Preliminary insights into temporal expression patterns of α10β1, α5β1, and α2β1 integrins in freshly isolated chondrocytes: potential impacts on cartilage repair strategies

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SUMMARY

Autologous chondrocyte implantation is a cell-based therapeutic strategy for cartilage repair. This strategy is limited by the dedifferentiation of chondrocytes during culturing. Integrins, particularly α10β1, have emerged as potential markers for selecting chondrocytes with enhanced chondrogenic capacity. This study aims to explore the expression patterns of α10β1/CD11c+CD29, α5β1/CD49e+CD29, and α2β1/ CD49b+CD29 integrins in freshly isolated chondrocytes, thus potentially providing alternative strategies in improving ACI outcomes. Cartilage samples were collected from three osteoarthritic knee joints after obtaining consent. Chondrocytes were isolated via enzymatic digestion and assessed via fluorescence-activated cell sorting for integrin expressions CD-11c+CD29+, CD49e+CD29+, and CD49b+CD29+ immediately and at specific time points (0, 20-, 40-, 60-, 90-, and 120-minutes post-isolation).

Flow cytometry analysis of freshly isolated chondrocytes showed that CD11c+CD29+ expression was high (79.22±6.73%) and progressively and significantly declined (P<0.01), reaching near-zero levels by 120 minutes. CD49e+CD29+ maintained stable expression (91.67±3.34%) throughout the time course, whereas CD49b+CD29+ decreased by 60 minutes. This study highlights the rapid dedifferentiation observed with chondrocytes and, particularly, the CD11c+CD29+ levels, a critical collagen-binding receptor with a high affinity for collagen type II, the primary structural protein in cartilage. If there is a need to use sorting to identify and obtain these cells which potentially will have a higher capacity for chondrogenesis, one must sort these cells as soon as they are released. Future research focusing on α10β1 expression and its impact on cartilage repair could offer valuable insights into developing more efficacious cell-based therapeutics for cartilage pathologies.

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INTRODUCTION

Autologous chondrocyte implantation (ACI) is widely utilized as a cell-based therapeutic approach for repairing cartilage, attributed to the tissue's minimal self-repair capabilities (Brittberg, 2021). ACI, a surgical procedure to treat chondral defects, involves harvesting the patient's cartilage tissue, enzymatically digesting them to release chondrocytes, which are cultured to achieve cell number, and then implanting back into the defect, often covered by a biological membrane (Migliorini et al., 2022). The fresh chondrocytes, once released, undergo a phenotypic drift in terms of surface marker and gene expression, with changes heightened following monolayer expansion (Diaz-Romero et al., 2008; Hamada et al., 2013; Vinod et al., 2018). Retrospective analysis of patients who have undergone ACI shows positive outcomes, with reports indicating the presence of a fibrocartilage admixture repair rather than only hyaline-like repair (Armiento et al., 2019). This has been attributed to the dedifferentiation of the chondrocytes with extended culturing, causing the acquisition of a hypertrophic phenotype. Thus, alternative strategies to overcome these limitations have led to the use of selecting its progenitors using the expression of its heterodimeric receptors, namely integrins (Williams et al., 2010).

Integrins are transmembrane receptors that play a crucial role in mediating interactions between cells and the extracellular matrix (ECM), influencing key cellular processes such as adhesion, migration, proliferation, differentiation, and survival (Humphries et al., 2006; Hynes, 2002). Fibronectin (CD49eCD29 or α5β1) and laminin (CD49bCD29 or α2β1) have been used in adhesion assays to selectively isolate chondroprogenitors, with α5β1 positive subsets displaying higher chondrogenic potential than chondrocytes (Nelson et al., 2014; Vinod et al., 2021a). These are currently being evaluated as alternative cell-based therapeutics for cartilage repair (Vinod et al., 2021b). The α10β1 integrin, also known as CD11c/CD29, is a critical collagen-binding receptor with a high affinity for collagen type II, the primary structural protein in cartilage. It is predominantly expressed in chondrocytes and mesenchymal stem cells (MSCs) (Camper et al., 1998; Delco et al., 2020).

