Original article

Immunohistochemistry of the chondroitin sulfate epitope in various normal human tissues of fresh cadavers

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Objective The purpose of this study was to reveal the expression trend of the WF6 epitope in various normal human tissues.

Methods Ten types of tissue samples from the brain, adipose, skeletal muscle, tendon, liver, cartilage, cardiac muscle, lung, nerve, and, skin were used in this study. They were obtained from five fresh cadavers aged 20-70 years and stained with H and E for their general morphology. In addition, expression of the WF6 epitope was examined using immuno localization.

Results The results demonstrated that WF6 epitope expression in the ten tissues can be categorized into three levels, with the highest being found in the brain, skeletal muscle, cartilage, cardiac muscle and skin tissues. A moderate level was found in the adipose, liver, lung and nerve tissues, and the lowest level was found in the tendon tissue.

Conclusion The WF6 epitope was expressed at different levels in all of the various normal tissues. This reflects the important role of this biomolecule for all cells, which may be beneficial for further study in pathological tissues. **Chiang Mai Medical Journal 2016;55(1):1-7**.

Keywords: chondroitin sulfate, glycosaminoglycans, Immunohistochemistry, WF6 epitope

Introduction

Chondroitin sulfate (CS) is a component of proteoglycans (PGs) or glycosaminoglycans (GAGs) that are predominantly in the extracellular matrix (ECM) and plasma membrane of various human tissues[1]. It consists of glucuronic acid and N-acetylgalactosamine, which is involved in many important biological functions, inflammation, coagulation, enzymatic activity, apoptosis and the stem cell niche[2-4]. The WF6 epitope is part of CS, which is recognized by the WF6 monoclonal antibody (mAb WF6)[5]. The altered metabolism of the ECM in some diseases indicates that expression of the CS epitope is increased in conditions such as osteoarthritis[6] and ovarian carcinoma[7], but is quite rare in normal human tissues. Therefore,

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Received: January 19, 2016**, Accepted:** March 9, 2016**.**

the objective of this study was to reveal the baseline tendency of WF6 expression in normal human tissues.

Materials and methods

The tissue samples were obtained from five fresh cadavers aged 20-70 years old at the Gross Laboratory of Anatomy Department, Faculty of Medicine, Chiang Mai University. They were obtained for this study from normal areas of the following ten sources: the brain, adipose, skeletal muscle, tendon, liver, cartilage, cardiac muscle, lung, nerve and skin, with no mass, abscess or trauma. In order to confirm tissue normality, sections were stained with H and E and viewed under a microscope. The study was approved by the Ethics Committee from the Faculty of Medicine, Chang Mai University, on 1 February 2011, no. 042/2011.

For tissue preparation, the samples were cut into 1x1x0.5 cm portions, then fixed with 4% paraformaldehyde in PBS buffer, pH 7.4 at 4 °C overnight. The tissues were washed 3x10 minutes in the same buffer. Subsequently, each sample was dehydrated with ethanol in a series of 50, 70, 80, 90, and 95% at 4 °C for 15 minutes before infiltrating in paraffin. The samples were cut at 5 µm thickness for paraffin sections.

Hematoxylin and Eosin Staining (H&E): The paraffin sections were deparaffinized 3x10 minutes in xylene, and rehydrated through a graded series of ethanol (3x3 minutes in 100%, 2x1 minute in 95%, 1x3 minutes in 80%, 1x3 minutes in 70%, and 1x3 minutes in 50%) before washing in distilled water for one minute. Tissue sections were stained in Mayer's hematoxylin solution for 10 minutes and washed in distilled water before counterstaining in Eosin-phloxine solution for 3 minutes. The sections were then rehydrated through a series of ethanol (1x2 minutes in 50%, 1x3 minutes in 70%, 1x1 minutes in 95% and 3x1 minutes in 100%), cleared 3x10 minutes in xylene, mounted with xylene-based mounting medium, viewed under light microscopy (Nikon Eclipse E200) and photographed using a Nikon DS-Fi 1, Program NIS Element, and Imaging software version 2.31.

