Original article

The chondroitin sulfate expression profile in human amniotic fluid cells: a time course study

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Abstract

Object The purpose of this study is to investigate the trend of expression of chondroitin sulfate epitope (WF6) in association with a continuous culture of hAFCs for a period of 30 days.

Methods The hAFCs were obtained from the amniotic fluid of pregnant women at 18 weeks of gestation (n=5). The cells were cultured in an RPMI 1,640 medium containing 20% FCS, Amnio-MAX-C100 16%, 0.03 mg/mL ampicillin and 0.1 mg/mL streptomycin (sigma, Buchs, Switzerland). The expression of chondroitin sulfate epitope (WF6) was detected continuously using immunocytochemistry and signal fluorescence evaluated with the use of Image-pro Plus version 6.0.

Results The levels of the mean area of the chondroitin sulfate WF6 was correlated with those of mean density. The first cycle showed a gradual increase from day 0 to day 12 and then a continuous decrease from day 12 to day 18. In the second cycle the level increased from day 18 to day 27 and decreased after day 27 to day 30.

Conclusion A reasonable conclusion is that the expression of chondroitin sulphate WF6 epitope in hAFCs gradually increased and then decreased in a cyclical pattern. **Chiang Mai Medical Journal 2016;55(2):49-55.**

keywords: expression, chondroitin sulfate, WF6 epitope, human amniotic fluid cells

Introduction

A biological molecule, chondroitin sulfate (CS) is one type of glycosaminoglycan (GAG) which occurs at the cell surface and in the extracellular matrix. It is involved in a wide variety of biological processes including cell proliferation, differentiation, migration, organogenesis, infection and wound repair[1, 2]. Chondroitin sulfate consists of glucuronic acid and N-acetylgalactosamine and is intrinsic to many important biological functions, some major ones being inflammation, coagulation, enzymatic activity and apoptosis and is also involved in the stem cell niche[3-5]. The WF-6 epitope is the part of chondroitin sulfate which is recognized by the monoclonal antibody WF-6 (mAb WF-6)[6]. The knowledge of the use of antibodies which

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are complementary to this epitope on CS is applied extensively in biomolecular research[7]. The hAFCs are an accessible source of stem cells[8-13]. Amniotic fluid stem cells are multipotent and can differentiate into adipogenic, osteogenic, myogenic, endothelial, hepatic and also neuronal cells[1,2]. From our preliminary study, it was found that the hAFCs differentiated distinctly within a continuous culture of hAFCs over a period of 20 days. It is reasonable to assume that CS has a role in this swift differentiation. The objective of this study is to investigate the pattern of the expression of the chondroitin sulfate epitope (WF6) by hAFCs in the 30 day culture.

Materials and methods

The amniotic fluid (AF) was obtained from pregnant women at 18 weeks of gestation (n= 5). The amniotic fluid was extracted by amniocentesis from patients who came for routine prenatal diagnosis at Maharaj Nakorn Chiang Mai Hospital, Human Genetic Laboratory of Anatomy Department, Faculty of Medicine, Chiang Mai University. The collection was carried out after obtaining informed written consent by the patients and was approved by the Ethics Committee of the Faculty of Medicine, Chiang Mai University on 26 September 2011. Application no. 374/2011.

10-15 mL of the AF samples were centrifuged at 1,200 rpm for 10 minutes, then the pellets were removed and suspended in 1 mL RPMI 1,640 medium. The hAFCs were cultured in Petri dishes of diameter 1.5 inches. Cells were grown in a RPMI 1,640 medium containing 20% FCS, 16% AmnioMAX-C100 (Gibco/Invitrogen, Basel, Switzerland), 0.03 mg/ml ampicillin and 0.1 mg/ml streptomycin (Sigma, Buchs, Switzerland) in an incubator with 5% CO_2 at 37 °C.

