30 further minutes at 37 °C in a 30 mM CuCl2 solution and then plastically compressed (30 kPa, 15 min). The dense collagen sheet was then rolled up along its longest side to form a rod-shaped construct, which was then conditioned in deionized water for 24 h at 37 °C to remove the excess of copper ion diffused in the gel. Collagen fibrillar density was then calculated as previously described. Dense collagen rods obtained using 10% DMEM or PBS and non-treated in 30 mM CuCl2 were used as controls. Copper content in the gels (n = 3) was calculated by atomic absorption (Atomic Absorption Spectrometer 3110, Perkin Elmer). 

2.11. Thermal stability analysis

The thermal stability of dense collagen gels obtained in 10% DMEM or PBS, non-treated and treated in 30 mM CuCl2, was investigated. Shortly, disk-shaped (r = 7.5 mm) dense collagen gels (n = 5) were immersed in heated bath under gentle agitation. A ramp of 1 °C/min was applied to the bath temperature. Collagen has two denaturation temperatures. When the first denaturation temperature is reached (Ts), collagen gels suddenly and visibly shrink, as a consequence of the release of collagen triple helix. When the second denaturation temperature is reached (Td), the gels disaggregate. This is an irreversible step that destroys the gels. Ts and Td were therefore recorded for the different types of gels investigated.

2.12. Collagensase assay

The stability of dense collagen gels obtained in 10% DMEM or PBS, non-treated and treated in 30 mM CuCl2, was investigated through an enzymatic assay, as previously reported. Collagenase (MP Biomedicals, Cat#2810305) was diluted in PBS buffer to 1 U/ml. Gels of different CFDs were placed into 3 ml collagenase solution and digested at 37 °C. 50 μl sample of each condition was taken at time points 0, 10, 20, 30, 45, 60, 90, and 120 min. The amount of collagen was then detected by Protein Assay Kit (Pierce, cat# 23225). The amount of collagen digested for each sample was calculated by measuring the absorbance of the working solution at 562 nm (EL800 Universal microplate Reader, Bio-Tek Instrument) and using a standard curve as reference. The change of collagen level over time was obtained by plotting absorbance against time.

2.13. Statistical analysis

Data are presented as representative images, representative experiments or as means ± standard error of the mean, with n indicating the number of independent experiments. Differences were assessed by either Student t-test or one way ANOVA test with a Tukey mean test and accepted as statistically significant at p < 0.05.

3. Results

3.1. Copper ions enhance bone regeneration and collagen deposition in an orthotopic model

Previously we found that localised copper delivery in a subcutaneous site enhanced collagen deposition in porous scaffolds in vivo. We hypothesised that this collagen likely served to support new growing structures such as bone vessels and may explain reported proangiogenic effects and possibly also the suggestion that endothelial cells translocate copper during tubulogenesis [24,25]. If this was the case, then one would expect the effect to be independent of implantation site. To test this we implanted calcium phosphate cylinders loaded with CuSO4 solution (560 ng) in an orthotopic long bone model in rabbits. First, we qualitatively found an accelerated bone formation and bone growth for CuSO4-loaded implants (Fig. 1a to iv). In addition, the percentage of bone area when compared to the total implant area (Fig. 1c i) was significantly increased (p < 0.001) at weeks 3 and 9 post implantation by copper release in the osseous site. Bone formation in cylinders not loaded with CuSO4 was negligible for all the time points considered, with less than 10% of bone covering the area of the implant at week 9. On the contrary, CuSO4-loaded implants showed a more marked bone formation as almost one quarter of the defect was filled by bone after 9 weeks of implant. A similar trend was measured for the percentage of contact between bone and available implant outline when compared to the total implant area (Fig. 1c ii), where loading of CuSO4 in the brushite implants resulted in a substantial increase of the bone/implant contact at week 3 (p < 0.01) and 9 (p < 0.01) post implantation. Bone growth was also accelerated in the presence of CuSO4-loaded implants (Fig. 1c iii), which resulted in a 70% and 30% increase in the new bone formation (p < 0.01) at week 1 and 9, respectively. Histological sections at an early time point (Fig. 1b) had clear evidence of marked accumulation of collagen (stained in red) in CuSO4-loaded implants. High magnification images focusing on the implant surface inside the hole indicated the increased collagen deposition at the interface of bone — CuSO4-loaded implant when compared to the control (Fig. 1b ii and iv) and the percentage of the total implant area occupied by collagen at week 1 was more than three times higher in presence of CuSO4 (Fig. 1d). In addition, the percentage of contact between the deposited collagen and the total implant surface was more than four times for CuSO4-loaded implants (Fig. 1e).