The α10β1 integrin has been reported to be essential for cartilage development and function, facilitating the interactions between chondrocytes and the surrounding ECM, a process fundamental to proper cartilage formation (Camper et al., 2001). Moreover, α10β1 integrin has been identified as a unique phenotypic marker for chondrocyte differentiation, with research suggesting its potential as a predictor of the chondrogenic differentiation state in MSCs (Goldring, 2012). Integrin α10-enriched MSCs exhibited potential in treating cartilage injuries and osteoarthritis by effectively targeting and repairing cartilage defects in a rabbit model, demonstrating promising therapeutic potential (Andersen et al., 2023). Previous reports studying the expression patterns of α5β1 on chondrocytes, report marked timeline increase in their percentage expression (Kachroo et al., 2020). This established the standard of limiting 20 minutes for the selection of fibronectin adhesion assay-derived progenitors from the heterogeneous pool of chondrocytes (Vinod et al., 2020).

The available information on the correlation of α10β1 and chondrogenesis, the recent research assessing its potential for isolating cells with higher chondrogenesis, and the lack of information on its expression pattern once released from its microenvironment require further evaluation. This study focused on analyzing the temporal expression patterns of integrins, especially α10β1/CD11c+CD29+, in chondrocytes released through collagenase digestion of cartilage, using fluorescence-activated cell sorting (FACS) analysis. The motivation behind this investigation was to understand the potential of α10β1 in selecting chondrocytes with enhanced chondrogenic capability, addressing the gap in knowledge regarding its expression timeline in freshly isolated chondrocytes.

MATERIALS AND METHODS

Sample procurement

The study was conducted following the guidelines established by the Institutional Review Board. Three osteoarthritic knee joints were ob-

tained from patients (Age: 63.3±7.8 years) undergoing knee replacement surgery after securing their written informed consent. The joints were transported in phosphate-buffered saline (PBS) prior to cartilage harvest.

Isolation of chondrocytes

To isolate chondrocytes, the joints were rinsed in PBS to remove any residual synovial fluid and stripped of soft tissue. The cartilage samples were harvested from the non-weight bearing areas of the joint which contained relatively healthy areas. Cartilage slices were obtained using a scalpel blade finely minced and subjected to enzymatic digestion using 0.15% Collagenase Type II (Worthington) for 8 hours. The resulting cell suspension was strained through a 40-µm cell strainer to remove any undigested cartilage debris. The filtered suspension was then centrifuged to obtain the chondrocyte pellet, which was further washed and resuspended. The freshly isolated chondrocytes were immediately processed for fluorescence-activated cell sorting (FACS) analysis.

FACS

The chondrocytes were assessed for the percentage expression of the following integrins: CD-11c+CD29+, CD49b+CD29+, and CD49e+CD29+ at specified time points using FACS. The initial assessment for all three groups was done at 0 hours, and subsequent measurements focused primarily on CD11c+CD29+, with assessments conducted at 20 minutes, 40 minutes, 60 minutes, 90 minutes, and 120 minutes after the initial time point. At the 60-minute mark, all three integrins were reassessed (Fig. 1). Staining was carried out following the technical data sheet specified for each antibody. Data acquisition was performed using the BD FACS Cyto-