To avoid maternal cell contamination only male fetuses were selected for this study. Chromosome analysis of hAFCs was carried out using the following methods. To obtain chromosomes at metaphase 0.1 mL of colcemid solution (10 µg/mL; Invitrogen) was added to the hAFCs culture 1 h prior to harvest. This prevented migration of the chromosomes and inhibited progression to anaphase. Harvested hAFCs were treated with 0.075 M KCl solution for 25 min and were fixed in freshly prepared 3:1 methanol: acetic acid. The metaphase chromosomes were stained using a standard G-banding technique and were examined using a Zeiss Axioskop microscope (Zeiss, Jena, Germany) under an oil immersion lens. Ten cells were examined and metaphase images were captured and karyotyed using KaryoImmager software. The chondroitin sulfate expression of the hAFCs at days 0, 3, 6, 9, 12, 15, 18, 21, 24, 27 and 30 was examined using immunofluorescence. In brief, the hAFCs were fixed with 4% paraformaldehyde for 60 min and collected from the petridish by manual surface scraping with a cell scraper. The cells were placed into Eppendorf tubes and washed with 0.1 M PBS then smeared on histoGrip adhesive slides (Zymed, San Francisco, CA). The smeared slides were dried at room temperature. To make the plasma membrane of hAFCs permeable Triton X-100 solution (0.1%) was applied in drops to cover the whole smear and incubated for 5 mins. The slides were blocked with 3% (W/V) BSA for 15 mins then incubated with the monoclonal antibody WF6 (anti-chondroitin sulfate epitopes) diluted in PBS containing 1% (W/V) BSA (37 °C, 90 min). After incubation, the slides were washed three times with 0.1%(W/V) BSA in PBS before being incubated with FITC conjugated anti-mouse Igs (Sigma–Aldich, USA) (1:100) for 30 min. The slides were then secondarily fixed with 4% formaldehyde in PBS for 15 min, counterstained with To-Pro-3 (1:500) for 1 h, mounted with antifade and observed under a Fluorescence microscope with an oil immersion lens from a Zeiss Axioskop microscope (Zeiss, Jena, Germany). Then cells were examined and captured using the Isis FISH Imaging System (MetaSystem, Altuussheim, Germany). The area and density of fluorescence of the WF6 epitope in cytoplasm of hAFCs were calculated by using Image–Pro Plus version 6.0 (Media Cybernetics, Inc., Maryland, U.S.A). The mean area which showed of areas positive signal fluorescence (green color) and mean density, the mean of the intensity of positive signal fluorescence (green color) were calculated digitally calcul.

Results

The karyotyping of 5 samples showed normal diploid chromosomes (46, XY) (Figure 1). The immunofluorescence localization of WF6 epitope expression in the cytoplasm of human amniotic fluid cells (hAFCs) in continuous culture from day 0 to day 30 were represented by both signal fluorescence (green color of FITC), the average area of fluorescence (mean area) and the average of fluorescence density (mean density) (Figure 2). The data of the mean area and mean density of WF6 epitope expression was different (Table 1). The pattern of the mean area of the WF6 expression

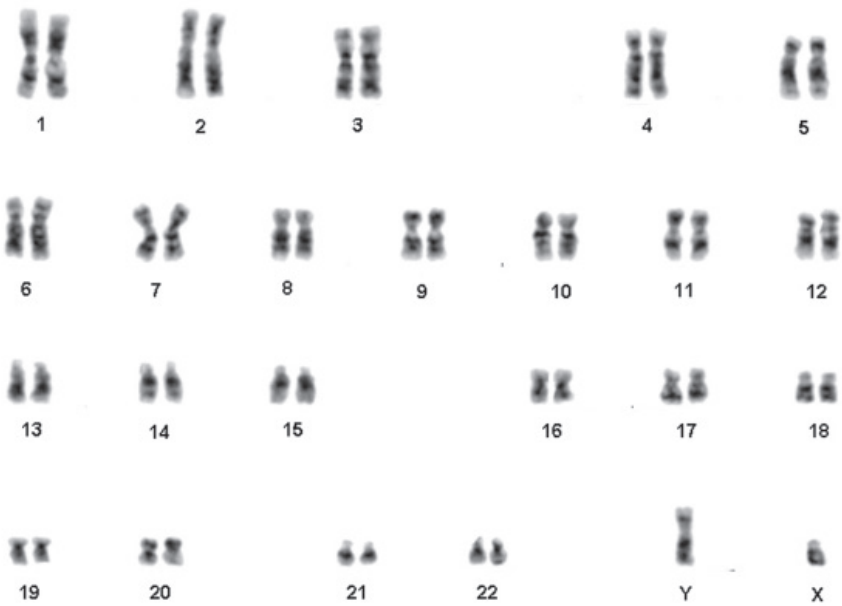

Figure 1. Photograph of the karyotype of the hAFC sample of male fetus showing 46, XY.

