Journal of Cellular Biochemistry 95:750–762 (2005)

Transforming Growth Factor-β1 (TGF-β1) Regulates ATDC5 Chondrogenic Differentiation and Fibronectin Isoform Expression

Fei Han,[1] Christopher S. Adams,[1] Zhuliang Tao,[1] Charlene J. Williams,[2,3] Raihana Zaka,[2] Rocky S. Tuan,[4] Pamela A. Norton,[3] and Noreen J. Hickok[1,3]*

[1]Department of Orthopaedic Surgery, Thomas Jefferson University, Philadelphia, Pennsylvania
[2]Department of Medicine, Division of Rheumatology, Thomas Jefferson University, Philadelphia, Pennsylvania
[3]Department of Biochemistry and Molecular Pharmacology and Jefferson Center for Biomedical Research, Thomas Jefferson University, Philadelphia, Pennsylvania
[4]Cartilage Biology and Orthopaedics Branch, NIAMS, NIH, Bethesda, Maryland

Abstract Regulated splicing of fibronectin (FN) occurs during the mesenchymal to chondrocyte transition and ultimately results in the relative enrichment of an extra domain B (EDB) exon-containing FN isoform with the suggestion that FN isoforms may play a functional role in chondrogenesis. Promotion of chondrogenesis can also be achieved by treatment with transforming growth factor-β (TGF-β), which also regulates FN isoform expression. We have examined the effects of TGF-β treatment on the assumption of the chondrogenic phenotype in the teratoma-derived cell line ATDC5 and tested whether these effects on chondrogenesis are paralleled by appropriate changes in FN isoform expression. ATDC5 cells were maintained in a pre-chondrogenic state and, in this state, treated with 10 ng/ml TGF-β. The cells started to elaborate a matrix rich in sulfated proteoglycans, such that within the first 12 days of culture, TGF-β1 treatment appeared to slightly accelerate early acquisition of an Alcian blue-stained matrix, and caused a dose- and time-dependent decrease in collagen type I expression; changes in collagen type II expression were variable. At later times, cells treated with TGF-β became indistinguishable from those of the controls. Interestingly, TGF-β treatment caused a significant dose- and time-dependent decrease in the proportion of FN containing the extra domain A (EDA) and the EDB exons. These data suggest that TGF-β induces the early stages of chondrogenic maturation in this pre-chondrogenic line and that TGF-β treatment increases expression of FN isoforms that lack the EDA and EDB exons. J. Cell. Biochem. 95: 750–762, 2005.

Key words: fibronectin; TGF-β1; collagen type II; collagen type I; alcian blue; alternative splicing

Endochondral bone formation arises from the staged replacement of the cartilaginous rudiment. These cartilaginous elements arise from mesenchymal cells that condense to form the embryonic limb bud and then undergo maturation that is governed by a series of transcriptional and post-transcriptional events [Hickok et al., 1998]. A number of transcription factors and extracellular matrix components have been suggested to be important for the transition from a mesenchymal cell through a pre-chondrogenic pre-cursor to a chondrocyte. Among these suggested regulators is the extracellular matrix protein fibronectin (FN).

Multiple isoforms of FN occur and are expressed in a tissue- and developmentally-specific manner [Kornblihtt et al., 1985; Bennett et al., 1991; ffrench-Constant, 1995; Gehris et al., 1996b; MacLeod et al., 1996]. These isoforms

Grant sponsor: NIH; Grant numbers: AR45181, AR39740, AR44360, AR46821, DE-13310, DE-10875, DE-05748; Grant sponsor: Department of the Army; Grant number: DAMD17-03-1-0713; Grant sponsor: US Army Medical Research Acquisition Activity; Grant number: 21702-5014.

*Correspondence to: Noreen J. Hickok, PhD, Department of Orthopaedic Surgery, Thomas Jefferson University, 1015 Walnut St., Suite 501, Philadelphia, PA 19107.
E-mail: Noreen.Hickok@jefferson.edu

Received 13 July 2004; Accepted 17 December 2004
DOI 10.1002/jcb.20427

arise from alternative splicing associated with two type III repeats, and complex splicing of a third exon called V or IIICS that connects two type III regions [Kornblihtt et al., 1985; Paul et al., 1986; Norton and Hynes, 1987]. The first two exons are referred to as extra domain A and B (EDA or EDB) or EIIIA or EIIIB. The protein segments encoded by these exons reside between type III repeats 11 and 12 (EDA) and between repeats 7 and 8 (EDB) [Schwarzbauer et al., 1983, 1987; Paul et al., 1986; Norton and Hynes, 1987]. Inclusion or exclusion of these two exons results in the production of four of the FN isoforms.

