Acta Histochemica 114 (2012) 805–812

Contents lists available at SciVerse ScienceDirect

Acta Histochemica

journal homepage: www.elsevier.de/acthis

β-Defensin-4 (HBD-4) is expressed in chondrocytes derived from normal and osteoarthritic cartilage encapsulated in PEGDA scaffold

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ARTICLE INFO

Article history:
Received 26 July 2011
Received in revised form 28 January 2012
Accepted 1 February 2012

Keywords:
Osteoarthritis
Knee
Chondrocytes
β-Defensin-4
Hydrogels (PEGDA)
Human

ABSTRACT

Defensins are antibiotic peptides involved in host defense mechanisms, wound healing and tissue repair. Furthermore, they seem to play an important role in protection mechanisms in articular joints. The aim of this study was to investigate β-defensin-4 expression in chondrocytes taken from articular cartilage of knees of patients with osteoarthritis (OA) compared to normal cartilage, *in vivo* in explanted tissue, and *in vitro* in chondrocytes encapsulated in construct PEGDA hydrogels. The present investigation was conducted to try and elucidate the possible use of β-defensin-4 as a relevant marker for the eventual use of successive scaffold allografts, and to provide new insights for hydrogel PEGDA scaffold efficacy in re-differentiation or repair of OA chondrocytes *in vitro*. Articular cartilage specimens from OA cartilage and normal cartilage were assessed by histology, histochemistry, immunohistochemistry and Western blot analysis. The results showed strong β-defensin-4 immunoexpression in explanted tissue from OA cartilage and weak β-defensin-4 expression in control cartilage. The chondrocytes from OA cartilage after 4 weeks of culture in PEGDA hydrogels showed the formation of new hyaline cartilage and a decreased expression of β-defensin-4 immunostaining comparable to that of control cartilage. Our results suggest the possibility of applying autologous cell transplantation in conjunction with scaffold materials for repair of cartilage lesions in patients with OA using β-defensin-4 as a relevant marker.

Introduction

Osteoarthritis (OA) is a multifactorial joint disease characterized by a progressive erosion of articular cartilage (Buckwalter and Mankin, 1998; Lorenz and Richter, 2006; Pritzker et al., 2006) that may be caused by mechanical and biological processes that modify cartilage homeostasis (Goldring and Goldring, 2004; Aigner et al., 2006).

Articular cartilage homeostasis is the result of an intricate interplay between anabolic and catabolic, anti- and pro-inflammatory, anti- and pro-apoptotic activities (Loreto et al., 2009; Musumeci et al., 2011a). Chondrocytes represent the versatile regulators of this cartilage equilibrium (Burrage et al., 2006). Any alteration of this fine regulation may result in cartilage damage and secondary OA (Burrage et al., 2006).

Several *in vitro* and *in vivo* studies have indicated an important role of tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6) in the initiation and progression of articular cartilage destruction (Goldring, 2000; Pelletier et al., 2001). Scientific evidence has demonstrated a clear link between inflammation and the expression of specific molecules called antimicrobial peptides (AMPs). AMPs are abundant and widely distributed effectors of the innate immune response that are able to kill microbes by destroying their cell membranes (Zasloff, 2002). The constitutive or inducible expression of AMPs is dependent on the examined tissue and is based on appropriate stimulation (Harder et al., 1997, 2000; Singh et al., 1998; Zasloff, 2002). Defensins, a major subclass of APMs, are a complex group of 4-kDa open-ended cysteine-rich, cationic peptides divided into α- and β-defensins based on the location and the connectivity of six-conserved cysteine residues. Some researchers have demonstrated that mesenchymal tissues, such as synovial membranes or cartilage, are also able to produce a specific spectrum of defensins (Paulsen et al., 2001, 2002; Varoga et al., 2004,