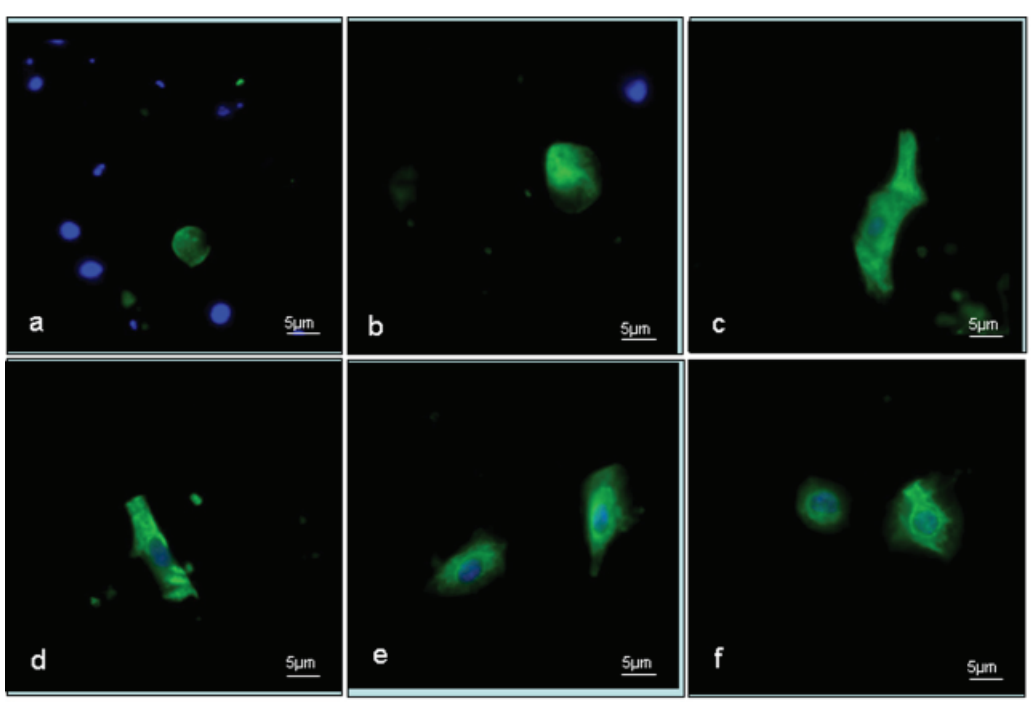

Figure 2. Immonofluorescent localization of WF6 epitope of the hAFCs in continuous culture from day 0 (a), day 6 (b), day 12 (c), day 15 (d), day 24 (e) and day 27 (f) is represented by green color signal.

Table 1. The mean area and mean density of the immunofluorescence localization of WF6 fluid cells in continuous culture from day 0, day 3,day 6, day 9, day 12, day 15, day 18, day21, day 24, day 27 and day 30.

Group	Area (mean±SE)	Density (mean±SE)
D0	1419.500 (±233.005)	66.880 (±8.988)
D3	1004.571(±124.578)	104.568 (±16.511)
D6	2526.571 (±847.628)	140.639 (±5.185)
D9	2917.846 (±686.629)	88.865 (±11.717)
D12	4490.941 (±1064.935)	109.770 (±7.734)
D15	2872.476 (±544.361)	111.555 (±7.593)
D18	1199.409 (±108.923)	71.502 (±5.806)
D21	1838.115 (±214.693)	79.754 (±5.475)
D24	2438.625 (±264.375)	97.518 (±4.347)
D27	3980.690 (±415.931)	99.228 (±5.762)
D30	3998.056 (±1055.718)	80.900 (±6.945)

fluctuated as follows: from day 0 to day 12 it increased gradually then it declined from day 12 to day 18. Interestingly, it increased gradually again from day 18 to day30 (Figure 3). There were significant differences ($p \leq .05$) between the mean area of WF6 epitope expression at day 0 when comparing it with day 12, day 27 and day 30 (Table 1). Moreover, the pattern of the mean density of WF6 expression of hAFCs in continuous culture increased from day 0 to day 6 and declined at day 9. It increased gradually from day 9 to day 12 and dropped on day 18. From day 18 to day 27, it increased gradually. If showed a slight decline on day 30 (Figure 4). Surprisingly, when comparing the mean density of WF6 epitope expression at day 0 with day 6, day 12, day 15, day 24 and day 27, there was a significant difference ($p \leq .05$) (table1). Similarly, at day 12 and day 27 there were significant differences in both the mean area and mean density of WF6 epitope expression when compared with day 0.

