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Hydrostatic Pressure Increases Apoptosis in Cartilage-Constructs Produced From Human Osteoarthritic Chondrocytes

Reid Wenger [1], Mark G. Hans [1], Jean F. Welter [2,3], Luis A. Solchaga [2,3], Yun R. Sheu [4], and Charles J. Malemud [4,5]

[1] Department of Orthodontics, Case Western Reserve University School of Dentistry, [2] Department of Orthopaedics, [4] Department of Medicine/Division of Rheumatic Diseases and [5] Department of Anatomy, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106-4946, [3] Skeletal Research Center, Department of Biology, Case Western Reserve University

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1. ABSTRACT

Tissue-engineering is considered a promising avenue for developing human articular cartilage implants that can be employed for resurfacing damaged cartilage in the early stages of osteoarthritis. In the present study, human cartilage-constructs were produced from human osteoarthritic chondrocytes maintained on a scaffold of HYAFF®-11 in perfusion mini-bioreactors or after implantation and recovery from nude or SCID mice after 3 weeks. The human cartilage-construct extracellular matrix reacted positively with anti-Type II collagen monoclonal antibody, but not with anti-Type I or anti-Type X collagen monoclonal antibodies. A significant portion of the cartilage-construct extracellular matrix stained metachromatic with Toluidine blue-O indicative of sulfated-proteoglycan deposition. Cyclic hydrostatic pressure applied for 4 hrs at 5 MPa using a 1 Hertz sinusoidal frequency significantly increased ($p<0.02$) the proportion of apoptotic cells in the cartilage-constructs (41% ± 4.2%; mean ± SD) compared to control cartilage-constructs (28.5 ± 8.4%).

2. INTRODUCTION

Human articular cartilage injury is a serious clinical challenge because of the inherent inability of adult cartilage to undergo intrinsic repair. Tissue engineering is an emerging approach for developing cartilage implants which could be employed for surgical therapy in small areas of cartilage degeneration and, perhaps, even in the larger surface lesions of articular cartilage typically found in human osteoarthritis (OA) (1). Recently, experimental tissue-engineering strategies were used so that human bone marrow-derived mesenchymal stem cells (hMSC) were combined with artificial scaffolds to produce composite implants of 3-4 mm in diameter (2). However, for these implants to achieve success in human synovial joints, larger cartilage-constructs will have to be employed since the cartilage defects are much larger when the clinical diagnosis of OA is made. In that regard, previous studies (3) suggested that large cartilage-constructs failed in experimental animal trials because they lacked the phenotypic characteristics of mature cartilage. In addition to these considerations, the response of human cartilage-

constructs (HCC) to biomechanical stress should first be studied in an experimentally-controlled *in vitro* environment, since the potential use of HCC as cartilage implants for early OA are likely to be employed in the weight-bearing areas (4) of human knees and hips.

In the present study, human chondrocytes isolated from OA cartilage were expanded in primary culture and then seeded onto a hyaluronan-based carrier scaffold of HYAFF®-11 to produce human cartilage-constructs. The carriers were maintained either in perfusion bioreactors (2, 3) or implanted and retrieved from nude or SCID mice. Experiments were also conducted to determine the extent to which programmed cell death (i.e. apoptosis) was induced by cyclic hydrostatic pressure (CHP) since it had previously been shown that *in vitro* chondrogenesis was modulated by CHP in hMSC pellet cultures (5) but that CHP induced apoptosis in adult human OA chondrocyte suspension cultures (6). Thus, we determined whether the elaboration of an extracellular matrix (ECM) by chondrocytes in the HCC had a protective effect on apoptosis induction by CHP.

3. MATERIALS AND METHODS

3.1. Antibodies

The following collagen antibodies were commercially obtained: anti-Type I collagen, clone col-1, Sigma, St. Louis, MO; anti-Type II collagen, II-II63B, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA. The anti-Type X collagen was obtained from Dr. Gary Gibson, Breech Research Laboratory, Henry Ford Hospital, Detroit, MI. Mouse pre-immune IgG, and FITC-conjugated goat anti-mouse IgG were purchased from Vector Laboratories, Burlingame, CA and MP Biomedical, Irvine, CA, respectively.