Abbreviations: ANOVA, analysis of variance; AMPs, antimicrobial peptides; DAB, 0.1% 3,3′-diaminobenzidine; DMEM, Dulbecco's modified Eagle's medium; ERT, estrogen replacement therapy; EtOH, ethyl alcohol; ES, extent score; ECM, extracellular matrix; FBS, fetal bovine serum; HBD-4, β-defensin-4; HCL, hydrochloric acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; H&E, hematoxylin and eosin; IS, intensity of staining; IL-1, interleukin-1; IL-6, interleukin-6; kDa, kilo-Dalton; MRI, magnetic resonance imaging; MMPs, metalloproteinases; NSAIDs, non-steroidal anti-inflammatory drugs; OA, osteoarthritis; PBS, phosphate buffered saline; PEGDA, polyethylene glycol diacrylate; NaOH, sodium hydroxide; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α.

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doi:10.1016/j.acthis.2012.02.001

2005). These molecules are induced by inflammation processes, *e.g.* transforming growth factor-β (TGF-β) (Sorensen et al., 2003), tumor necrosis factor-α (TGF-α), interleukin-1 (IL-1) (Singh et al., 1998) and during processes of articular cartilage tissue remodeling by increasing activities of metalloproteinases (MMPs) (Varoga et al., 2004) thus playing an important role in articular cartilage homeostasis. β-Defensins appear to augment catabolic pathways in articular cartilage, ultimately leading to a breakdown of the extracellular matrix (Varoga et al., 2006).

As far as we are aware there are no published studies on the molecular expression of β-defensin-4 in normal and OA cartilage. The purpose of our investigation was to determine the immunohistochemical expression of β-defensin-4, in human autologous chondrocytes derived from normal and OA cartilage and encapsulated in a scaffold, in order to understand the role of this molecule in the articular defence mechanisms and cartilage homeostasis. The authors also speculate on the hypothesis of the possible use of β-defensin-4 as a relevant marker for the subsequent use of hydrogel poly(ethylene glycol) diacrylate PEGDA scaffold allografts, which have considerable importance as a scaffold material for cartilage tissue engineering (Jeon et al., 1991; Hubbell, 1995; Elisseeff et al., 2000; Mauck et al., 2000; Masuda et al., 2003).

The aim of the study was to elucidate new insights on hydrogel PEGDA scaffold efficacy in re-differentiation or repair of OA chondrocytes *in vitro* and the possibility of novel future approaches for the medical treatment of OA. This in turn could provide further insights into pathological processes of cartilage and indicate novel therapeutic strategies and new materials for OA treatment.

Materials and methods

Patients

Osteoarthritic cartilage was obtained from 25 patients (15 males and 10 females), with similar height and weight, who underwent surgical knee joint replacement because of pain and functional impairment at the Orthopaedic and Traumatology Unit, Hospital of Suzzara, Mantova, Italy, after obtaining the required informed consent from the patients. The mean age of the OA patients was 58 years (range 36–76 years). Tissue samples were graded according to the Mankin histopathological scores (van der Sluijs et al., 1992) and included only samples of moderate-to-severe OA (Mankin score 6–14). The patients were non-smokers, occasionally taking NSAIDs (non-steroidal anti-inflammatory drugs) in addition to the classical treatment for knee OA composed of hot packs, therapeutic ultrasound and terminal isometric exercises. The female patients did not take estrogen replacement therapy (ERT) that may influence the physiological homeostasis of the joint.

Diagnosis, based on clinical and radiological criteria, included physical examination, X-ray imaging and MRI of the injured knee. According to Ahlbäck classification (grade IV, moderate bone attrition) and Kellgren and Lawrence classification, patients had OA of the knee of grade 3 or 4 with moderate diminution of joint space or joint space greatly impaired with sclerosis of subchondral bone (Petersson et al., 1997). Samples used as the control were obtained from 16 patients of similar height and weight to patients with OA knee (8 males and 8 females), lacking any history of primary or secondary arthritis, from the same hospital, after obtaining the required informed consent from the patients. The mean age of these patients was 42 years (range 37–58 years). Knee joint tissues were removed because of traumatic events. Macroscopic and microscopic examination did not demonstrate signs of degenerative or inflammatory joint disease. These patients represented the control group as they did not show symptoms of arthritic processes at the time of the traumatic event.