TGF-β is an important growth factor implicated in regulation of many genes and proteins during chondrogenic maturation [Wu et al., 1992] and is used to induce chondrogenesis in high-density cultures of mesenchymal precursor cells [Lust et al., 1991]. Regulation of FN levels and isoform expression may occur independently of the Smad pathway [Hocevar et al., 1999]. TGF-β1 treatment favors inclusion of EDA in fibroblasts [Balza et al., 1988; Inoue et al., 1999], bovine granulose cells [Colman-Lerner et al., 1999], and tubular epithelial cells [Viedt et al., 1995; Burger et al., 1998] and inclusion of EDB in fibroblasts [Borsi et al., 1992].

The FN isoforms are expressed in a characteristic pattern during chondrocyte maturation [Bennett et al., 1991; Gehris et al., 1996b; Peters and Hynes, 1996]. FNs containing these exons appear to be more readily incorporated into the extracellular matrix, suggesting a role in matrix stability [Guan et al., 1990]. Specifically, FN A+ B+ are characteristic of mesenchymal pre-cursor cells. As these cells undergo chondrocytic differentiation, splicing favors exclusion of the EDA exon and inclusion of the B exon [Bennett et al., 1991]. Exclusion of EDA during chondrocyte maturation occurs consistently over several species [Zhang et al., 1995], whereas the inclusion rate of the EDB exon appears to be dependent on the tissue and on the species studied [Bennett et al., 1991; Magnuson et al., 1991; Zhang et al., 1995]. Studies of the EDA exon implicate it in altered cellular spreading, migration, and transit through the cell cycle [Hashimoto-Uoshima et al., 1997; Manabe et al., 1997, 1999]. Animals in which splicing of the EDA exon was ablated appeared normal except for deficits in wound healing and a decreased life span [Muro et al., 2003; Tan et al., 2004]. In vitro studies using mini-genes containing the EDB exon suggest an RGD- and integrin-independent role in cell adhesion [Chen and Culp, 1998]. The EDB exon has been ablated and results in mice that are of normal size, exhibit Mendelian frequencies for offspring, and show no obvious abnormalities in cartilage or fracture repair. However, fibroblasts from these animals showed a small decrease in proliferative rate and decreased FN matrix assembly [Fukuda et al., 2002].

The data therefore suggest that the different FN isoforms may have unique roles in the initiation of cellular signaling and determination of cellular morphology. During chondrogenesis, the transition from a more fibroblastic mesenchymal cell to a rounded cell is necessary for differentiation [Loty et al., 1995], and based on reported studies, we and others have hypothesized that the production and deposition of these FN isoforms may play an important role in the induction and maintenance of the chondrogenic phenotype [Bennett et al., 1991; Burton-Wurster et al., 1997; White et al., 2003].

In order to begin to examine interrelationships between TGF-β1 regulation of FN isoform expression and chondrogenesis, we have chosen to use the mouse teratoma cell line ATDC5. This line is maintained in its committed, proliferative, pre-chondrogenic state by culturing in DMEM/F-12 containing serum, 37°C, 5% CO_2. When switched to α-MEM containing serum supplements with insulin, transferrin, and selenium (ITS), 3% CO_2, the cells differentiate to recapitulate the chondrocyte condensation to hypertrophy program [Atsumi et al., 1990]. When these chondrogenic cells (in medium containing ITS) are treated with members of the TGF-β superfamily, they appear to progress to the proliferating chondrocyte without the transition through cellular condensation [Asahina et al., 1996; Shukunami et al., 1998; Kawai et al., 1999]. Effects of TGF-β1 on the prechondrogenic cells (without ITS) are not known. We have asked if TGF-β1 is sufficient to induce chondrogenesis and appropriate maturation-dependent FN-isoform expression in ATDC5 cells maintained without ITS. Our data suggest that (1) TGF-β1 effects on chondrogenesis were separable from effects on FN splicing and (2) TGF-β1 appears to only accelerate early chondrogenic maturation while suppressing FN EDA and EDB inclusion.