Immunolocalization for the WF6 Epitope: The paraffin sections were dewaxed, hydrated and then rehydrated in PBS buffer before treating with 0.3% (v/v) H2O2 in absolute methanol for 30 minutes in order to remove endogenous peroxidase activity. The sections were blocked with 3% (w/v) BSA for 15 minutes and then incubated with primary antibody (epitope WF6 1:500) diluted in PBS containing 1% (w/v) BSA (37 ° C, 60 minutes). The WF6 mAb was constructed and patented by Prof. Dr. Prachya Kongtawelert *et al* at the Biochemistry Department, Faculty of Medicine, Chiang Mai University. The sections were washed after incubation 3 times with 0.1% (w/v) BSA in PBS before incubating with specific secondary antibody conjugated with peroxidase (37 °C, 60 minutes). Subsequently, they were washed 3 times with 1% (w/v) BSA in PBS. For negative control sections, the primary antibody was omitted and incubated in the dilution buffer. The reaction sites were then visualized by conventional diaminobenzidine (DAB)-H_2O_2 reaction. The reaction was stopped by rinsing well with distilled water, then mounted and covered with cover slips. Finally, the samples were observed under light microscopy magnification using objective lens × ocular lens, 10×10 Nikon Eclipse E200, with photographs taken by the Nikon DS-Fi 1, Program NIS Element. The antigen-antibody reaction was evaluated from the brown color of the whole area of each photograph. The scoring levels of immunoreactive intensity were classified from the density of brown color into weak, moderate and strong expression.

Results

The H&E staining of various normal tissue characteristics are shown as follows: brain, adipose, skeletal muscle (Figure 1A, 1B, 1C, respectively), tendon, liver, cartilage (Figures 2A, 2B, 2C, respectively), cardiac muscle, lung, nerve and skin (Figures 3A, 3B, 3C and 3D, respectively).

The immunohistochemistry of WF6 epitopes of various normal tissues was positive, as represented by the brown color in the brain, adipose, skeletal muscle (Figures 1D, 1E, 1F, respectively), tendon, liver, cartilage (Figures 2D, 2E, 2F, respectively), cardiac muscle, lung, nerve and skin (Figures 3E, 3F, 3G and 3H, respectively). A strong level was expressed specifically around the chondrocytes in the cartilage (Figure 2F), blood vessels of the lung (Figures 3F) and epidermis of the skin (Figure 3H), as indicated by arrows, but this was not observed in the negative control groups. The tissues with a strong (+++) level of WF6 expression were from the brain, skeletal muscle, cartilage, cardiac muscle and skin. Those with a moderate (++) level were from the adipose, liver, lung and nerve, and one tissue with a weak level was from the tendon. Additionally, the WF6 epitope was expressed at different levels in all tissue types (Table 1).

Discussion

Chondroitin sulfate (CS) proteoglycans are major components of the cartilage and many connective tissues. However, CS chains have structural variability and their biological functions are not well understood. Generated WF6 monoclonal antibodies (mAbs) recognize specific features of CS chains or the epitope[8]. The structure of the two octasaccharides, ΔD-C-C-C and ΔC-C-A-D [A = GlcUAβ1–3GalNAc(4-O-sulfate), C = GlcUAβ1–3GalNAc (6-O-sulfate), D = GlcUA (2-O-sulfate)β1–3GalNAc(6-O-sulfate), ΔC = Δ4,5HexUAα1–3GalNAc (6-O-sulfate), and ΔD = Δ4,5HexUA(2-O-sulfate) α1–3GalNAc(6-O-sulfate)] was recognized by a WF6 monoclonal antibody[9].