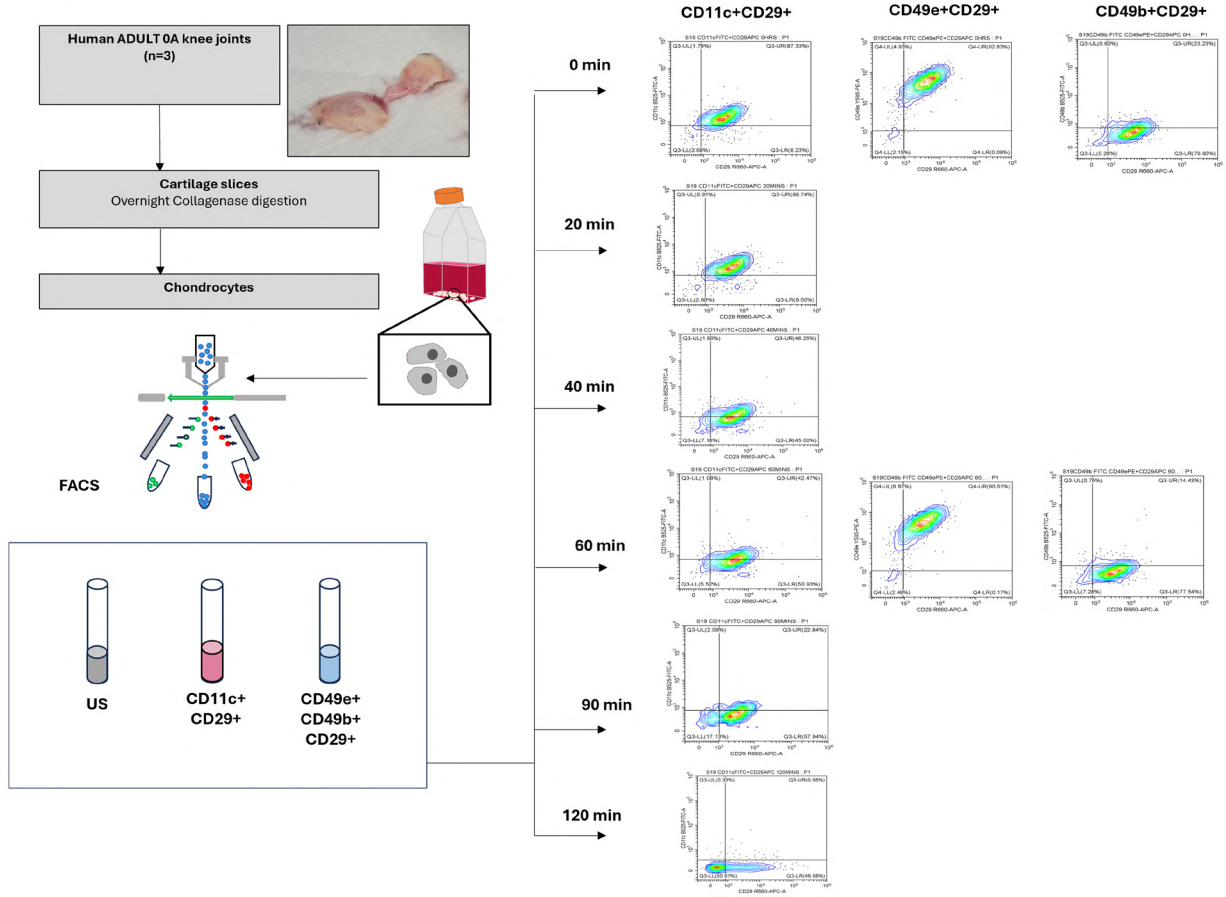

Fig. 1.- Study algorithm depicting temporal analysis of surface marker expression in human chondrocytes. Cartilage was obtained from adult human knee joints (n=3) and subjected to overnight collagenase digestion to isolate chondrocytes. Isolated cells were analyzed using flow cytometry to determine the expression of CD11c, CD49e, CD49b, and CD29 at different time points (0, 20-, 40-, 60-, 90-, and 120- minutes) post-isolation. Representative flow cytometry dot plots show the expression patterns of CD11c+CD29+, CD49e+CD29+, and CD49b+CD29+ populations over time, providing insight into the temporal dynamics of chondrocyte surface marker expression.

flex cytexpert, with unstained controls concurrently processed. Subsequent data analysis was executed through BD FACS Diva v 8.0.1.1 and Flo Jo software.

Statistical analysis

Offline analysis of data was performed using SPSS version 21.0 and pictorial representation of the data using GraphPad Prism 8.0.2. A longitudinal data analysis was used to compare each time point and the changes from the baseline for CD11c, CD29 and CD11c+CD29+ expression and Generalized Estimating Equation (GEE). Wilcoxon signed ranked test was used to compare the difference in expression between the two time points (0 and 60-min) for CD49e, CD49b, CD29, CD49e+CD29+ and CD49b+CD29+. Data was expressed as mean ± standard error mean. A P-value of 0.05 was considered significant.

RESULTS

Flow cytometry analysis of fresh chondrocytes at 0 min revealed the CD11c+CD29+ expression was 79.22 ± 6.73, the CD49e+CD29+ expression was 91.67 ± 3.34, and the CD49b+CD29+ expression was 35.45 ± 13.95 (Fig. 2). The expression of CD11c+CD29+ in all three donors revealed a linear drop in expression, reaching zero to low levels by 120 min (**P=0.002**). When the individual expression patterns were analyzed, the CD11c lost all its expression by 120 min, while CD29 levels achieved a 50% decrease. Notable reduction in CD49b+CD29+ levels was observed within 60 minutes, while there was no significant reduction in CD49e+CD29+ levels during the same period.

DISCUSSION

The challenge of chondrocyte dedifferentiation in culture, which potentially influences the repair quality, underscores the need for a refined selection of chondrocytes. Herein, the focus on integrins, especially the α10β1 integrin (CD11c+CD29+), emerges as a promising strategy. This integrin's prominence in chondrocytes and MSCs (Camper et al., 1998), coupled with its crucial role in cartilage development and potential as a marker for chondrogenic differentiation, establishes a critical avenue for enhancing ACI outcomes.