3.2. Human Cartilage Specimens

Seventeen human OA cartilage specimens (61.9 ± 9.9 years, mean ± SD) were obtained from the Cooperative Human Tissue Network at the University of Pennsylvania, Philadelphia, PA and studied individually. Chondrocytes were isolated by enzymatic dissociation (6) and the liberated chondrocytes expanded in primary culture to produce high-density monolayer cultures (7). Approximately 10^6 chondrocytes were seeded into 7 mm in diameter x 3 mm thick HYAFF®-11 sponges (Fidia Advanced Biopolymers, Abano Terme, Italy). HYAFF®-11 is composed of a linear derivative of hyaluronan modified by the esterification of the carboxylic function of glucuronic acid with benzyl groups (2).

3.3. Preparation of Human Cartilage-Constructs (HCC)

The chondrocyte-containing HYAFF®-11 carriers were maintained in perfusion bioreactors (2, 3) at 37°C in complete culture medium as previously described (6) and harvested after 3 wks. Additional studies were performed on HCC produced from human OA chondrocytes grown in HYAFF®-11 after implantation and retrieval after 3 wks from nude (8) or SCID mice. In this model, the athymic or SCID mice served as *in vivo* bioreactors.

3.4. Toluidine Blue-O staining of HCC

Eight micron paraffin-embedded sections were employed for staining with Toluidine Blue-O as previously described (2, 3) to assess the extent to which sulfated-proteoglycans were deposited into the HCC ECM.

3.5. Immunohistochemical Analysis of Collagen Isotypes in HCC

Sections of paraffin-embedded HCC and hMSC pellet culture were stained with fluorescein-5-isothiocyanate (FITC)-conjugated anti-Type I, Type II and Type X monoclonal antibodies as previously described (5). Human MSC (hMSC) pellet cultures were employed to provide a positive control for Type II using antibody II-II63B and Type X collagen using the anti-Type X collagen antibody as previously described (5). Paraffin-embedded human skin sections were used as a positive control for Type I collagen using the anti-Type I collagen antibody. Sections were also incubated with mouse pre-immune IgG as a negative control. Deparaffinized sections were mounted on slides in 5% N-propyl gallate in glycerol to slow down photobleaching. Slides were photographed using a SPOT-RT digital camera attached to a fluorescence microscope.

3.6. Cyclic hydrostatic pressure (CHP)

CHP was applied to HCC at 5 MPa using a 1 Hz sinusoidal frequency for up to 4 hrs as previously described (5, 6).

3.7. Apoptosis Detection

For analysis of apoptosis frequency, paraffin-embedded sections were stained with 4', 6-diamidino-2-phenylindole (DAPI) or monoclonal antibody F7-26 [subclass IgM)] (Chemicon International, Temecula, CA) which identify chondrocyte nuclei and apoptotic cells containing single-stranded DNA, respectively (9). The frequency of apoptosis in response to CHP was determined by counting 5 random fields of DAPI-stained sections merged with F7-26-stained sections at 10X magnification (9). The percent of apoptotic cells was calculated from inspection taking into account inter-observer variation. The apoptosis frequency in HCC subjected to CHP was compared to control HCC by the 2-tailed T-test where $p<0.05$ was considered significant to reject the null hypothesis.

4. RESULTS

Human chondrocytes were isolated from 17 OA cartilage samples with a yield of 4.8 ± 1.9 x10^6cells/gm of tissue, (mean ± SD). After expansion in primary culture, chondrocytes were seeded onto HYAFF®-11 and maintained in perfusion bioreactors for 3 wks or seeded onto HYAFF®-11, implanted and then retrieved from nude or SCID mice after 3 wks.