In this study, results of the WF6 epitope being detected by the WF6 monoclonal antibody demonstrated that the WF6 epitope was expressed in all of the ten normal human tissues samples from the brain, adipose, skeletal muscle, tendon, liver, cartilage, cardiac muscle, lung, nerve and skin. This correlated with previous studies, indicating that CS was found in various human and other mammal tissues[10,6]. Cells synthesize CS within the Golgi apparatus[11-13]. After synthesis, proteoglycans (PGs) are transported from the Golgi apparatus before being sent to the plasma membrane or intracellular organelles, or secreted to the ECM[13]. Different WF6 epitope levels of CS may reflect the ability to synthesize CS in active stage cells in each tissue. This study noted a high WF6 epitope level in the ECM around the chondrocytes, which may have the ability to synthesize CS, as described in a previous

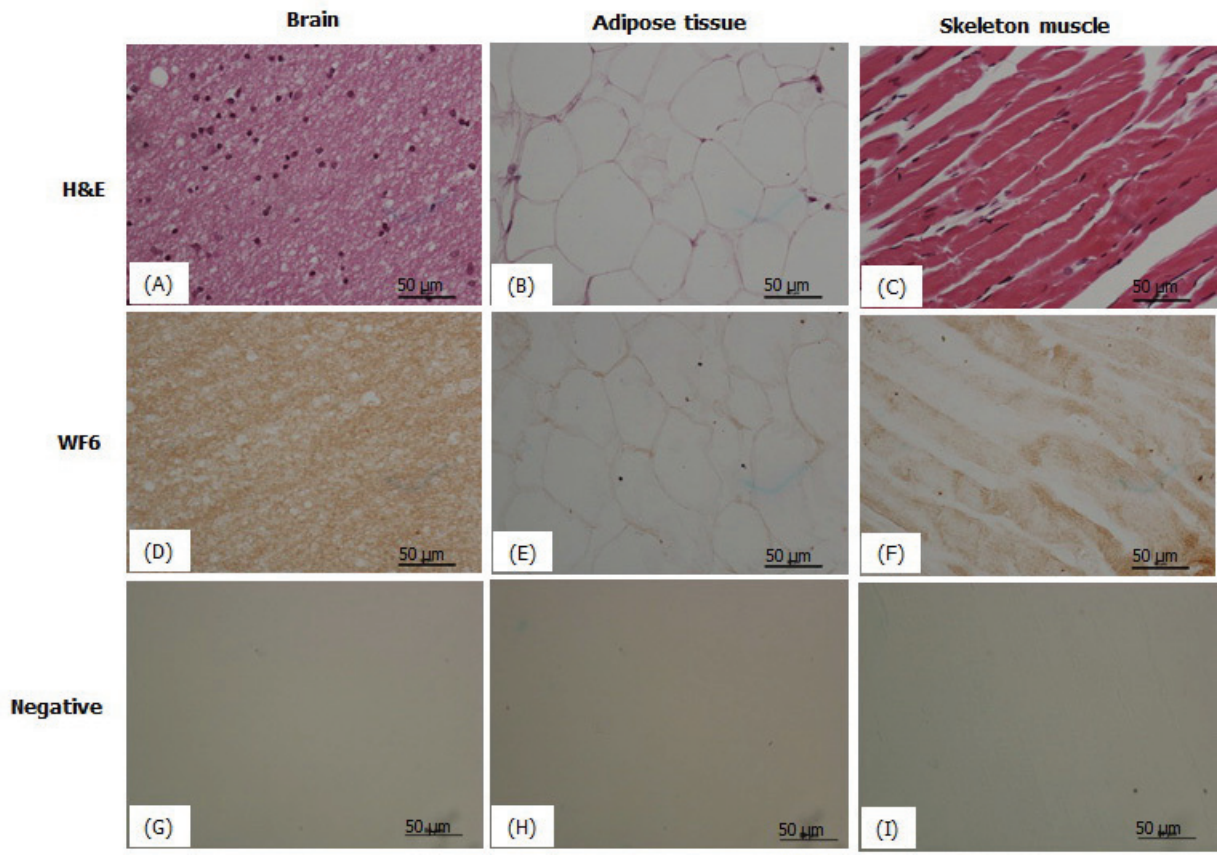


Figure 1. H&E staining showing normal morphology of the brain, adipose and skeletal muscle (A, B, and C, respectively). The brown color represents a positive expression for the WF6 epitope of CS, which was found in the ECM and plasma membrane of the brain, adipose and skeletal muscle (D, E, and F, respectively). There was no signal in any of the negative controls (G, H, and I).