Discussion

In this study, all samples were collected only when the fetus was male. This was done to avoid any confusion with maternal cells. Their karyotypes all showed 46, XY. The WF-6 epitope expression of the hAFCs in continuous culture from day 0 to day 30 was detected using the monoclonal antibody WF6; the results demonstrated that the WF-6 epitope was expressed over the whole of the culture period.

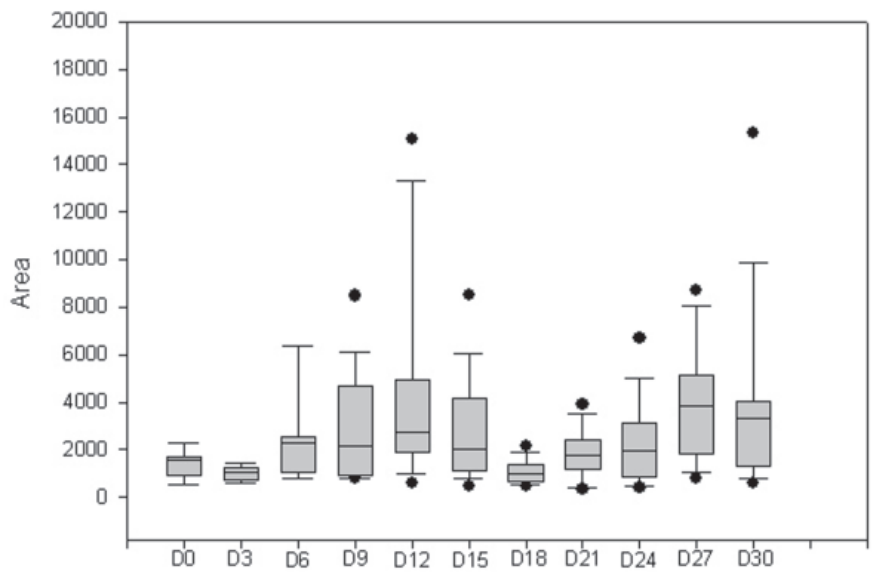

Figure 3. A comparison of the mean area of WF6 epitope expression of hAFCs in continuous culture from day 0, day 3,day 6, day 9, day 12, day 15, day 18, day 21, day 24, day 27 and day 30. Boxes represent mean (-), whiskers indicate 5th and 95th percentiles. Asterisk (*) represents significant differences ($p \leq .05$)

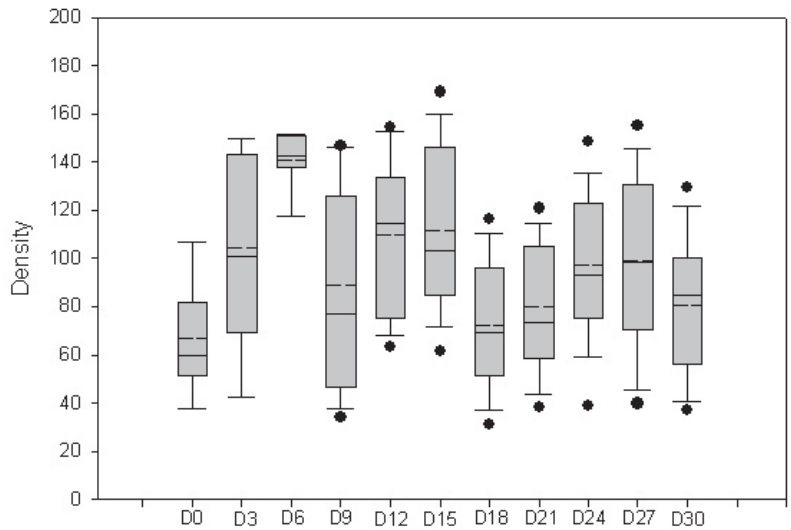

Figure 4. A comparison of the mean density of WF6 epitope expression of hAFCs in continuous culture from day 0, day 3,day 6, day 9, day 12, day 15, day 18, day 21, day 24, day 27 and day 30. Boxes represent median (--), mean (-), whiskers indicate 5th and 95th percentiles. Asterisk (*) represents significant differences ($p \leq .05$)