HCC produced on HYAFF®-11 showed evidence of a heterogeneous but highly metachromatic ECM, although areas near the periphery of the sectioned HCC stained orthochromatic (Figure 1). In addition, HCC

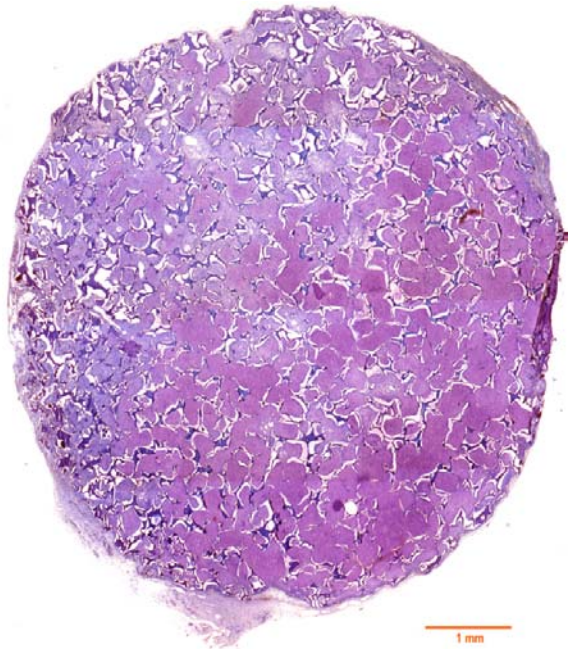

Figure1. HCC/HYAFF®-11 stained with Toluidine blue-O. A 7 mm in diameter x 3 mm thick HYAFF®-11 disk seeded with culture-expanded human chondrocytes was retrieved from a SCID mouse after 3 wks. The HCC ECM stained principally metachromatic (purple) indicative of sulfated-proteoglycan deposition. Some orthochromatic areas (blue) were also identified, but these were primarily at the periphery of the section. Bar=1mm

maintained on HYAFF®-11 also retained some of the HYAFF®-11 scaffolding (Figure 1).

HCC ECM produced on HYAFF®-11 and retrieved from nude mice after 3 wks reacted only with the Type II collagen antibody (Figure 2). For comparison, hMSC pellet cultures maintained in culture for 3 wks reacted with both Type II and Type X collagen antibodies (Figure 2). While the anti-Type I collagen antibody strongly reacted with human skin sections (data not shown), neither HCC nor hMSC pellet culture sections stained positively with anti-Type I collagen antibody (Figure 2). HCC ECM produced on HYAFF®-11 in perfusion mini-bioreactors also reacted only with the Type II collagen antibody (Figure 2). The immunofluorescence at the periphery of the section after staining with anti-Type X collagen antibody (Figure 2) was also evident in the control section (no primary antibody) and was therefore considered to be a non-specific reaction.

HCC/HYAFF®-11 sections stained with DAPI or F7-26 were merged and the percent of apoptotic cells enumerated by inspection at 10X magnification (Figure 3). CHP applied at 5 MPa with a 1 Hz sinusoidal frequency significantly increased the percent of apoptotic chondrocytes in HCC after 4 hrs (Table 1). The percentage of apoptotic chondrocytes in control HCC was 28.5±8.4%; (mean ±SD, n=5) and 41.0±4.2% in HCC subjected to CHP ($p<0.02$).

5. DISCUSSION

These results showed that HCC produced from OA chondrocytes on a scaffold of HYAFF®-11 synthesized a sulfated-proteoglycan and Type II collagen-containing ECM. These results suggested that even if chondrocytes derived from the remaining cartilage of OA joints contained the fibrocartilaginous or hypertrophic cartilage markers of Type I or Type X collagen proteins, respectively, *in situ* (10), these collagen isotypes were down-regulated or suppressed by the tissue-engineering strategies employed in this study. In this regard, recent evidence suggested that HYAFF®-11 up-regulated Type IIB collagen mRNA (i.e. adult Type II collagen) by human chondrocytes derived from the epiphyseal cartilage of 3 subjects (avg. age, 71.7 years) (11) but these cartilage-constructs also transcribed Type I and Type X collagen mRNA as evidenced by RT-PCR analysis. In that study (11), immunohistochemistry confirmed the presence of the Type II collagen isotype in the pericellular ECM, but the presence or absence of the Type I and Type X collagen isotypes were not reported. Further studies are indicated to determine whether the activity of the Type X collagen transcription factor Runx2/Cbfa1 (12) or other related factors were down-regulated and may have contributed to Type X collagen gene suppression in HCC/HYAFF®-11.