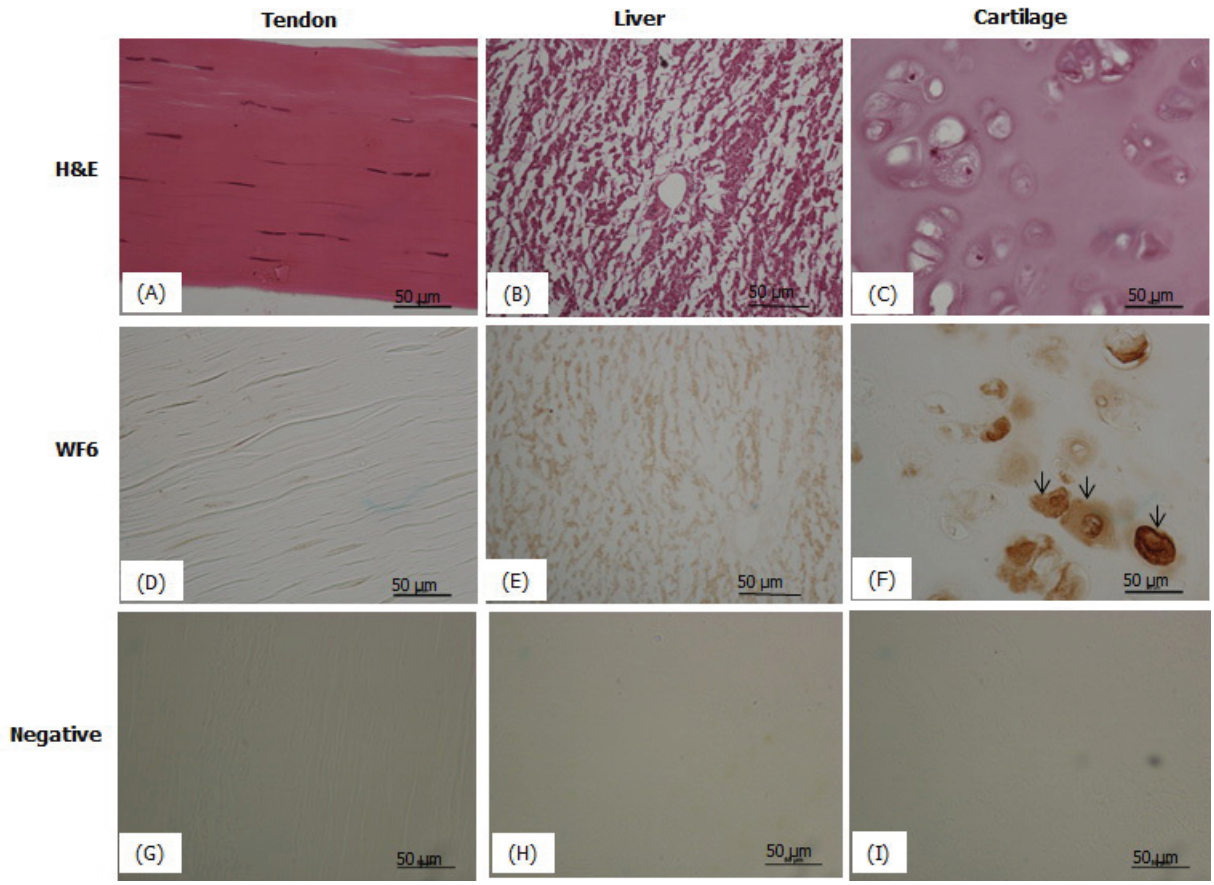


Figure 2. H&E staining indicating normal morphology of the tendon, liver and cartilage (A, B, and C, respectively). The brown color represents a positive expression for the WF6 epitope of CS, which was found in ECM and plasma membrane of the tendon and liver (D and E, respectively), with a strong expression in the cartilage around the lacunae (see arrows in F). There was no signal in any of the negative controls. (G, H, and I).