The expression patterns of integrins such as α10β1, α2β1, and α5β1 in chondrocytes offer intriguing insights into their distinct and complementary roles in cartilage biology. For instance, the decrease in expression of α10β1 integrin as seen in this study with available information of this decrease attributed to chondrocytes maturing and transitioning towards hypertrophic stages and subsequent cartilage matrix mineralization (Goldring, 2012). This understanding not only facilitates the selection of cells for ACI but also provides biomarkers for tracking the progression and effectiveness of cartilage repair therapies. Consequently, in investigations necessitating the collection of chondrocytes that exhibit elevated expression of both CD11c and CD29, it is imperative to promptly subject these cells to sorting subsequent to their harvesting. This is due to the observed tendency of these cells to experience a significant diminution in their expression levels by the 40- min timeframe.

On the other hand, the evaluation of other integrins showed that fresh chondrocytes displayed a relatively stable expression of α5β1 integrin (CD49e+CD29+) over time, a marker implicated in cellular adhesion, migration, and matrix remodeling (Hynes, 2002). Furthermore, there was a decrease in the expression of α2β1 integrin (CD49b+CD29+). A recent report evaluating the phenotypic change in the expression of α5β1 in negatively sorted cells showed a positive increase over time, presenting an effect opposite to that observed with α10β1 as noted in this study (Kachroo et al., 2020). This indicates the complexity of integrin roles and signaling pathways, which require further evaluation. Additionally, the role of α10β1 integrin (CD11c+CD29+) in mediating interactions between chondrocytes and ECM underscores the significance of integrin-ECM interactions in the early stages of chondrogenic differentiation. A lot of research now provides proof that the cartilage resident cells contain not only chondrocytes but also a minor subset of chondroprogenitors with their ECM (Dowthwaite et al., 2004; Grogan et al., 2009; Koelling and Miosge, 2009). These progenitors have heightened chondrogenesis and lower hypertrophic tendency, thus offering potential for genuine hyaline cartilage-like repair. The subjection of the cartilage tissue to digestion causes the

release of a heterogeneous group of cells, which requires selective adhesion assays. The varied expression patterns of these integrins suggest the need to refine cell selection within a crucial time frame, with the current study reporting the need for select them within the 40 min timeframe.

This study highlights the rapid dedifferentiation of chondrocytes, specifically focusing on the α10β1/CD11c+CD29+ receptor, which plays a critical role in chondrocyte differentiation and maturation. Research investigating whether the sorting of CD11c+CD29+ cells can enhance their chondrogenic potential and serve as an improved cell-based therapeutic strategy for generating durable hyaline-like cartilage repairs in response to cartilage-related pathologies, compared to unsorted chondrocytes, will provide valuable insights for clinical applications.

Additionally, as a receptor for collagen type II, α10β1 could potentially be used as a marker to assess chondrogenic maturation following in vitro differentiation studies or to evaluate the chon-