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 three groups and cultured as follows: Group one: DMEM/Ham's F-12 (1:1) containing 5% FBS, 37°C, 5% CO_2 as a control; Group two: α-modified Eagle Medium (αMEM) (Invitrogen) containing 5% FBS, 37°C, 3% CO_2; Group three: αMEM containing 5% FBS, and 1 or 10 ng/ml human transforming growth factor-β 1(TGF-β1, Atlanta Biologicals, Atlanta, GA) In a series of short-term experiments, cells were cultured as above in the presence and absence of serum for up to 4 days. The cells proliferate as pre-chondrocytic mesenchymal cells when cultured in serum-containing DMEM/F-12, the pre-chondrogenic maintenance medium or α-MEM, the medium permissive for chondrogenesis. Please note that, according to published accounts, addition of 10 μg/ml bovine insulin (I), 10 μg/ml human transferrin (T), 3×10^{-8} mol/L sodium selenite (S) (ITS, Biowhittaker, Walkersville, MD) is necessary for them to undergo the complete program of chondrocyte maturation; in the absence of ITS, according to published accounts, they should mature at most into the condensing chondrocyte [Atsumi et al., 1990]. Medium was 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 0.1N HCl overnight at room temperature. Plates were rinsed with PBS and extent of staining detected by digital imaging.

RNA Extraction and Transcription-Polymerase Chain Reaction

Total RNA was isolated from ATDC5 cells (Qiagen RNeasy kit (Qiagen, Chatsworth, CA)), measured spectrophotometrically, 1.0 μg was reverse transcribed into cDNA (SuperScript First-Stand Synthesis System (Invitrogen)), and amplified in 50 μl of PCR reaction. The primers used are shown in Table I, with amplification conditions in Table II. The 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.)

DNA Sequencing of PCR Products

PCR-amplified FN EDB isoform cDNAs were fractionated by electrophoresis, bands isolated and eluted (Qiagen Gel Extraction kit (Qiagen)), and sequenced using a TGG CCT GGA GTA CAA CGT CAG primer that resides 21 bp 3′ of the original sense primer used to generate the cDNA fragment. Sequencing was performed using the central sequencing facility at Thomas Jefferson University.

DNA Measurement

ATDC5 proliferation was measured based on cellular DNA content using the PicoGreen dsDNA quantitation kit (Molecular Probes, Eugene, OR); PicoGreen binds to double-stranded DNA to yield a fluorescent signal that is proportional to DNA content. ATDC5 cells were incubated in 96-well flat bottom plates (Corning, Corning, NY). Different cell densities (5,000, 1,000, 500, and 100/well) were added, and cultured in (MEM containing 5% FBS and 0, 1, or 10 ng/ml TGF-β1. Cells were harvested, lysed, and fluorescence measured, relative to a

TABLE I. Amplification Primers

Name	Forward (5′–3′)	Reverse (5′–3′)	Reference
Collagen type I, (col α1 (I))	GAATTCGGACTAGACATTG	GGTAAGGTTGAATGCACT	
Collagen type II, (col α1(II)),	GTGAGCCATGATCCGC	GACCAGGATTTCCAGG	489 bp 285 bp
SR protein40 (SRp40)	GAATTTATCCTCAAGAGTCAG	ACTTCTGGTCCGAGATCGTGA	Kuo et al. [2002]
FN EDA exon	GAAATGACCATTGAAGGTTTG	TTCTTTCATTGGTCCTGTCTT	Gehris et al. [1996a]
FN EDB exon	CATGCTGATCAGAGTTCCTG	GGTGAGTAGCGCACCAAGAG	Gehris et al. [1996a]
β-actin	GTGCTATGTTGCCCTGGATT	TGCTAGGGCTGTGATCTCCT	

TABLE II. Amplification Conditions

	Denaturation		Annealing		Extension		Cycles
Col α1(I)	94°C	1 min	47°C	1.5 min	72°C	1 min	26,27
Col αI(II)	94°C	1 min	48°C	1.5 min	72°C	1 min	30,33
SRp40	94°C	1 min	55°C	1.5 min	72°C	1 min	27,28,30
FN EDA	94°C	1 min	50°C	1.5 min	72°C	1 min	28,35
FN EDB	94°C	1 min	53°C	1.5 min	72°C	1 min	28,35
β-actin	94°C	1 min	55°C	1.5 min	72°C	1 min	20

standard curve of DNA concentration using SpectreFluorPlus (Tecan, Austria) plate reader.