study[14]. Many studies have reported that CS is significant in the process of cell division and development of the nervous system[15,16]. Astrocytes synthesize CS in culture medium[17]. This study found that the WF6 epitope had the highest level in the brain, which might be reflected in the nerve cells, or glia cells that can synthesize CS. Likewise, expression of the WF6 epitope was found to be highest in the epidermis, sebaceous gland and hair (data not shown)[5,18]. This may reflect from proliferation of epithelial cells in the stratum basale layer and stratum spinosum of the epidermis as well as active cells of the sebaceous gland and hair. In addition, the WF6 epitope of CS expression was at a high level in blood vessels that may reflect from CS accumulation in the blood, or secretion from endothelial cells, which correlated to a study by Fine JD *et al*, 1988. Their findings suggested that circulation or accumulation of CS in serum might be reflected in the high level of WF6 epitope in the serum of patients with more CS production. The changes in ECM metabolism caused an increase in WF6 serum in early rheumatoid arthritis (RA), ovarian cancer and neurological disease[19,5,20].

The findings of this study showed that the WF6 epitope was expressed at different levels in all of the normal human tissue samples. This reflects the important role of this biomolecule for cells, which may be beneficial for further study in pathological tissues.

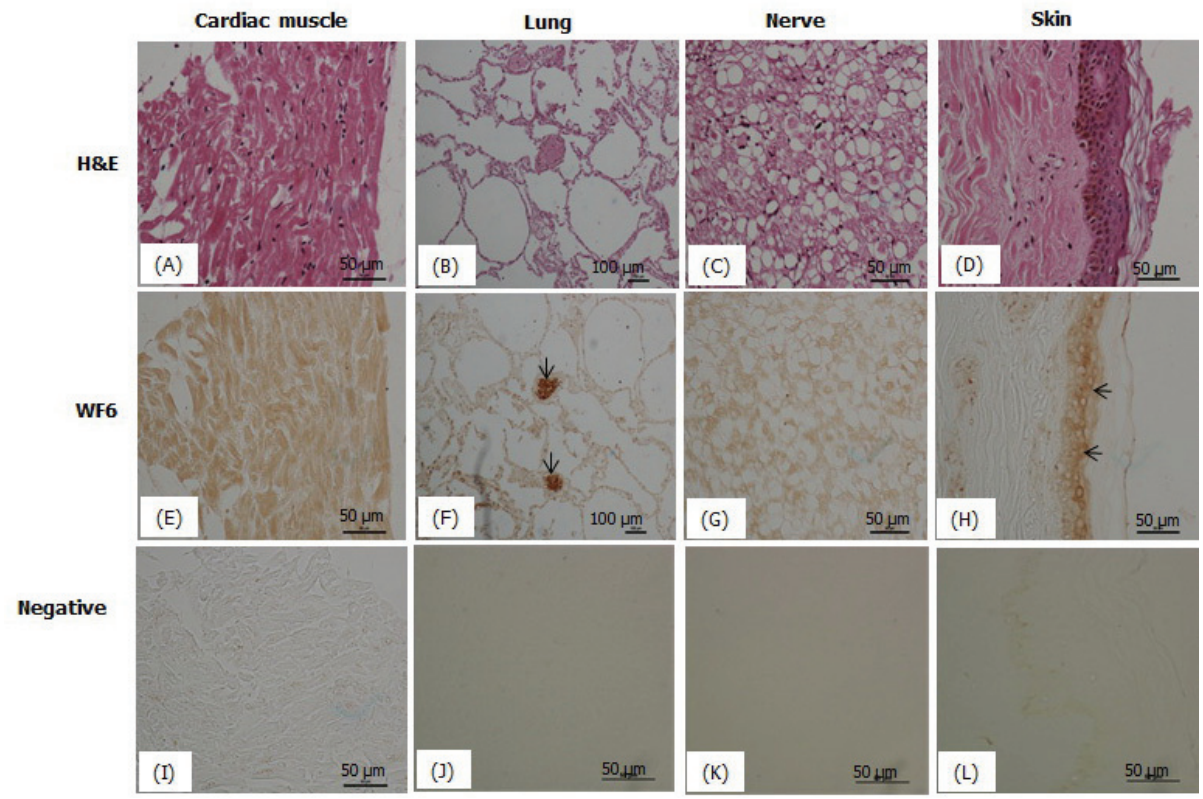


Figure 3. H&E staining demonstrating the normal morphology of the cardiac muscle, lung, nerve and skin (A, B, C and D, respectively). The brown color represents a positive expression for the WF6 epitope of CS, which was found in ECM and plasma membrane of the cardiac muscle, lung, nerve and skin (E, F, G and H, respectively). Additionally, a strong expression was noted in the blood vessels (see arrows in F) and basale layers of the epidermis (arrows in H). There was no signal in any of the negative controls. (I, J, K and L).