3.2. Copper ions do not affect human fibroblast collagen gene expression or collagen formation

To test if the enhanced collagen deposition in the CuSO4-loaded orthotopic sites was the result of a copper-regulated enhancement in collagen synthesis, we measured type I soluble and insoluble collagen production by human fibroblasts when CuSO4 (1 μg/ml) was added to the culture media. The concentration of copper ions was chosen based on our previous studies [14,23]. Interestingly, exposure of human fibroblasts to copper ions had no significant observable effect on soluble or insoluble collagen synthesis (p > 0.05), when compared to cells grown in normal culture media.
Copper accelerates bone healing and collagen deposition in vivo. Rabbits were bilaterally implanted in the tibia and femur with brushite cylinder loaded or not with CuSO4 solution (560 ng) and sacrificed after 3 and 9 weeks. a) Bone formation and bone growth were observed and assessed on histological section within the implant after methylene blue/basic fuchsin staining. i) and ii) Representative images of transversal sections of control implants after 3 and 9 weeks of implantation. iii) and iv) Representative images of transversal sections of CuSO4-loaded implants after 3 and 9 weeks of implantation. Black arrows indicate bone formation. Calibration bar applies to all images. b) Collagen deposition is increased in the presence of copper. Collagen formation was observed and assessed on histological section within the implant after sirius red staining. Representative images of transversal sections of control (i) and CuSO4-loaded (ii) implants after 1 week of implantation. High magnification images focusing on implant surface inside the hole are presented in images (iii) and (iv), respectively. Black spots represent the nuclei of cells as a result of the picrosirius red staining. Calibration bar applies to all images. c) Increase bone deposition is increased in the presence of copper. Collagen formation was observed and assessed on histological section within the implant after sirius red staining. Representative images of transversal sections of control (i) and CuSO4-loaded (ii) implants after 1 week of implantation. High magnification images focusing on implant surface inside the hole are presented in images (iii) and (iv), respectively. Black spots represent the nuclei of cells as a result of the picrosirius red staining. Calibration bar applies to all images. c) Increase bone deposition is increased in the presence of copper. Collagen formation was observed and assessed on histological section within the implant after sirius red staining. Representative images of transversal sections of control (i) and CuSO4-loaded (ii) implants after 1 week of implantation. High magnification images focusing on implant surface inside the hole are presented in images (iii) and (iv), respectively. Black spots represent the nuclei of cells as a result of the picrosirius red staining. Calibration bar applies to all images.

3.3. Copper ions stabilize and crosslink reconstituted collagen gels post-fibrillogenesis

As the cellular in vitro results did not support the suggestion that copper was directly regulating collagen synthesis, we investigated the effects of copper ions on collagen itself. Since extracellular matrix deposition rate is a balance between synthesis and degradation [28], we hypothesized that collagen synthesis observed in vivo was a consequence of a non-cellular copper-mediated processes. Reconstituted, tissue-equivalent collagen gels were exposed to copper ions (30 mM CuCl2, 30 min at 37 °C) and densified through compression (50 kPa, 15 min) to increase collagen fibrillar density (CFD) to physiologically relevant values (CFD> 5 wt%) (Fig. 3a). We then rinsed the dense collagen (DC) gels in deionized water for 24 h to remove non-bound copper. The final copper content in the gels 78 ± 19 ppm, within the usual physiological concentration in tendon and bones (0.02–120 ppm) [27]. The CuCl2 concentration of 30 mM was then chosen as trade-off between CFD achievable and physiological residual copper content in the collagen gels (Fig. S1). Surprisingly, this treatment resulted in collagen constructs with increased fibrillar density (CFD>30 wt% Fig. 3b, c and Fig. S2) and enhanced mechanical properties (4.5-fold increase in the apparent modulus and 2.7-fold increase in the ultimate tensile strength – UTS, Fig. S3), when compared with neat collagen gels (10 wt% < CFD < 16 wt%). This behaviour was independent of the solution used to titrate collagen pH (we tried DMEM and PBS) and was not affected by the presence of physiological levels of glucose in the solution – titration with PBS was glucose-free (Fig. 3c). Spectroscopic investigation of collagen gels treated with 30 mM CuCl2 did not show differences in the infrared spectra of Amide I, II and III resonances, indicating of no denaturation of the protein had occurred. In addition, the differences in the absorbance at 976 and 942 cm−1 suggested the formation of interfibrillar crosslinks through copper-bridged cysteine or histidine complexes (Fig. S4), which was also sustained by the previously reported existence of copper-complexing sites in native collagen [28]. The crosslinking was dependant on the concentration of copper in the solution as there was a quasi-linear increase in copper chelation by collagen fibrils, which corresponded with an increased collagen fibrillar density (Fig. S1). In addition, the exposure of collagen gels to other transition metal ions did not produce the same stability effects measured with copper, indicating a unique role of copper cations (Table S1).