The application of 5MPa static pressure for 4 hrs was previously shown to significantly increase proteoglycan synthesis when bovine chondrocytes were maintained in agarose gels. Pulsatile loading applying the identical pressure and sinusoidal frequency employed in the present study tended to decrease proteoglycan synthesis, but not significantly (13). In a similar manner, although static compression applied for 30 min initially increased aggrecan and Type II collagen mRNA levels in bovine cartilage explants, by 4-24 hrs, aggrecan and Type II collagen synthesis had significantly decreased (14). In HCC/HYAFF®-11, there was no indication that metachromasia or the intensity of anti-Type II collagen staining was altered by CHP after 4 hrs (data not shown).

We previously showed that CHP (5 MPa, 1 Hz sinusoidal frequency) applied for 4 hrs induced apoptosis in human chondrocyte suspension cultures that was characterized by up-regulation of p53 and c-myc as well as Bax-α and suppression of Bcl-2 (6). Although apoptotic cells were identified in control HCC and the apoptosis frequency was higher than previously reported in control chondrocyte suspension cultures (6), the level of apoptosis measured in the HCC not subjected to pressure as assessed by monoclonal F7-26 immunostaining was similar to the apoptosis frequency reported in native OA cartilage (i.e. 20 - 25%) by either the terminal deoxynucleotidyl transferase mediated dUTP nick-end labeling (TUNEL) or annexin-V methods (15).

The present study did compare TUNEL, annexin-5 and F7-26-based apoptotic nuclei enumeration techniques. Several *in vitro* studies employing human cartilage (16, 17) questioned the reliability of the TUNEL