Data Analysis

All gels were digitally imaged and assembled using Adobe PhotoShop. Within any series, all adjustments were made in parallel to all gels used for comparison. Statistical significance was determined using a two-way analysis of variance (ANOVA) with time and treatment as covariates. A Tukey multiple comparison procedure was used to determine differences between groups. Each experiment shown is a representative experiment of at least three experiments.

RESULTS

TGF-β1 is a growth factor that has been implicated in chondrocyte condensation, as well as regulating FN splice site selection. We therefore investigated the effects of TGF-β1 on the pre-chondrogenic, teratoma-derived cell line, ATDC5. ATDC5 cells were cultured in DMEM/F-12, the medium used to maintain cells in their committed pre-chondrogenic state; effects of TGF-β1 were assessed in this medium for some dose-response studies. In parallel, in all dose-response and time course studies, cells were maintained in αMEM containing 5% FBS, 3% CO_2. In this medium, ATDC5 cells are reported to remain as pre-chondrocytes with minimal differentiation; work from others shows that chondrocyte maturation does not occur until addition of insulin, transferrin, and selenium (ITS) [Atsumi et al., 1990]. Thus, in this report, ATDC5 cells were maintained as pre-chondrocytes. Using these conditions, we determined whether TGF-β1 treatment caused the assumption of a chondrogenic phenotype, characterized by a reduction in cellular proliferation, acquisition of a sulfated proteoglycan matrix and modification of the expression pattern of collagens type I and II. We then tested the hypothesis that TGF-β1 treatment alters FN EDA or EDB inclusion in parallel with effects on chondrogenesis and as a function of dose and time.

TGF-β1 Does Not Affect Early Cell Proliferation

To measure cell proliferation, DNA content was measured in cells maintained in αMEM containing 0, 1, or10 ng/ml TGF-β1 (Fig. 1A). No proliferative differences were apparent at 1, 2, or 3 days of culture. At day 4, cells cultured in the presence of TGF-β1 showed a trend towards a decrease in proliferation in comparison to cells treated with 1 or 0 ng/ml TGF-β1.

TGF-β1 Treatment Accelerates Accumulation of an Early Sulfated Proteoglycan Matrix

We next examined the effect of 10 ng/ml TGF-β1 on the acquisition of a sulfated proteoglycan matrix over a 42-day time course. As shown in Figure 1B, through day 17, Alcian blue staining appeared slightly bluer in cultures containing TGF-β. Beyond that time point, staining appeared equivalent between control and TGF-β1-treated cells. However, after day 14, cultures treated with αMEM alone showed focal clusters of intensely stained cells that were not seen in the cultures treated with αMEM supplemented with TGF-β1 (See Fig. 1B, close-ups). Therefore, αMEM, even in the absence of ITS, appeared to support some degree of chondrocyte maturation; based on the appearance of these cultures, the inclusion of TGF-β1 early in the culture period facilitated this process. At later time points, cells cultured in αMEM alone continued to mature whereas inclusion of TGF-β1 in the αMEM culture medium appeared to inhibit complete chondrogenic differentiation as marked by the absence of focal Alcian blue staining. Based on densitometry of the cultures (Fig. 1C), total Alcian blue staining only showed a time-dependence.