The formation of interfibrillar crosslinks in collagen gels exposed to copper ions and then plastically compressed was further
confirmed by the enhanced resistance of CCC gels to thermal shrinkage (increased from 42°C to 46°C) and to thermal disaggregation (from 53 to 61°C), when compared to the non-treated ones (Fig. 3d).

We further assessed the effect of interfibrillar copper-crosslinking on the enzymatic degradation of reconstituted collagen gels (Fig. 4). Confocal laser scanning microscopy of 15.4 wt% collagen gels before and after exposure to collagenase I and MMP-9 (gelatinase) showed an increased proteolytic resistance of collagen gels treated with copper. Bicinchoninic acid (BCA) assay for degradation (with collagenase I) of 15.4 wt% collagen gels assembled with and without copper corroborated the microscopy results. In particular, to exclude the inhibitory effects copper ions on the activity of collagenase I, 80 ppm of copper ions (circa equal to the amount of copper measured in the collagen gels) were added in the collagenase solution, as control. Copper treatment of collagen gels, but not the inclusion of 80 ppm of copper in the collagenase solution, significantly (p < 0.05) reduced collagen digestion.

3.4. Copper crosslinked collagen sustains human fibroblast viability, migration and modulate Mmps expression

To establish the effects of copper-mediated collagen crosslinking on cell–material interactions we cultured human fibroblasts on tissue-equivalent collagen gels treated and non-treated with copper and we investigated cell viability, migration and gene expression. Confocal laser scanning microscopy coupled with Live/Dead® assay showed that human fibroblasts remained alive on both the materials evaluated (Fig. 5a) but the metabolic activity of human fibroblast was decreased when cultured on collagen gels treated with copper ions (Fig. 5b). Migration assay (scratch test) showed no difference in the mobility of human fibroblasts on collagen treated and non-treated with copper, indicating that the copper treatment did not affect the dynamic of focal adhesion of fibroblasts on the collagens (Fig. 5c). To establish the remodelling of the copper crosslinked collagen gels by human fibroblasts, we quantified the relative expression of several MMPs, when compared to the one of human fibroblast cultured on non-treated collagen gels (Fig. 5d). Interestingly, qRT-PCR showed an up-regulation (p < 0.05) of Mmp-1 and Mmp-3 and a downregulation (p < 0.05) of gelatinases (Mmp-2 and Mmp-9) and of Mmp-13. Expression of Mmp-9 was not affected by the copper treatment (p > 0.05).

4. Discussion

Our work indicates that enhanced collagen deposition in response to localised copper exposure in vivo is not site specific. Human fibroblasts cultured in the presence of non-toxic quantities of copper ions did not upregulate Col1a1 genes and did not deposit...
more collagen matrix. Finney et al. have reported a large-scale relocalization of cellular copper in the extracellular space during angiogenesis [24], with local copper concentrations increasing to few millimolar, i.e. far above the physiological copper content in the extracellular space, depending on the tissue [2]. Furthermore, superphysiological concentrations of copper were found in the milieu of neo-formed capillaries and in wound-healing sites. The accumulation of copper ions in the extracellular space has also been linked to fibrosis, where imbalanced collagen remodeling results in increased protein deposition [29,30]. Non-physiological amounts of copper in the extracellular space has also been reported in many tumours [4,25], mainly attributed to the essential role of the cation for blood vessel formation [31]. In addition, the architecture of the collagenous extracellular matrix is