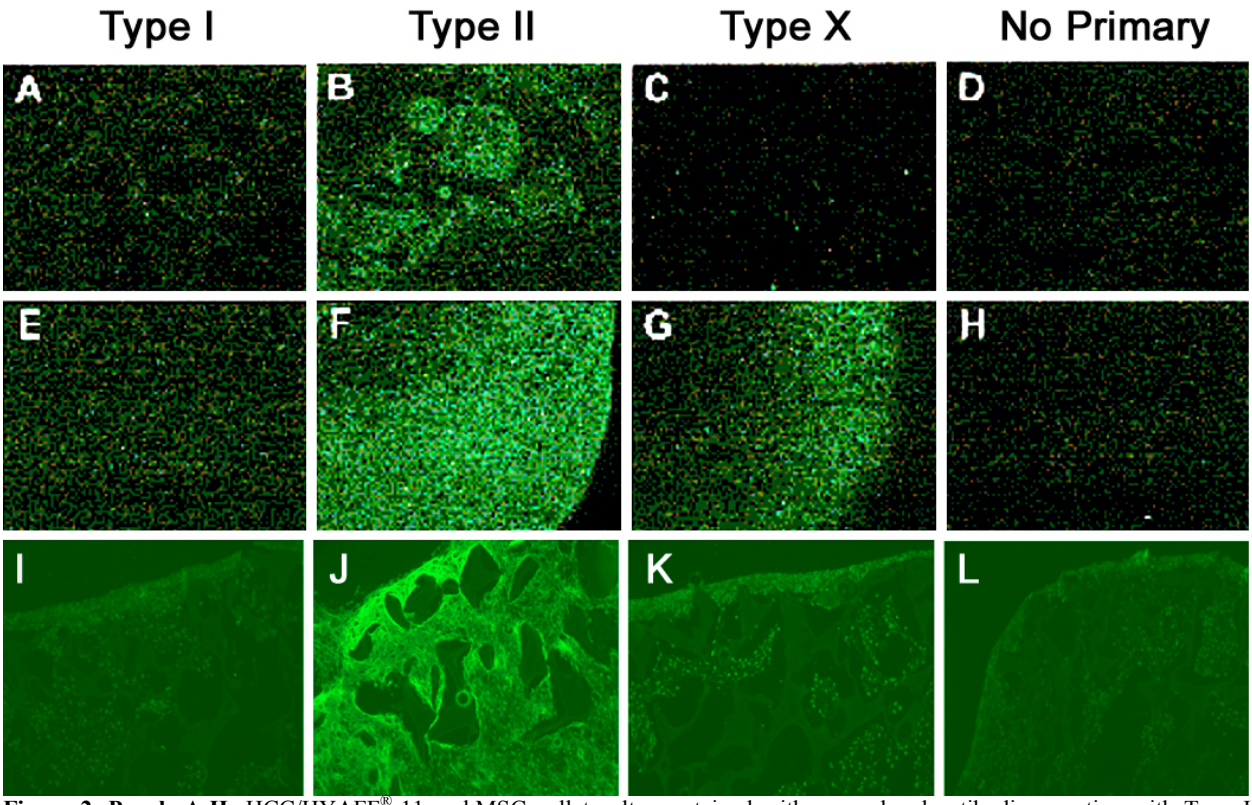

Figure 2. Panels A-H: HCC/HYAFF®-11 or hMSC pellet cultures stained with monoclonal antibodies reactive with Type I collagen (A and E), Type II collagen (B and F) and Type X collagen (C and G) or HCC/HYAFF® or hMSC pellet cultures without primary monoclonal antibody (D and H). Retrieved implant after 3 wks: Eight micron paraffin section of HCC/HYAFF®-11 retrieved from a nude mouse. hMSC pellet after 3 wks: Eight micron paraffin section of hMSC pellet culture harvested after 3 wks. **Panels I-L:** Eight micron paraffin-embedded section of HCC/HYAFF®-11 maintained in a perfusion bioreactor for 3 wks and stained with monoclonal antibodies reactive with Type I collagen (I), Type II collagen (J), Type X collagen (K) or without primary antibody (L).

Table 1. CHP Increases Apoptotic Chondrocytes in HCC

Group	% $DAPI^+/F7\text{-}26^+$ Cells	Mean ± SD	*p* Value
Control	60/12	28.4 ± 8.4	
	31/8		
	70/24		
	75/30		
	50/12		
CHP	59/23	41.0 ± 4.2	$p < 0.02$
	55/26		
	53/23		
	21/9		
	76/30		

CHP was applied to HCC at 5 MPa and 1 Hz sinusoidal frequency for 4 hrs in a servopneumatic pressure device as previously described (5, 6). Control HCC were maintained in the device for 4 hrs but not subjected to pressure (6). Eight micron paraffin-embedded HCC sections were stained either with DAPI or with monoclonal antibody F7-26. The percentage of apoptotic cells (i.e. apoptosis frequency) was calculated by counting 5 microscopic fields at 10X magnification using a DAPI/F7-26 merge technique as previously described (9).

method for detecting chondrocyte apoptosis. At the very least, evidence of chondrocyte apoptosis by the TUNEL method appeared to give artificially high rates of apoptosis in normal and OA human cartilage (16). Studzinski and Benjamins (9) showed that F7-26 immunostaining of single-stranded DNA in apoptotic nuclei of oligodendroglial cells induced by staurosporine after treatment with cyclic AMP correlated with DNA laddering consistent with apoptosis as well as caspase-3 activation. We previously showed that apoptosis induced by CHP correlated with evidence of DNA laddering, caspase-3 activation and poly-ADP-ribose polymerase degradation (6), a substrate for caspase-3. In the present study, CHP significantly increased the apoptotic frequency of chondrocytes in HCC/HYAFF®-11. The extent to which CHP modulated the expression of mitochondrial proteins, Bcl-2 and Bax-α that are critical to the apoptosis cascade is currently being evaluated.

