Alloreactivity and immunosuppressive properties of articular chondrocytes from osteoarthritic cartilage

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Alloreactivity and immunosuppressive properties of articular chondrocytes from osteoarthritic cartilage

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ABSTRACT

Purpose. To determine whether articular chondrocytes derived from osteoarthritic knee joints could evoke alloreactive proliferation of peripheral blood mononuclear cells (PBMC) and inhibit mitogenic activity of polyclonally activated CD4+ major histocompatibility complex (MHC) class II–restricted T cells in vitro.

Methods. Osteoarthritic cartilages of 17 patients aged 61 to 85 years were harvested during total knee arthroplasty. Chondrocytes were cultured for experiments. PBMCs, CD4+ T cells, CD8+ T cells, and CD14+ monocytes from healthy subjects were also used. To investigate the allogeneic response and immunosuppressive properties of chondrocytes, assays for one-way mixed lymphocyte reaction (MLR), apoptosis, activated CD4+ T-cell proliferation, and cytotoxic CD8+ T-cells were performed. Chondrocyte cell-surface antigens were examined using flow cytometry.

Results. Chondrocytes failed to trigger an allogeneic PBMC reaction and did not induce apoptosis of allogeneic PBMCs in the MLR assay. Chondrocytes inhibited the proliferation of polyclonally activated CD4+ T cells via cell-cell contact and escaped the allogeneic cytotoxic reactivity of CD8+ T cells. Chondrocytes expressed MHC class I but not MHC class II molecules or B7-1/-2−positive co-stimulatory molecules.

Conclusion. Chondrocytes from osteoarthritic knees in older patients exhibited similar immunomodulatory properties in vitro to those in juveniles or adults.

Key words: chondrocytes; immune system; osteoarthritis

INTRODUCTION

Articular cartilage injury can progress to irreversible degradation and osteoarthritis if left untreated. Fresh-frozen osteochondral allografts can be used to repair large articular cartilage defects. Allogeneic or xenogeneic whole cartilage can remain viable for a long period but is eventually rejected. This may be due to the lack of vessels and lymph ducts, as well as the thick extracellular matrix that prevent host immune recognition and contact with immunocompetent
cells. Allogeneic juvenile cartilage (DeNovo NT, Zimmer, Warsaw [IN], USA) and allogeneic juvenile 3-dimensional neocartilage (RavaFlex/DeNovo ET, ISTO Tec, St Louis [MO], USA) have been used for cartilage repair,5–7 but their immune response remains unclear. The use of autologous chondrocyte implantation (ACI) is limited by the waiting period for cell culture and low cell number and density for large defects or subsequent surgeries. Allogeneic chondrocyte implantation can solve these problems, but the immunogenicity of chondrocytes remains controversial. Human articular chondrocytes suppress proliferation of activated peripheral blood mononuclear cells (PBMC),8 and are non-alloreactive in an mixed lymphocyte reaction (MLR) assay under serum-free conditions, and exert immune modulatory functions to inhibit the proliferation of polyclonally activated CD4+ T cells via cell-cell contact.9–13 These chondrocytes express negligible levels of major histocompatibility complex (MHC) class II molecules and lack B7-1 and B7-2 antigens, which are positive co-stimulators of CD4+ T-cell proliferation.9–13 This suggests that the presence of negative co-stimulatory molecules is indicative of immune privilege in chondrocytes. Nonetheless, expression of MHC molecules by transplanted allogeneic chondrocytes is upregulated at an early stage, and the tissue-fluid environment in vivo affects their immunological properties.14

This study aimed to determine whether articular chondrocytes derived from osteoarthritic (OA) knee joints could evoke alloreactive proliferation of PBMCs and inhibit mitogenic activity of polyclonally activated CD4+ MHC class II–restricted T cells in vitro.

MATERIALS AND METHODS

This study was approved by the ethics committee of our hospital (No.162-1400).

Chondrocyte culture

OA cartilages of 17 patients aged 61 to 85 years who had International Cartilage Repair Society grade 0 or 1 OA knees were harvested during total knee arthroplasty. The cartilage was minced and digested by sequential treatment with Pronase for 90 minutes, and then incubated overnight in HL-1 medium (BioWhittaker) with 0.5 mg/ml CLS4 collagenase (Worthington), 0.3 mg/ml hyaluronidase (type IV hyaluronidase; Sigma), and 50 μg/ml ascorbic acid. After enzymatic dissociation, the chondrocytes were cultured in Dulbecco’s Modified Eagle’s Medium containing 10% foetal bovine serum and used for experiments at passage 1 or 2.