Table 1. Expression of the WF6 epitope of chondroitin sulfate in various human tissues

Level of WF6 epitope expression	Tissues
Strong (+++)	Brain
	Skeletal muscle
	Cartilage
	Cardiac muscle
	Skin
Moderate (++)	Adipose
	Liver
	Lung
	Nerve
Weak (+)	Tendon

Acknowledgements

This work was supported by the Faculty of Medicine Research Fund, Chiang Mai University, Chiang Mai, Thailand.

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อิมมูโนฮีสโตเคมีสตรี ต่อบริเวณจำเพาะ WF6 อิพิโทปในเนื้อเยื่อชนิดต่าง ๆ ของร่างสดมนุษย์

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วัตถุประสงค์ เพื่อศึกษาแนวโน้มการแสดงออกของ WF6 อิพิโทป ในเนื้อเยื่อชนิดต่าง ๆ ของมนุษย์

วิธีการ เนื้อเยื่อของมนุษย์จำนวนสิบชนิดได้แก่ สมอง ไขมัน กล้ามเนื้อลาย เอ็น ตับ กระดูกอ่อน กล้ามเนื้อหัวใจ ปอด เส้นประสาท และ ผิวหนัง โดยตัวอย่างที่ใช้ในการทดลองได้มาจากร่างศพที่ยังไม่ผ่านการดอง ซึ่งอายุระหว่าง 20 ถึง 70 ปี จะทำการย้อมเนื้อเยื่อทั้งหมดด้วยสีฮีมาทอกซิลิน และอีโอซิน เพื่อศึกษาลักษณะทั่วไป นอกจากนั้นจะศึกษาการแสดงออกของ WF6 อิพิโธป ด้วยวิธีอิมมูโนวิทยา

ผลการศึกษา ผลศึกษาระดับการแสดงออกของ WF6 อิพิโธป ในเนื้อเยื่อทั้งสิบชนิดพบว่ามีการแสดงออกในเนื้อเยื่อทุกชนิดโดยสามารถแบ่งออกได้เป็นสามระดับได้แก่ กลุ่มที่มีระดับสูงสุดได้แก่ เนื้อเยื่อ สมอง กล้ามเนื้อลาย กระดูกอ่อน กล้ามเนื้อหัวใจ และผิวหนัง กลุ่มที่มีระดับปานกลางได้แก่ เนื้อเยื่อ ไขมัน ตับ และเส้นประสาท ส่วนกลุ่มที่มีระดับต่ำสุดได้แก่ เนื้อเยื่อ เอ็น

สรุป แนวโน้มการแสดงออกของ WF6 อิพิโทป ในเนื้อเยื่อปกติของมนุษย์ชนิดต่าง ๆ พบว่ามีการแสดงออกในเนื้อเยื่อทุกชนิดโดยมีระดับที่แตกต่างกัน สะท้อนให้เห็นความสำคัญของสารชีวะโมเลกุลนี้นี้ต่อเซลล์ทุกชนิด ซึ่งอาจเป็นประโยชน์ต่อการศึกษาเกี่ยวกับเนื้อเยื่อที่มีพยาธิสภาพในอนาคต **เชียงใหม่เวชสาร 2559;55(1): 1-7.**

คำสำคัญ: คอนดรอยตินซัลเฟต ไกลโคซามิโนไกลแคน อิมมูโนฮีสโตเคมีสตรี WF6 อิพิโทป