HCC produced from OA chondrocytes cannot be expected to fully recapitulate the topographical distribution of human chondrocytes found in normal articular cartilage. Specifically, morphological and Type II collagen differences (18, 19) as well as proteoglycan and lubricating glycoprotein (20) differences have been identified in the superficial or tangential zone compared to the intermediate zone and basal zone of normal human articular cartilage. Perhaps the apparent failure to demonstrate morphological variation in these HCC may also be related to the putative

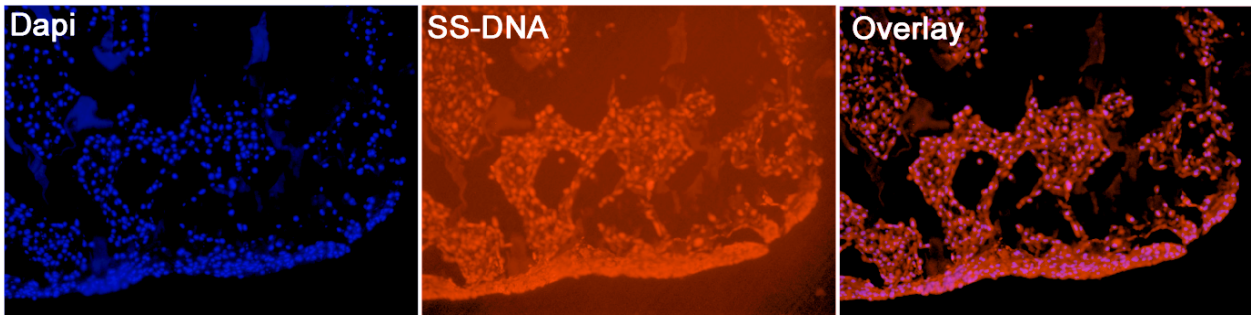

Figure 3. HCC/HYAFF®-11 stained with DAPI (Dapi) or monoclonal antibody F7-26 reactive with single-stranded DNA (SS-DNA). DAPI/F7-26 sections were merged (Overlay) to determine the frequency of apoptotic nuclei in 5 representative fields.

erosion of the uppermost superficial zone in the "resident" cartilage sampled from human OA joints (18) so that the eventual ECM structure of HCC produced under these conditions basically reflected the contribution of chondrocytes derived principally from the growth or intermediate zone. Nevertheless, the ECM composition of HCC produced under these conditions may be adequate in structure and composition for them to be employed as implants. Only experimental biomechanical and transplantation studies will determine their fitness for that purpose.

In summary, these results showed that although HCC could be produced from the "resident" chondrocytes isolated from human OA cartilage, their suitability for repairing lesions in the weight-bearing zones of articular cartilage in OA will require further study. Our results are in contrast to the results of other studies that suggested that CHP had a strong, and in some cases, transient positive effect on hMSC and chondrocyte anabolic metabolism (5, 13, 14). Further, our findings indicated that the increased susceptibility of chondrocytes derived from OA cartilage to damage by CHP as evidenced by the increased apoptosis frequency is substantially retained in HCC. Although the ECM elaborated by chondrocytes within the developing HCC is biochemically similar to that of normal cartilage, the 3-dimensional architecture of the HCC ECM is essentially isotropic which is in sharp contrast to the highly organized ECM of normal cartilage. The extent to which selective modifications in the HCC ECM may be achieved or progressive adaptation to increased cyclic or intermittent pressures and manipulation of HCC by transient over-expression of anti-apoptosis genes could result in apoptosis-resistance must be assessed *in vitro* prior to their use in experimental implant protocols. In addition, the effect of adding exogenous active matrix metalloproteinases to HCC prior to CHP should also be evaluated since increased apoptotic frequency was demonstrated in zones of human cartilage degeneration in OA (21). These assessments are likely to be critical for determining the suitability of HCC for cartilage repair in human clinical trials.

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Send Correspondence to: Charles J. Malemud, Ph.D. Professor of Medicine and Anatomy, Case Western Reserve University School of Medicine, 2061 Cornell Road, Room 207, Cleveland, OH 44106-5076, Tel: 216-844-7846, Fax: 216-844-2288, E-mail: cjm4@cwru.edu

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