Purification of CD14+ monocytes and CD4+CD14+ T cells

PBMCs from healthy volunteers were isolated and enriched using Histopaque-1077 (Sigma). CD4+CD14+ T cells (CD4+ T cells), CD8+ T cells, and CD14+ monocytes were isolated using the magnetic-activated cell sorting system, and microbeads conjugated to anti-human CD4, CD8, and CD14 mAbs, respectively.

Mixed lymphocyte reaction assay

Alloreactivity was examined using the MLR assay. Chondrocytes were stimulated by irradiation with 3000 rad. As a positive control for stimulation, CD14+ monocytes were irradiated with 1500 rad, and then stimulated with 200 U/ml of interferon-γ and 1 μg/ml of lipopolysaccharide as described previously.15 Next, 4×10^3, 2×10^4, or 1×10^5 stimulator cells and 5×10^5 PBMCs (as responder cells) were co-cultured in triplicate for 5 days in 96-well flat bottom plates with AIM-V serum-free media (BioWhittaker). [3H] thymidine (1 μCi/well; Amersham) was added to the culture medium for the last 18 hours, and lymphocyte (responder cell) proliferation was measured based on radiolabel incorporation. Results were expressed as counts per minute, and a stimulator index of >10 was considered a positive response.

Cell-cycle analysis by flow cytometry

Cell-cycle analysis was performed by propidium iodide staining followed by flow cytometry. PBMCs were fixed with ice-cold 70% ethanol and resuspended in 300 μl of phosphate-buffered saline containing 200 μg/ml RNase A and 25 μg/ml propidium iodide staining. The mixture was incubated at room temperature in the dark for 30 minutes. Nuclear changes were detected by flow cytometry on the FACSCalibur (Becton Dickinson). Analysis was made using the CellQuest software. Cells that exhibited DNA fragmentation were considered to be dead.

Activated lymphocyte proliferation assay with or without cell-cell contact

To measure the immunosuppressive properties of chondrocytes, purified CD4+ T cells were activated by simultaneous ligation of the T-cell receptor complex and co-stimulatory molecules with 10 ng/ml CD3 mAb and 5 μg/ml CD28 mAb as previously
described. Irradiated chondrocytes were co-cultured with activated lymphocytes at responder/stimulator ratios ranging from 1/1 to 25/1. After 3 days of co-culture, proliferation of activated lymphocytes was measured using the MLR assay. To disrupt cell-cell contact, chondrocytes and activated CD4+ T cells were co-cultured separately in the Transwell system for 3 days, after which proliferation of activated CD4+ T cells was measured in a similar manner.

**Cytotoxicity assay**

Allogeneic PBMCs and CD8+ T cells were prepared as effector cells. Chondrocytes were labelled with 51Cr for 4 hours and used as target cells. Effector and target cells were incubated in triplicate cultures in the AIM-V medium at effector/target cell (E/T) ratios of 5/1 or 1/1. Briefly, 1x10^4 target chondrocytes were incubated with 5x10^5 PBMCs (E/T ratio, 5/1) or 1x10^5 CD8+T cells (E/T ratio, 1/1). After a 4-hour incubation, supernatants were collected, and the amount of 51Cr released from targets destroyed by effectors was measured using a gamma counter. To measure the amount of 51Cr that was spontaneously released from the targets, chondrocytes were incubated alone in medium. To determine the maximum isotope that could be released from the target cells, chondrocytes were incubated in medium containing 1% Triton X-100. In general, maximum release should be at least 3 times greater than spontaneous release.

**Flow cytometry**

To examine MHC class I and II expression on chondrocytes, cell-surface molecules were examined by immunofluorescence. FITC-conjugated mouse anti-human HLA-ABC mAb (IOTets), purified mouse anti-human HLA-DR, DP, and DQ mAbs (BD Pharmingen), and fluorescein-conjugated goat anti-mouse IgG (Meloy Laboratories) were used. To confirm expression of B7-1 (CD80) and B7-2 (CD86) antigens on chondrocytes, FITC-conjugated mouse anti-human CD-80 mAb (IOTets) or PE-conjugated mouse anti-human CD-86 mAb (IOTets) was used. Non-specific mouse IgG (R&D Systems) was used as an isotype-specific negative control. Quantitative flow-cytometric analyses were performed on the FACSCalibur. A total of 10000 live cells were examined, and data were analysed using the CellQuest software.

**Statistical analysis**

Comparisons were made using the Student’s t-test. A p value of <0.05 was considered statistically significant.
RESULTS

Chondrocytes fail to elicit an allogeneic response

In contrast to the potent stimulatory capacity of CD14+ cells, chondrocytes failed to elicit a dose-dependent positive reaction (Fig. 1). This indicated that chondrocytes could elude immune surveillance at a responder/stimulator ratio of 1/5.