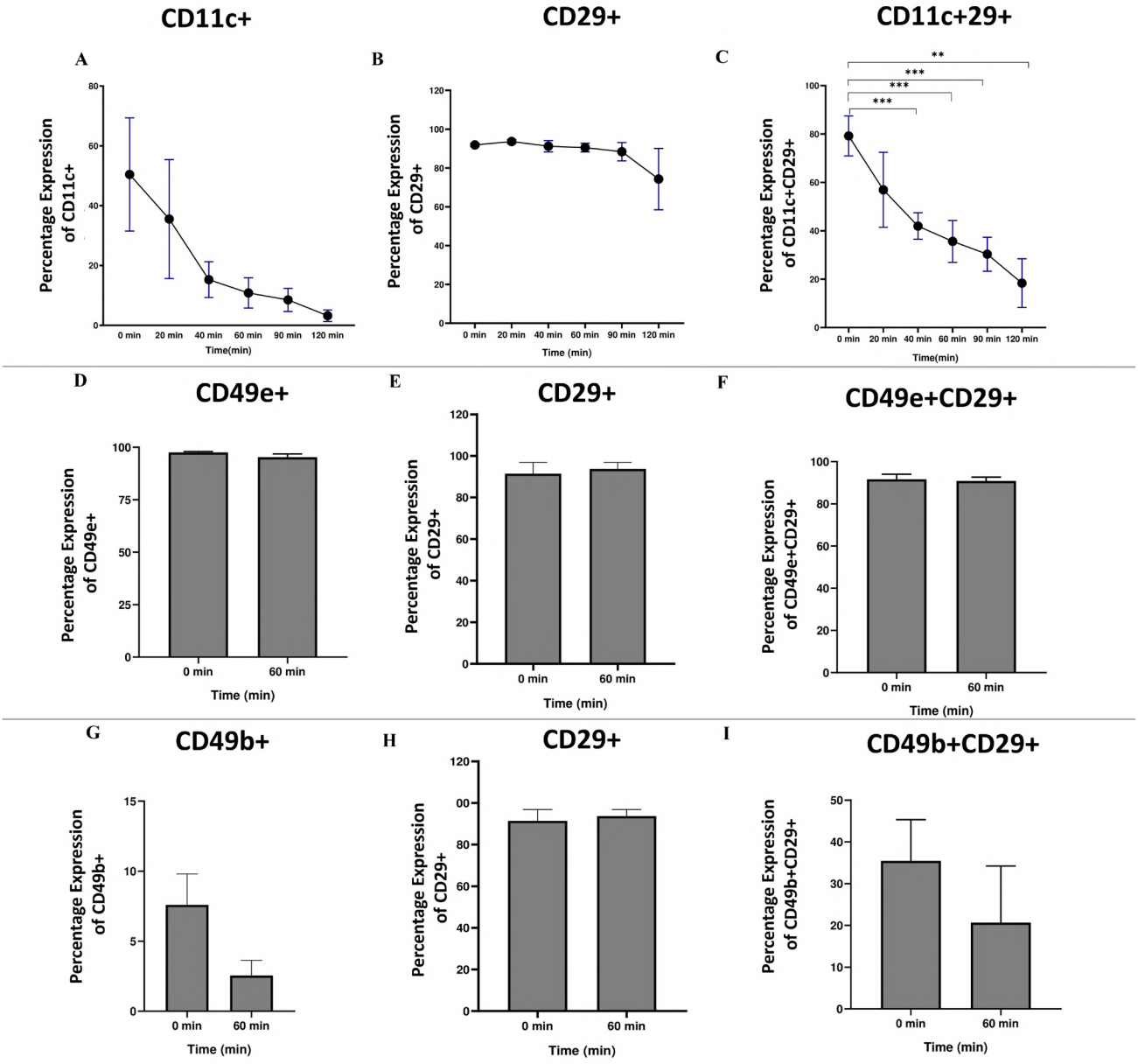

Fig. 2.- Percentage expression of surface markers on cells over time and at different intervals. Panels **A-C** show time-course expression of CD11c, CD29, and co-expression of CD11c/CD29. Panels **D-I** compare marker expression at 0 and 60 minutes. Error bars indicate standard error mean, and asterisks denote significance (*p<0.05, **p<0.01, ***p<0.001). A) CD11c+, B) CD29+, C) CD11c+ and CD29+ double-positive: 0, 20, 40-, 60-, 90- and 120-min D) CD49e+, E) CD29+, F) CD49e+ and CD29+ double-positive: 0- and 60-min G) CD49b+, H) CD29+, I) CD49b+ and CD29+ double-positive: 0 and 60 min.

drogenic status of the chondrocyte donor. Future research focusing on this novel strategy and assessing the comparative chondrogenic potential of α10β1-positive chondrocytes using gene expression analysis for markers of chondrogenesis such as SOX-9, Aggrecan, collagen type II could offer valuable insights into alternative isolation strategies aimed at harnessing cells with an enhanced capacity for cartilage repair.

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STATEMENT OF ETHICS

All techniques observed were, according to the Institutional Review Board (Christian Medical College, Vellore, IRB Min No. 14385 dated 22.12.2021) and Ethics Committee regulations, as per the declaration of Helsinki following the obtainment of informed written consent. This study is supported by the Science and Engineering Research Board (SERB) under grant number DST No: CRG/2022/004277.

AUTHOR CONTRIBUTIONS

E.V, A.R.C, G.P were involved in the study conception and design. E.V, A.R.C, G.P, M.P, A.S, C.J performed the experiments and were involved in data collection. G.R, E.V, A.R.C, M.P, A.S were involved in data analysis and interpretation. E.V provided financial support. All authors were involved in preparing the final version of the manuscript.

DATA AVAILABILITY STATEMENT

The data will be made available on request.

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