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**Fig. 3. Effect of copper ions on the physical properties of dense collagenous gels.**

a) Methodology used to obtain tissue-equivalent collagen gels by plastic compression procedure and by conditioning the samples in a 30 mM CuCl2 solution for 30 min: a) collagen acidic solution was neutralized with 10x DMEM and NaOH and incubated for 30 min at 37 °C in a rectangular-shaped mould. The so obtained prismatic gel was conditioned for 30 further minutes at 37 °C in a 30 mM CuCl2 solution and then plastic compression was applied (50 kPa, 15 min). The obtained dense collagen sheet was then rolled up along its shorter side. b) pictures of dense collagen rolls i) non-treated and ii) treated in 30 mM CuCl2. c) As made gels had a collagen fibrillar density (CFD) of 0.2 wt%. Plastic compression was used to increase the fibrillar density to physiological values. DMEM (10x) and PBS were used to dilute and titrate the collagen acidic before self-assembly. Copper ions increased the efficacy of plastic compression as the collagen fibrillar density of the gels treated with 30 mM CuCl2 was higher after 12 h in deionized water (p < 0.05). d) Thermal shrinkage (Ts) and thermal disaggregation (Td) of collagenous gels described in a). One way ANOVA test showed that both copper affected the collagen gel thermal stability (i.e. Ts and Td).
severe altered in cancer tissues, where collagen deposition is elevated, and had been reported to be non-physiologically cross-linked, and resistant to proteolytic fragmentation [32].

We tentatively suggest that the aforementioned accumulation of copper in the extracellular space might serve to crosslink of collagen fibrils local to the developing blood vessel, increasing stability and altering susceptibility to MMPs acting somewhat as an inorganic tissue inhibitor of metalloproteinase. Since collagen deposition is a balance between formation and degradation, the enhanced copper formation observed in vivo may occur as a result of this apparent resistance to collagenases. It may explain the apparent success in treating some fibrotic disorders with copper chelators [33–35]. Pathologically, copper-mediated stabilization of collagen could result in an altered ECM mechanical properties and remodelling. Another ECM protein, fibronectin, has also been reported to be stabilised by copper ions [36,37].

Collagen crosslinking processes are tissue specific and can be enzymatic and non-enzymatic [38]. The first occurs in immature and

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**Fig. 4. Effect of copper ions on the enzymatic degradation of dense collagenous gels.** Collagen gels were plastically compressed to disks with a fibrillar density of 15 wt% without or with copper in the self-assembly solution. a) CLSM microscopy of 15.4 wt% collagen gels before and after exposure to collagenase I and MMP-9. Enzymatic degradation was investigated by adding a drop (10 μl) of Collagenase I (20 U/ml) and recombinant rat MMP-9 (20 U/ml) on the dense collagen disks and incubating for 3 h at 37 °C. Collagen gels assembled without copper showed sign of degradation at CLSM, while degradation was delayed in gels fibrillized with copper. b) The plot represents the amount of collagen digested by collagenase (1 U/ml) in gels with different CFDs as a function of time, as calculated through a BCA assay. One way ANOVA test showed that the treatment in 30 mM CuCl₂, to which corresponded CFD values > 30 wt%, significantly (p < 0.05) reduced collagen digestion. c) BCA assay for collagen degradation (Collagenase I – 1 U/ml) of 15.4 wt% collagen gels assembled with and without copper. To evaluate the effects of copper ions on the activity of collagenase, 80 ppm of copper ions (equal to the amount of Cu measured in the collagen gels) were added in the collagenase solution, as control. One way ANOVA test showed that the treatment in 30 mM CuCl₂, but not the inclusion of 80 ppm of copper in the collagenase solution, significantly (p < 0.05) reduced collagen digestion.
Fig. 5. Effect of copper ions on the viability, metabolic activity, migration and gene expression of human fibroblasts cultured on dense collagenous gels. a) CLSM Live/Dead® assay of h-fibroblasts cultured on 15.4 wt% collagen gels fibrillized with and without copper. Cells were found alive on both the materials considered. Scale bar = 200 μm. b) Alamar Blue® assay of h-fibroblasts cultured on 15.4 wt% collagen assembled with and without copper. Metabolic activity of cells was decreased on cells cultured in the presence of copper ions. c) Migration assay of h-fibroblasts cultured on 15.4 wt% collagen assembled with and without copper. Cell migration was monitored upon scratching the dense gels with a razor blade. Scale bar = 400 μm. d) RT-qPCR measurement of the expression of several Mmps by h-fibroblasts at day 7 of culture in 15.4 wt% collagen assembled with and without copper.
mature collagen and gives an optimum functioning tissue by involving the formation of divalent and then trivalent intermolecular crosslinks at defined sites. The second type involves non-enzymatic reactions, the chemistry of which is slowly being unravelled, to form additional interfibrillar crosslinks [19,39]. These glucose mediated crosslinks (i.e. collagen glycation), are peculiar of the mature state of the protein, increasing the fibres’ brittleness and are responsible for the formation of advanced glycation end products (AGEs), which play a critical role in microvascular pathologies in several studies that transition metal ions, and copper in particular, catalyze the formation of AGEs (Table S2). The proposed reaction involves two steps [21]. The glucose-induced glycation of collagen is the initiating factor, to which follows the metal-catalyzed oxidation of glycated collagen, responsible for its crosslinking. This second step is catalyzed by: a) metal-catalyzed autoxidation of glucose in the presence of oxygen that yields an α-ketoaldehyde with formation of superoxide radical (e.g. H2O2), the reduction of which generates hydroxyl radicals, responsible for the formation of fibril crosslinks and b) generation of hydroxyl radicals due to the ease autoreduction to which transition metal salts undergo. Nevertheless, the collagen crosslinking herein described did not require glucose and did not appear to be related to collagen glycation.