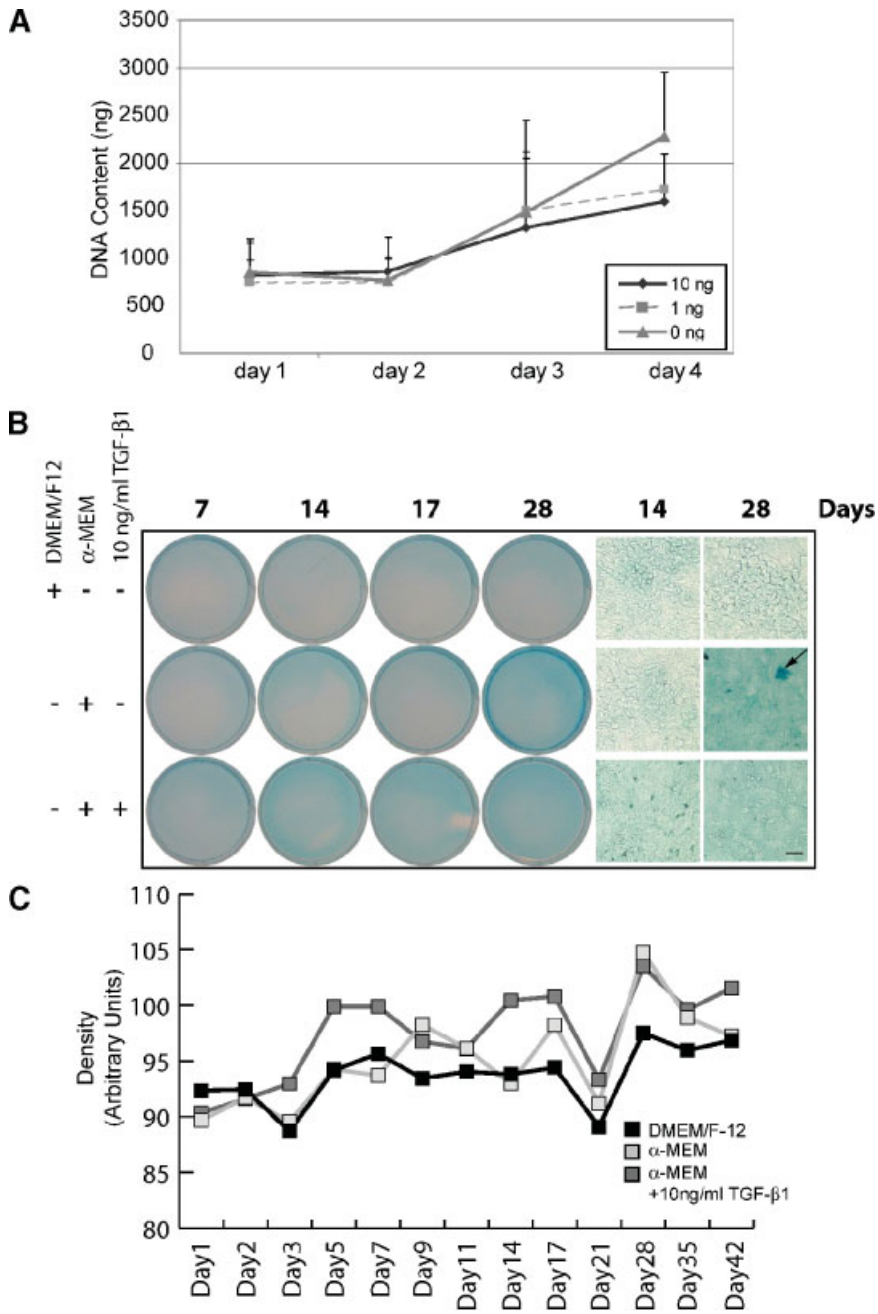

Fig. 1. TGF-β1 does not affect ATDC5 cell proliferation and accelerates Alcian blue staining. **A**: ATDC5 cells were maintained in αMEM containing 10 (◆), 1 (■), or 0 ng/ml (▲) TGF-β1 and cells stained for DNA content using the PicoGreen kit on the indicated days. **B**: ATDC5 cells were maintained in DMEM/F-12, αMEM or αMEM + 10 ng/ml TGF-α1 for times up to 42 days and sulfated proteoglycan content detected by Alcian blue staining. Representative samples for 7, 14, 17, and 28 days are shown, as are close-up views for the 14 and 28 day cultures, where the presence of focal clusters of cells can be readily observed (arrow, day 28 cultures). **C**: Relative densities of the stained plates with time. (■) DMEM/F-12; (□Î) αMEM; (■) αMEM + 10 ng/ml TGF-β1.

TGF-β1 Regulates Collagen Type II and Type I Accumulation in a Time-Dependent Manner

To examine the time course of maturation of the ATDC5 precursor cells, collagen type I and collagen type II mRNA levels at times through 42 days were measured by semi-quantitative RT-PCR. β-actin amplified in parallel was used as a control. Based on normalization of the data, two groups were analyzed. The first group

corresponds to the proliferating, condensing pre-chondrocyte, and the second group has the characteristics of the post-confluence pre-hypertrophic chondrocyte [Atsumi et al., 1990]. While statistical analysis showed no significant differences between groups in either the early or late culture periods (Fig. 2B), TGF-β1 treatment appeared to consistently depress col II expression early. Effects of TGF-β1 at times after 12 days showed the same trends as controls.

