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Culture expanded primary chondrocytes have potent immunomodulatory properties and do not induce an allogeneic immune response

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SUMMARY

Objective: Allogeneic cell therapies, such as mesenchymal stromal cells (MSC), which have potent regenerative and anti-inflammatory potential are being investigated as a therapy for osteoarthritis (OA) and cartilage injury. Here we describe another potential source of regenerative and anti-inflammatory allogeneic cells, culture expanded primary chondrocytes (CEPC). In direct comparison to allogeneic MSC, we extensively assess the immunological interactions of CEPC in an allogeneic setting.

Methods: Chondrocytes were isolated from rat articular cartilage and cultured in normoxic or hypoxic conditions. *In vitro* co-culture assays with allogeneic lymphocytes and macrophages were used to assess the immunomodulatory capacities of the chondrocytes, followed by immune response analysis by flow cytometry, ELISA and qPCR.

Results: CEPC showed reduced induction of proliferation, activation and cytotoxic granzyme B expression in allogeneic T cells. Importantly, exposure to pro-inflammatory cytokines did not increase CEPC immunogenicity despite increases in MHC-I. Furthermore, CEPC had a potent ability to suppress allogeneic T cell proliferation, which was dependent on nitric oxide production. This suppression was contact independent in hypoxia cultured CEPC. Finally, chondrocytes were shown to have the capacity to modulate pro-inflammatory macrophage activity by reducing MHC-II expression and TNF-α secretion.

Conclusion: These data indicate the potential use of allogeneic chondrocytes in OA and cartilage defects. The lack of evident immunogenicity, despite exposure to a pro-inflammatory environment, coupled with the immunomodulatory ability indicates that these cells have the potential to evade the host immune system and suppress inflammation, thus potentially facilitating the resolution of OA induced inflammation and cartilage regeneration.

Introduction

Cartilage injury and osteoarthritis (OA) are common joint conditions, with 25% of people over 55 reporting knee pain annually with no curative therapy available[1,2]. Due to the debilitating nature of OA, coupled with the poor regenerative capacity of articular cartilage, treatments with long-term efficacy are required[3,4]. Because of its unique composition, being avascular and composed of up to 70% water with sparsely dispersed chondrocytes, recapitulation of articular cartilage is a significant challenge[3].

Due to their inherent synthesis of cartilage matrix, articular chondrocytes are potentially an optimal cell type for cartilage regeneration. Indeed, Autologous Chondrocyte Implantation (ACI) is the current gold standard cell therapy for cartilage defects[5]. ACI involves expanding chondrocytes from a non-weight bearing

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region of the joint[2]. These cells are injected into the defect under a collagen or periosteal flap or implanted in a matrix (MACI)[2]. ACI shows good to excellent results on follow up with outcomes reported as being equivalent or superior to microfracture[2,4,6].

However, autologous therapies require tissue to be explanted from a healthy area of cartilage[2,4], raising the possibility of donor site morbidity. 70% of patients have been reported to have reduction in donor knee function after 12 months indicating that this may be a significant concern[7]. In addition to donor site morbidity, two separate procedures are required for ACI which increases patient discomfort and overall costs. An allogeneic chondrocyte therapy would remove the risk of donor site morbidity, lower patient risk and reduce costs while maintaining the regenerative component of the therapy.

However, as with any allogeneic therapy an understanding of the immunological consequences is required for successful translation to the clinic[8]. To address immunogenicity (capacity to induce anti-donor adaptive immune responses) this characterisation should include: (a) Expression of histocompatibility antigens and co-stimulatory molecules such as MHC-I, MHC-II, CD80 and CD86 which are required for T cell allo-recognition[8,9], (b) Characterisation of donor-specific T cells responses in *in vitro* stimulation assays, (c) Assessment of the *in vivo* allo-immune response to transplanted cells in appropriate pre-clinical models. As OA has a significant immune-mediated component, any potential modulation of the immune cells by the allogeneic cellular therapy should also be investigated.

The avascularity of articular cartilage is important for its mechanical integrity, as its strength would be compromised by a fragile vasculature[10]. Avascularity also means that articular cartilage is hypoxic, with partial pressure of oxygen (pO_2) between 1% and 10%[10–12]. Hypoxia is important for the normal function of chondrocytes, including regulation of chondrocyte specific genes such as Sox9, collagens and aggrecan through hypoxia-inducible factors (HIFs)[10,11,13] and the response of chondrocytes to inflammatory stimuli[14]. As chondrocytes naturally reside in a hypoxic environment, the immunologically relevant effects of pro-inflammatory stimuli on these cells under hypoxic conditions is of particular importance.