Metal ion-mediated stabilization and crosslinking of collagen fibrils by intra- and interfibrillar mechanisms have been previously reported at high concentrations. In particular, the role of titanium, chromium (which is used as tanning agent in the formation of leather), manganese, iron, silver, aluminum but not copper (alone, without any additional reagent) has been studied [28,40–43]. Wu et al. have also described the formation of Cr3+ clusters outside the collagen fibrils, which were responsible for the formation of interfibrillar crosslinks [40]. A similar mechanism was also proposed by Fathima et al. to described the stabilization of collagen against collagenase upon exposure of the protein to an iron complex [44]. In our study, the proposed interfibrillar crosslinks induced by copper-collagen complexes was found to be unique, since although solutions made with chromium, manganese, iron, nickel, zinc and silver stabilized the collagen matrix against thermal degradation they did not prevent the re-swelling of the compressed gels (indicating intermolecular crosslinking). It has previously been reported that chromium—collagen complexes involve the aspecific protonation of the carboxyl groups present in the protein, resulting in collagen intermolecular crosslinking [40]. In addition, it has been proposed that copper may be involved in interfibrillar stabilization by binding the imidazole of one of the five histidine residues present in the tropocollagen molecule (Nimid) and three Npept of adjacent peptidic groups (e.g. glycine on the peptide backbone supply ample binding sites) [45,46].

Here, we have demonstrated both the enhanced deposition of collagen in response to copper in an orthotopic model at 2 weeks and the unprecedented ability of copper ions to crosslink type I collagen fibrils at very low concentrations (70 ppm) under mechanical stimuli, resulting in the formation of collagenous constructs with enhanced mechanical properties and with increased resistance to proteolytic enzymes (i.e. collagenase I and MMP9). Together these results depict how sensitive ECM is to free copper and may provide new insights on the mechanisms governing the pathological accumulation of collagen in the extracellular space.

5. Conclusions

We have found that reconstituted type I fibrillar collagen crosslinks in the presence copper II ions when under compression. This interplay between cation and collagen seemed particular to copper amongst the cations investigated and was distinct from glucose dependent Fenton reaction-like crosslinks. Our data showing that copper crosslinking of collagen effectively inhibited fibroblast proliferation and rendered the collagen resistant to collagenase indicated that very subtle changes in structure at the superfibrillar level can dramatically alter cell behaviour, since equivalent amounts of copper in solution had no effect. The levels of copper involved are practically undetectable without complete destruction of the tissue and so if this crosslinking occurs physiologically, and it is not unreasonable to suppose it does, it may explain some differences in biological responses to this ECM with age, pathology and anatomy. This newly uncovered interaction may also be correlated with our previously reported observation of enhanced collagen deposition in response to localised copper delivery and may provide direction for therapeutic approaches for some fibrotic conditions.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2015.03.018.

References