Isolation of chondrocytes and culture conditions

To isolate the chondrocytes from OA and non-OA articular cartilage, cartilage pieces from deep layers close to the subchondral bone were incubated in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) containing 0.2% collagenase (Worthington Biochemical Corporation, Lakewood, NJ, USA) and 5% FBS (fetal bovine serum, Gibco, Grand Island, NY, USA) for 14–16 h at 37 °C and 5% CO_2. The resulting cell suspension was then filtered through 70 μm nylon filters (Cell Strainer; Falcon, Franklin Lakes, NJ, USA) and washed 3 times with phosphate buffered saline (PBS) containing 100 U/ml penicillin and 100 μg/ml streptomycin. The number and size distribution of the isolated cells were then determined with a Z2 Coulter Counter and a Size Analyzer (Beckman Coulter, Palo Alto, CA, USA). After isolation, the chondrocytes were plated onto separate 10 cm tissue culture dishes at a density of 10×10^6 cells/cm^2. Cells were incubated at 37 °C and 5% CO_2 in chondrocyte medium composed of DMEM containing 10% fetal bovine serum, 0.4 mM proline, 50 μg/ml ascorbic acid, 10 mM HEPES, 0.1 mM non-essential amino acids, 100 U/ml penicillin and 100 μg/ml streptomycin. The culture medium was changed twice weekly. Cells were observed with an Axioplan Zeiss light microscope (Germany) and photographed with a digital camera (Power Shot G5 Canon, Japan) and calibrated by means of a micrometer slide.

Cell encapsulation in PEGDA hydrogels

Cells were encapsulated in PEGDA hydrogels for 2, 3 and 4 weeks of culture as described previously (Terraciano et al., 2007). Briefly, chondrocytes (passage 3 or 4) were suspended in 10% (w/v) PEGDA (SunBio, Orinda, CA, USA) solution in sterile PBS with 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, Grand Island, NY, USA). The photoinitiator, Irgacure 2959 (Ciba Specialty Chemicals, Tarrytown, NY, USA), was used at 0.05% (w/v) final concentration. The photoinitiation and encapsulation processes have been previously determined to be biocompatible. Chondrocytes were resuspended in 95 μl of polymer solution at a concentration of 20×10^6 cells per ml. The cell-polymer solution was added to a cylindrical silicone-tubing mold with an internal diameter of 4.75 mm and a construct height of 5 mm. The tubing was attached to the glass microscope slide using a silicon lubricant. The prepolymer (macromer) was exposed to UV light (365 nm) for 5 min to induce gelation. Constructs were removed from the molds and cultured at 37 °C, 5% carbon dioxide in chondrocyte medium. The culture medium was changed twice weekly and the cells were observed with an Axiovert 25 Zeiss inverted microscope (Carl Zeiss, Oberkochen, Germany). The scaffolds were photographed with a digital camera (Canon, Japan).

Fixation, dehydration and embedding of hydrogels

The hydrogel was fixed by incubating it in 500 μL of 4% paraformaldehyde (w/v) in PBS (Invitrogen), pH 7.4, overnight (~12 h). Then the hydrogel was dehydrated using the following sequence: 70% EtOH × 1 h, 80% EtOH × 1.5 h, 95% EtOH × 12 h, 100% EtOH × 1.5 h-repeat twice, xylene × 1 h. The hydrogel was placed in a metal mold and embedded in paraffin at 60 °C overnight (~12 h). The paraffin was poured off and replaced with fresh paraffin. An embedding cassette (Fisher) was placed on top of the mold and additional paraffin was added to fill the cassette. Blocks were cooled for 1 h using the cold surface of the embedding station (EG1150, Leica Microsystems, Wetzlar, Germany). Sections 4–5 μm thick were cut from paraffin blocks using a microtome and mounted on silane-coated slides and stored at room temperature.