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Research Article

Transforming growth factor-β1 regulates fibronectin isoform expression and splicing factor SRp40 expression during ATDC5 chondrogenic maturation

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ABSTRACT

Fibronectin (FN) isoform expression is altered during chondrocyte commitment and maturation, with cartilage favoring expression of FN isoforms that includes the type II repeat extra domain B (EDB) but excludes extra domain A (EDA). We and others have hypothesized that the regulated splicing of FN mRNAs is necessary for the progression of chondrogenesis. To test this, we treated the pre-chondrogenic cell line ATDC5 with transforming growth factor-β1, which has been shown to modulate expression of the EDA and EDB exons, as well as the late markers of chondrocyte maturation; it also slightly accelerates the early acquisition of a sulfated proteoglycan matrix without affecting cell proliferation. When chondrocytes are treated with TGF-β1, the EDA exon is preferentially excluded at all times whereas the EDB exon is relatively depleted at early times. This regulated alternative splicing of FN correlates with the regulation of alternative splicing of SRp40, a splicing factor facilitating inclusion of the EDA exon. To determine if overexpression of the SRp40 isoforms altered FN and FN EDA organization, cDNAs encoding these isoforms were overexpressed in ATDC5 cells. Overexpression of the long-form of SRp40 yielded an FN organization similar to TGF-β1 treatment; whereas overexpression of the short form of SRp40 (which facilitates EDA inclusion) increased formation of long-thick FN fibrils. Therefore, we conclude that the effects of TGF-β1 on FN splicing during chondrogenesis may be largely dependent on its effect on SRp40 isoform expression.

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Introduction

Fibronectin (FN) is a fibril-forming, dimeric glycoprotein that, upon engagement of its integrin receptors, influences cellular signaling, adhesion, and migration [1]. Depending on the species, multiple isoforms (>10) of the protein are expressed through alternative splicing of two exons, termed EDA (or EIIIA) and EDB (or EIIIB), and one variable region, referred to as either V or IIICS. *In vitro* studies using mini-genes containing the individual exons have suggested exon-specific functions [2–4] and our interests have centered on the regulated splicing of the EDA and EDB exons during chondrogenesis. In the process of chondrocyte differentiation, fibronectin and collagen type II undergo alternative splicing to result in maturation-dependent changes in ECM composition. In the case of fibronectin, this switch has been suggested to be important for the induction and maintenance of the mature chondrocytic phenotype [5].

Specifically, the embryonic mesoderm expresses an FN isoform that includes EDA and EDB (FN A+B+). During condensation, the maturing cartilage switches to the production of FN isoforms that exclude the EDA exon and are enriched for inclusion of the EDB exon (FN A–B+) [6,7], suggesting that loss of the EDA exon is associated with chondrocyte maturation [8]. Specifically, the presence of this EDA exon facilitates cell spreading, which is characteristic of undifferentiated chondrocytes [3,9]. The change in FN isoform production appears to have an effect on the differentiation of the resident cells of these tissues. This is reproduced *in vitro* as embryonic limb bud mesenchymal cells, when plated on the different FN isoforms, exhibit modulation of their chondrogenic potential [10]. Although the endogenous signals that modulate the switch in isoform are unknown, the splicing factor SRp40 plays a critical role in determining EDA inclusion [11] [Liang et al., accompanying manuscript]. Interestingly, SRp40 is also alternatively spliced, where the short form stimulates the inclusion of EDA during FN RNA splicing, while the long form of SRp40 decreases the amount of FN A+, without a concomitant increase in FN A–. Thus, while the short form facilitates inclusion of the EDA exon, the actions of the long form to inhibit this inclusion could be either due to RNA splicing or to FN RNA expression [Liang et al., accompanying manuscript].

In cells in culture, transforming growth factor-β1 (TGF-β1) has been used to induce chondrogenesis [12–14]. Some studies have demonstrated that TGF-β treatment favors retention of the FN EDA exon [15–19]. In our studies using a pre-chondrocytic cell line, TGF-β1 promotes the loss of the EDA exon in the absence of chondrogenic stimuli (insulin, transferrin, selenium (ITS)) [20]. If, in fact, loss of the EDA exon is an important step during chondrogenesis, our studies suggest that TGF-β1 should promote acquisition of an early chondrocyte phenotype.

The goal of this study is to determine the effects of TGF-β1 on the maturation-dependent expression of the different FN isoforms in cells that are committed chondrocytes. Furthermore, we measure the effects of TGF-β1 on SRp40 isoform expression to determine if this may be a control point for FN splicing. Finally, we overexpress the cDNAs encoding the SRp40 proteins and ask if this overexpression affects FN and FN EDA organization in the presence and absence of TGF-β1.

Methods

Cell culture

ATDC5 cells were routinely grown in Dulbecco's modified Eagle medium (DMEM) Ham's F-12 (1:1) (Invitrogen, San Diego, CA) containing 5% fetal bovine serum (FBS) (Atlanta Biologicals, Atlanta, GA), and maintained at 37 °C and 5% CO_2. ATDC5 cells were inoculated into 6-well plastic cell culture plates (Corning, Corning, NY), and maintained in DMEM/Ham's F-12 (1:1) containing 5% FBS at 37 °C and 5% CO_2 until confluence. After confluence, plates were separated into four groups and cultured as follows: (Group 1) DMEM/Ham's F-12 (1:1) containing 5% FBS, 37 °C, 5% CO_2 as a control; (Group 2) α-modified Eagle medium (α-MEM) (Invitrogen, San Diego, CA) containing 5% FBS, 37 °C, 3% CO_2; (Group 3) α-modified Eagle medium (α-MEM) (Invitrogen, San Diego, CA) containing 5% FBS and ITS (10 μg/ml bovine insulin (I), 10 μg/ml human transferrin (T), 3×10^{-8} mol/l sodium selenite (S)) (Biowhittaker, Walkersville, MD), 37 °C, 3% CO_2; (Group 4) α-MEM containing 5% FBS, ITS, and 1 or 10 ng/ml human transforming growth factor-β1 (TGF-β1, Atlanta Biologicals, Atlanta, GA), 37 °C, 3% CO_2. The cells proliferate as pre-chondrocytic mesenchymal cells when cultured in the pre-chondrogenic maintenance medium (serum-containing DMEM/F-12), or in the medium permissive for chondrogenesis (serum-containing α-MEM); addition of ITS is necessary for the cells to assume a chondrogenic phenotype [21]. Medium is refreshed every 3 days.

Alcian blue staining

Cells were washed in PBS, fixed in 4% formalin in PBS for 10 min, and incubated with 1% Alcian blue (Fluka, Buchs, Switzerland) in 0.1 N HCl overnight at room temperature. Plates were rinsed with PBS and extent of staining was measured by digital imaging. The amount of Alcian blue staining was also determined spectrophotometrically after solubilization. Alcian blue was extracted from cultures by overnight incubation of wells with 6 M guanidine–HCl, followed by the measurement of absorbance at 620 nm [22].

RNA extraction and transcription-polymerase chain reaction

Total RNA was isolated from ATDC5 cells (Qiagen RNeasy kit (Qiagen, Chatsworth, CA)), 1.0 μg was reverse transcribed into cDNA (SuperScript First-Stand Synthesis System (Invitrogen, San Diego, CA)), and amplified in 50 μl of PCR reaction. Primers are listed in Table 1, with amplification conditions in Table 2. PCR products were fractionated by electrophoresis in 1% agarose gels containing ethidium bromide, and sizes compared to pGEM DNA markers (Promega, Madison, WI). Images were recorded digitally and relative densities determined using the Scion Image Program (Scion Technologies, Inc.).