In brief the cell up takes the building block for CS synthesis, monosaccharide and sulfate for GAG synthesis and trough specialized transporter complexes. The WF6 epitope was at a high level at day 12 and day 27 according to both parameters when compared to the other days of culture. Additionally, it was high on day 6 when mean density was used as the detection method (Figure 3). After that, the level of WF6 epitope was lowest at day 18 (Figure 3, Figure 4) according to both parameters. It was evident that the level of WF6 epitope gradually increased from day 0 to day 12 and then it was showed a continuous decrease in expression from day 12 to day 18. Later, it increased gradually from day 18 to day 27 and then from day 27 to day 30 it decreased. It is evident that the level of WF6 epitope expression has a cyclical pattern. There was a correlation between both parameters of the level of the chondroitin sulfate WF6 epitope in the hAFCs. It was possible that the chondroitin sulfate WF6 epitope was released from hAFCs into either the extracellular matrix or the media. However, the level of WF6 epitope was not detected in the media so this data was not complete and was disregarded in the analysis. In the new cycle, from day 18 to day 27, the pattern was very similar to that seen from day 0 to day 12 (Figure 3) and again there was a continuous decrease from day 27 to day 30 in common with the pattern seen from day 12 to day 18. The period of the culture was quite short so it may well mean we did not get the complete second cycle. The trend of the chondroitin sulphate or WF6 epitope expression showed a correlation with the trend of cell proliferation which was shown when the hAFC growth curve indicated a gradual increase from day 1 to day 3 of cell cultivation, exhibiting the log-phase characteristics. From day 5-15 of the culturing phase, the hAFCs proliferated dramatically by presenting a doubling percentage of reduction. Then, cell division began to decrease[17] which showed a correlation with the slow decline of WF-6 epitope. A reasonable conclusion is that the expression of the WF-6 epitope by hAFCs has a specific pattern. For further studies, the expression of WF-6 epitopes should be detected in both cells and the extracellular matrix. This study reflects the importance of the role of chondroitin sulphate in the biochemical activities of living cells, a role which needs further investigation.

Acknowledgements

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การแสดงออกของสารคอนดรอยตินซัลเฟตในเซลล์น้ำคร่ำของมนุษย์ในช่วงเวลาที่เพาะเลี้ยง

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วัตถุประสงค์ เพื่อศึกษาการแสดงออกของสารคอนดรอยตินซัลเฟต (WF6) ของเซลล์น้ำคร่ำมนุษย์ที่สัมพันธ์กับระยะการเลี้ยงเซลล์ที่ต่อเนื่องจากวันที่เริ่มต้นเลี้ยงจนถึงวันที่ 30

วิธีการวิจัย ตัวอย่างเซลล์น้ำคร่ำมนุษย์ได้มาจากหญิงตั้งครรภ์ที่มีอายุครรภ์ 18 สัปดาห์ จำนวน 5 ราย นำเซลล์มาเพาะเลี้ยงในน้ำยาเลี้ยงเซลล์ RPMI ผสม FBS 20 เปอร์เซ็นต์ สาร AmnioMAX 16 เปอร์เซ็นต์ และยาปฏิชีวนะ ทำการวัดการแสดงออกของสารคอนดรอยตินซัลเฟต (WF6) อย่างต่อเนื่องโดยวิธี อิมมูโนฮีสโตเคมีสตรี และประเมินผลสัญญาณ ฟลูออเรสเซนส์ โดยใช้โปรแกรม Image-pro Plus version 6.0

ผลการทดลอง ระดับของสารคอนดรอยตินซัลเฟต (WF6) ในเซลล์น้ำคร่ำมนุษย์ในการวัดโดยสองตัววัดมีความสัมพันธ์กัน โดยมีลักษณะการเพิ่มขึ้นลดลงและเพิ่มขึ้นลดลงอีก ทำให้มีลักษณะครบวงจรในรอบแรกโดยระดับสารจะค่อย ๆ เพิ่มขึ้นจากวันเริ่มต้นจนถึงวันที่ 12 และจะลดลงมาอย่างต่อเนื่องจนถึงวันที่ 18 ต่อมาสารจะเริ่มเพิ่มขึ้นอีกครั้งจากวันที่ 18 จนถึงวันที่ 27 แล้วจะลดลงมาจนถึงวันที่ 30 ของการเพาะเลี้ยง

สรุป การแสดงออกของสารคอนดรอยตินซัลเฟต (WF6) ในเซลล์น้ำคร่ำมนุษย์ มีรูปแบบการเพิ่มขึ้นและลดลงคล้ายเป็นรูปแบบวงจร **เชียงใหม่เวชสาร 2559;55(2):49-55.**

คำสำคัญ: expression, chondroitin sulfate, WF6 epitope, the human amniotic fluid cells