We have recently examined one allogeneic cell type, the differentiated allogeneic mesenchymal stromal cell (MSC). We found that chondrogenically differentiated MSC lose their immunosuppressive ability, take on a more immunogenic phenotype and are recognised by the host immune system in a rat subcutaneous implantation model[9]. As cultured autologous primary chondrocytes have been shown for over 20 years to produce effective cartilage providing a benefit to the patient[5,6,15], we have sought to investigate the potential for culture-expanded allogeneic primary chondrocytes to be therapeutically adapted in the absence of concomitant immunosuppressive therapy. Here, we have comprehensively investigated the basic immunological characteristics of culture-expanded rat primary chondrocytes (CEPC) under normoxic (CEPC-N) and hypoxic (CEPC-H) conditions. We show that CEPC maintain chondrogenic potential in monolayer. Using fully allogeneic rat strains, we demonstrate that CEPC do not induce T cell activation *in vitro*, even following pre-exposure to inflammatory stimuli. Furthermore, we find that CEPC are capable of modulating an ongoing T cell proliferative response through the release of nitric oxide (NO) and they are capable of modulating the inflammatory activity of allogeneic macrophages. Our results also indicate that CEPC-H may be more effective than CEPC-N, as they are capable of non-contact mediated immunosuppression. This study highlights the potential utility of allogeneic CEPC for use in inflammatory degenerative joint conditions such as OA and after injury to cartilage.

Method

Animals

Dark Agouti (DA) and Lewis (LEW) rats aged from 6 to 12 weeks were obtained from Harlan Laboratories UK and housed in an accredited animal facility. All procedures were approved by the Animal Care Research Ethics Committee of the National University of Ireland, Galway (Filing ID 011/11), under licence from the Irish Department of Health.

CEPC and MSC isolation and culture

Unless otherwise stated, all reagents were purchased from Sigma–Aldrich (Dorset, UK). Male DA rats were euthanized, femurs removed and transferred to a tissue culture hood. MSC were isolated from the bone marrow of femurs and tibias as previously described[16] and cultured in 19% O_2. MSC were passaged when they reached 85–90% confluency. The articular cartilage was scraped from the condyles of the knees and femoral heads. Cartilage chips were incubated with 2 mg/ml protease in serum free α-minimum essential medium (α-MEM) at 37°C for 90 min. Chips were washed twice with PBS and incubated with 1.5 mg/ml collagenase D (Roche, Mannheim, Germany) in serum free α-MEM for 18 h at 37°C. The digested cartilage chips and released cells were washed twice with PBS and plated in T-75 culture flasks in 19% (CEPC-N) or 2% (CEPC-H) oxygen at 37°C. MSC, CEPC-N and CEPC-H were cultured in tissue culture medium composed of a 1:1 mixture of α-MEM with L-glutamine and F-12 supplement mixture (both Life Technologies, Dublin, Ireland) with 10% foetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. 2% oxygen culture conditions were maintained in a New Brunswick Galaxy 170R incubator (Mason Technologies, Dublin, Ireland) (incubator set to 37°C, 5% CO_2, 2% O_2). In all experiments CEPC were used between passage 2 and 4. CEPC were cultured in T-175 flasks from passage 1 onwards and passaged when they reached 90% confluency. At least three CEPC (3–8 animals/isolation) and MSC isolations (8 animals/isolation) were used throughout the study. In each experiment, at least three independent lymphocyte donors were used.

Cell surface characterisation

Cell surface protein expression on MSC, CEPC-N and CEPC-H was quantified by flow cytometry with fluorochrome-conjugated monoclonal antibodies: mouse anti-rat MHC-I-FITC (AbD Serotec), mouse anti-rat MHC-II-PE, mouse anti-rat CD80-PE, mouse anti-rat CD86-FITC, mouse anti-rat CD90-PE, hamster anti-rat CD73-PE, mouse anti-rat CD45-PE, mouse anti-rat CD73-PE (all BD Biosciences, Oxford, UK) and mouse anti-rat CD44H-PE (eBioscience, Hatfield, UK). Data was acquired on a BD FACS Canto flow cytometer and analysed using FlowJo (FlowJo, Oregon, USA).

Lymphocyte co-culture assays

Immunogenicity of MSC, CEPC-N and CEPC-H was assessed by co-culturing with allogeneic lymphocytes for 5 days, followed by flow cytometric analysis of proliferation, activation marker and Granzyme B expression[9]. Contact dependent and independent immunosuppressive ability was assessed in similar assays with anti-CD3/CD28 stimulated lymphocytes for 4 days. A detailed outline of these experiments can be found in the Supplementary materials and methods.