Collagen type I expression decreased over time under all conditions (Fig. 3). Although, as consistent with collagen II, statistical analysis showed no significant differences between groups, TGF-β1 treatment tended to cause consistently lower expression of collagen I during the early time period. After day 12, collagen type I expression is slowly lost under all conditions.

TGF-β1 Regulates Alternative Splicing of Fibronectin in a Dose- and Time-Dependent Manner

Because TGF-β1 alters alternative splicing of FN [Balza et al., 1988; Borsi et al., 1992; Viedt et al., 1995; Burger et al., 1998; Inoue et al., 1999], we next investigated the effects of TGF-β1 on FN isoform expression in the condensing pre-chondrocytic cell. FN RNA was amplified by RT-PCR using primers that span the EDA or EDB splice sites. The amounts of any particular isoform, as measured by densitometry, were expressed as a percentage of the total amount of FN amplified in that experiment (i.e., ([FN A+]/([FN A+] + [FN A−])) × 100). This representation of FN alternative splicing has been validated as not only accurate but preferable for RT-PCR measurements of exon usage [Magnuson et al., 1991]. Inclusion of the EDA exon (A+) was

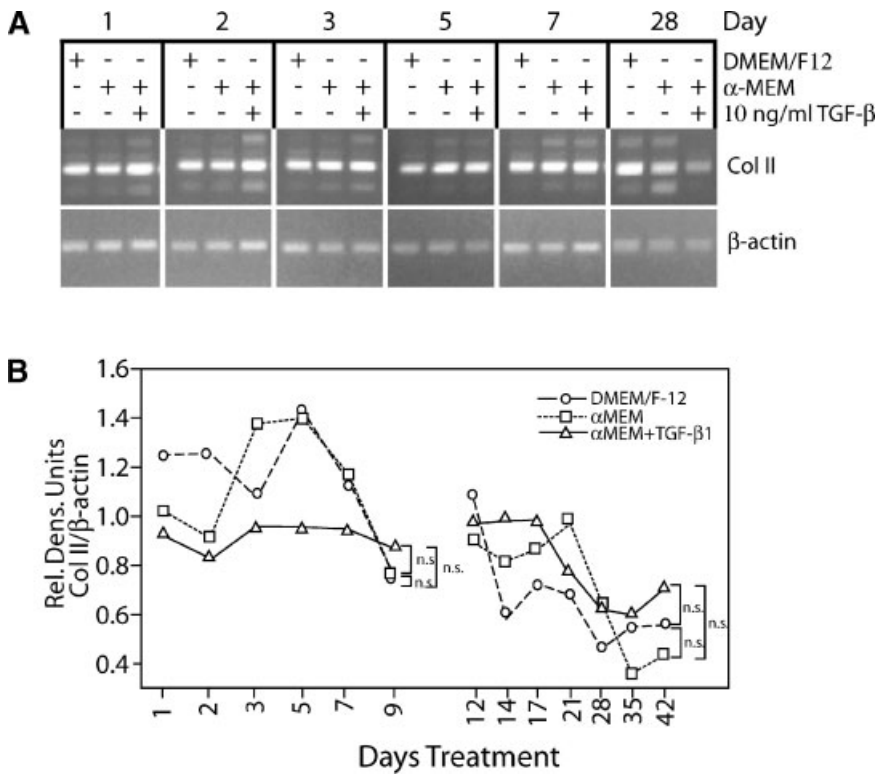

Fig. 2. Effects of 10 ng/ml TGF-β1 with time on collagen type II mRNA expression. ATDC5 cells were maintained in DMEM/F-12, αMEM, or αMEM + 10 ng/ml TGF-β1 at times out to 42 days. **A**: Representative gel for effects of TGF-β1 on collagen type II and β-actin at 1, 2, 3, 5, 7, and 28 days is shown. The samples come from different regions of the same gel for each of the three. **B**: Densitometry of the complete time course, normalized to β-actin that was amplified in parallel from the same cDNA synthesis. Statistical analysis of the data showed that it consistently broke into two groups comprising days 1–9 and 12–42. Statistical analysis of group values showed that different treatments did not cause significant changes in expression of collagen type II (ns in figure). (○) DMEM/F-12; (□) αMEM; (△) αMEM + 10 ng/ml TGF-β1.