Chondrocytes not induce apoptosis of allogeneic PBMCs

Based on their DNA content, cells were categorised as M1 (apoptotic), M2 (G0/G1 phase), or M3 (S to M phase). Their respective percentages were 5.9±0.1%, 84.3±0.4%, and 1.1±0.2% for PBMCs cultured alone, and 0.8±0.2%, 93.6±2.8%, and 0.7±0.5% when 5×10^5 PBMCs were co-cultured with 1×10^5 chondrocytes (Fig. 2). The percentages of PBMCs in respective categories did not differ significantly in cells cultured with or without chondrocytes, nor did the percentages of apoptotic cells. This indicated that OA chondrocytes did not induce apoptosis of allogeneic PBMCs in co-culture.

Chondrocytes inhibit proliferation of activated CD4+ T cells via cell-cell contact

Juvenile and adult chondrocytes suppress immune responses in a dose-dependent manner in vitro.\textsuperscript{12,15} Purified CD4+ T cells were activated by cross-linking the T-cell receptor/CD3 complex with anti-CD3 mAb and the B7-1/B7-2 receptor (CD28) with anti-CD28 mAb. This combination of antibodies strongly activated CD4+ T cells, which then proliferated. Its proliferation ratio (the rate of proliferation in the presence of chondrocytes divided by that without chondrocytes) was >1 (accelerated) when 1×10^5 activated CD4+ T cells were cultured with a smaller number (4×10^4 or 2×10^4) of chondrocytes, and <1 (inhibited) when 1×10^5 activated CD4+ T cells were cultured with an equal number of chondrocytes (Fig. 3). When there was no cell-cell contact, CD4+ T cell proliferation was restored (proliferation ratio >1). This indicated that OA chondrocytes inhibited proliferation of activated CD4+ T cells in vitro via a cell-cell contact mechanism. Chondrocytes might also secrete a soluble factor that promoted T-cell proliferation because of the proliferation ratio >1.

Chondrocytes fail to elicit allogeneic CD8+ T cell reactivity

CD8+ T cells are cytotoxic and directly destroy their target cells. When 1×10^5 chondrocytes were incubated with 5×10^5 PBMCs (E/T ratio, 5/1) or 1×10^5 CD8+ T cells (E/T ratio, 1/1) for 4 hours, the rate of release of ^51Cr from chondrocytes was not significantly higher than the rate of spontaneous release from chondrocytes cultured alone (Fig. 4). This indicated that chondrocytes failed to elicit allogeneic CD8+ T-cell reactions and eluded MHC class I–restricted immune surveillance.

Chondrocytes express MHC class I antigens

Flow-cytometric analysis of 10 samples demonstrated that 80.7±8.8% of OA chondrocytes expressed MHC class I antigens—HLA-ABC, whereas only 1.9±1.5% of
the chondrocytes expressed MHC class II antigens—HLA-DP, -DQ, and -DR, and only 0.4±0.7% and 0.3±0.5% of the chondrocytes expressed B7-1 (CD80) and B7-2 (CD86), respectively (Fig. 5).

**DISCUSSION**

Articular chondrocytes function as accessory cells for immune cells. Alloreactivity of chondrocytes remains unclear. Articular chondrocytes express molecules involved in heterotypic cell-cell interactions such as ICAM-1 and VCAM-1, MHC class I antigens, and MHC class II antigens under specific conditions, suggesting that articular chondrocytes can function as accessory cells for immune cells. If articular chondrocytes function as accessory cells, xenogeneic antigens in the serum could affect the MLR assay that used medium containing xenogeneic bovine serum. To avoid these potential effects, serum-free conditions are recommended.

In our study, serum-free medium was used in MLR assays. To investigate immunomodulatory function, activated lymphocyte proliferation assays were used to compare direct co-culture versus Transwell cultures. OA chondrocytes inhibited proliferation of activated CD4⁺ T cells via cell-cell contact. This finding is consistent with that for juvenile and adult healthy chondrocytes in previous studies. In contrast to the situation with juvenile cells, a large number of OA chondrocytes (chondrocyte/T-cell ratio of 1/1) was required to inhibit the proliferation of active CD4⁺ T cells. The contact requirement indicated that the surface molecules of chondrocytes play an important role in immunomodulatory function. We propose 3 potential mechanisms: (1) the presence of an inhibitory cell-surface molecule, (2) extracellular matrix production by chondrocytes results in transduction of negative proliferation signals to CD4⁺ T cells, and (3) secretion of an extracellular matrix-binding protein by chondrocytes that negatively affects CD4⁺ T-cell proliferation (Fig. 6).
To distinguish these mechanisms, cell-surface molecules were examined by flow cytometry. CD8<sup>+</sup> and CD4<sup>+</sup> T cells are respectively restricted by MHC class I and MHC class II. The inhibition of proliferation of activated CD4<sup>+</sup> T cells could be due to their negligible expression of MHC class II and B7-1/-2. Even when MHC class II or B7-1 and B7-2 were transfected into mesenchymal stem cells (MSCs), they still failed to elicit an allogeneic T-cell reaction. These results indicated that the lack of MHC class II and B7-1/-2 was not critical to the immunomodulatory mechanism. Immune-inhibitory B7 molecules such as B7-DC, B7-H1, and B7-H4 are expressed in both juvenile and adult chondrocytes.<sup>9–13</sup> Chondrocytes express other negative regulators of immune responses, including chondromodulin-I and indoleamine 2,3 dioxygenase (IDO).<sup>9</sup> In our study, the percentage of apoptotic lymphocytes did not increase, whereas the percentage of PBMCs in each category did not differ significantly (p>0.05) in cells cultured

**Figure 5** Flow-cytometric assay: filled bars represent (a) HLA-ABC; (b) HLA-DP, -DQ, and -DR; (c) CD80; and (d) CD86 staining. Open bars represent isotype control staining and the negative control.

**Figure 6** Potential mechanisms of inhibition by articular chondrocytes: (a) surface molecules on chondrocytes transduce signals to CD4<sup>+</sup> T cells that negatively influence proliferation; (b) extracellular matrix produced by chondrocytes transduces negative proliferation signals to CD4<sup>+</sup> T cells; and (c) extracellular matrix–binding protein secreted by chondrocytes negatively affects CD4<sup>+</sup> T-cells proliferation.
in the presence or absence of chondrocytes. Therefore, the surface molecules that induce lymphocyte apoptosis and destruction, such as B7-DC and B7-H1, are not primary candidates for immunomodulation produced by chondrocytes.

Intramuscularly transplanted cartilage in syngeneic rats is rejected in sensitised recipient rats previously exposed to allogeneic chondrocytes. Cartilage fragments generated from syngeneic rat chondrocytes and transplanted into the articular surface are rejected in rats sensitised with allogeneic chondrocytes. The synovium around the rejected fragment contains CD8+ lymphocytes and macrophages. This indicates that chondrocyte-specific antigens and CD8+ T cells may play a role in rejection. Nonetheless, in our study, OA chondrocytes failed to elicit allogeneic PBMC and CD8+ T-cell reactivity (E/T ratios of 1/5 and 1/1). Chondrocytes could elude MHC class I-restricted immune surveillance. Natural killer cells may also exert cytotoxic functions.

Transplantation of isolated allogeneic chondrocytes evokes a specific humoral and cellular immunological response. Infiltrating immune cells on the subchondral side of the transplant gradually destroy the reformed cartilage. Species and the technical details of chondrocyte isolation might influence the immunological response of the host animals. The immune privilege of articular cartilage depends on the donor source and transplant location. Therefore, to assess the safety of allogeneic chondrocyte implantation, additional in vivo studies are needed.

MSCs have immunomodulatory properties, including the ability to reduce immune cell infiltration and modulate inflammation. Their immunosuppressive ability is attributed to the secretion of immunomodulatory molecules, including prostaglandin E2, nitric oxide, IDO, and TGF, that suppress the proliferation and activation of immune cells including CD4+ T, CD8+ T, natural killer, B, and antigen-presenting cells. Allogeneic mesenchymal stromal cells have been investigated in the context of cartilage repair. Nonetheless, chondrogenic differentiation increases the immunogenicity of MSCs, and there is an increase in their expression of MHC class I, class II, and B7-1/-2. Therefore, the safety of allogeneic MSC transplantation for cartilage repair remains in doubt.

This study had limitations. Traditional methods were used, and the error of the thymidine uptake assay is larger than that of the MTT assay, as cells may be lost during collection in the thymidine uptake assay. Furthermore, blast cells fail to incorporate sufficient amounts of 51Cr and/or spontaneously release high levels of 51Cr. The lactate dehydrogenase-release assay should have been used as a cytotoxic assay as in a previous study. Negative controls using autologous lymphocytes may have enabled a more straightforward conclusion if ethic approval was obtained.

CONCLUSION

Cultured OA chondrocytes (1) expressed MHC class I but not MHC class II or positive co-stimulatory molecules, (2) failed to evoke both MHC class I and II mismatch allogeneic lymphocyte responses, and (3) inhibited proliferation of activated CD4+ T cells via cell-cell contact. Therefore, chondrocytes preserved their immunological characteristics in vitro, regardless of ageing or degenerative condition.

DISCLOSURE

No conflicts of interest were declared by the authors.

REFERENCES