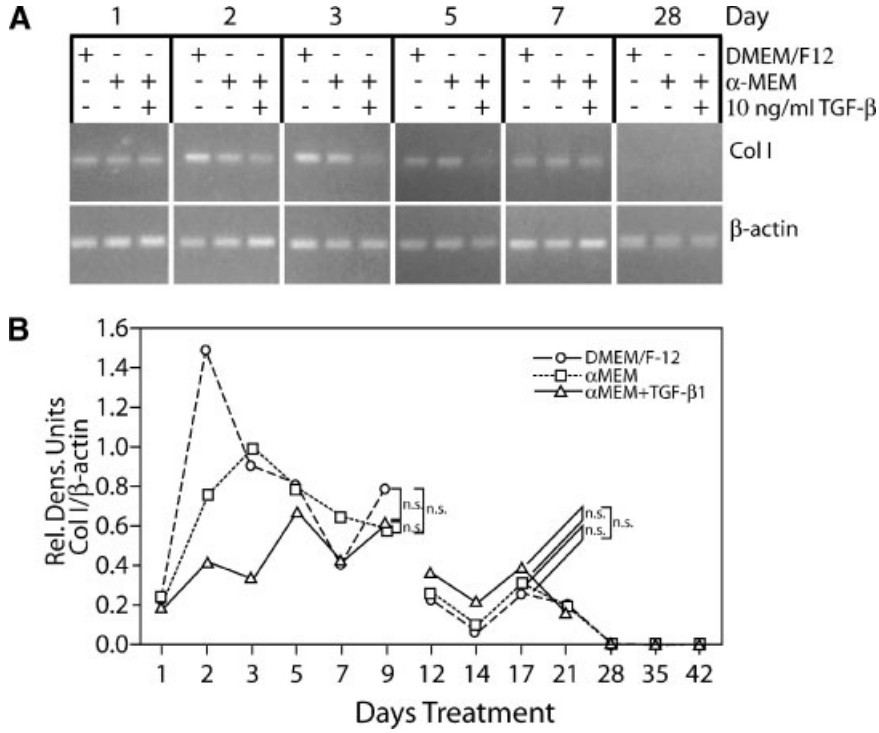

Fig. 3. Effects of 10 ng/ml TGF-β1 with time on collagen type I mRNA expression. ATDC5 cells were maintained in DMEM/F-12, αMEM, or αMEM + 10 ng/ml TGF-β1 at times out to 42 days. **A**: Representative gel for effects of TGF-β1 on collagen type I and β-actin at 1, 2, 3, 5, 7, and 28 days is shown. The samples come from different regions of the same gel for each of the three. **B**: Densitometry of the above gels, normalized to β-actin that was amplified in parallel from the same cDNA synthesis. The overall trend was for decreased expression, with no significant differences (ns) observed between the groups, although there was an early trend towards decreased col I expression in the presence of TGF-β1. (○) DMEM/F-12; (□) αMEM; (△) αMEM + 10 ng/ml TGF-β1.

decreased in a dose-dependent manner on both days 2 and 4 (Fig. 4); 1 ng/ml TGF-β1 showed little effect whereas the 10 ng/ml dose greatly decreased FN EDA splicing. Similar results were seen in the pre-chondrogenic DMEM and α-MEM that is permissive for chondrogenesis. In both media, sensitivity to the effects of TGF-β1 was greater on days 4 than on 2. Thus, TGF-β1 treatment of ATDC5 cells favored exclusion of the EDA exon both in αMEM and in DMEM.

Similarly, splicing of the EDB exon also showed a dependency on TGF-β1 dose (Fig. 5). Like EDA splicing, at 1 ng/ml TGF-β1, treatment reproducibly caused a small increase in EDB exclusion; 10 ng/ml caused a large effect such that much greater than 50% of the FN excluded the EDB exon. These effects were independent of the medium. However, sensitivity to the effects of TGF-β1 was increased on day 4, in comparison to day 2. Thus, TGF-β1 treatment increased exclusion of the EDA and of the EDB exon in both a time- and dose-dependent manner at both times tested.

We therefore tested the effects of TGF-β1 on FN expression throughout the 42 days that we used to characterize the response of collagen types I and II. Inclusion of the EDA exon was favored when ATDC5 cells were cultured in the presence of DMEM/F12 or αMEM (Fig. 6). As determined by densitometry (Fig. 6B), incubation with TGF-β1 significantly decreased levels of FN containing the EDA exon throughout the entire culture period. This exclusion of the EDA exon is consistent with increased commitment to chondrogenesis.

Inclusion of the EDB exon was significantly favored in ATDC5 cells cultured in DMEM/F-12, the maintenance medium (Fig. 7). In this and some other RT-PCR experiments amplifying the EDB region of FN, a band sometimes appeared that was larger than expected for EDB+. To confirm the assignment of the bands, all three bands were sequenced after purification. The top band corresponded to a non-specific product; the second band represented EDB+ and the smallest band EDB−, as

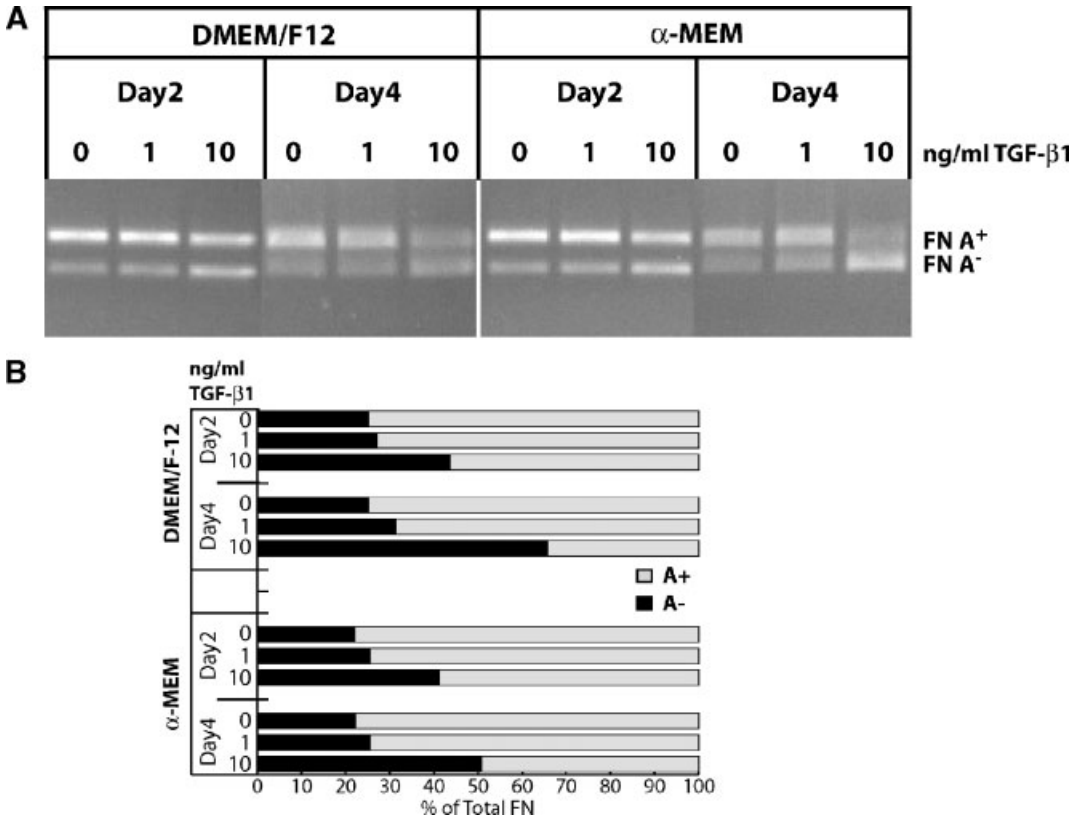

Fig. 4. TGF-β1 treatment favors exclusion of the FN extra domain A (EDA) exon and is dose-responsive. ATDC5 cells were maintained in αMEM or DMEM/F-12 containing 0, 1, or 10 ng/ml TGF-β1 for 2 or 4 days. RNA was collected and amplified by RT-PCR. **A**: Representative gel for effects of TGF-β1 on FN EDA. Inclusion of the exon results in a longer product, designated as A+; similarly the exclusion of the EDA exon results in a shorter project labeled A−. **B**: Densitometry of the above gels. Amounts for each of the splice products are expressed as a percentage of the total, that is, FN A+ = {[FN A+]/([FN A+] + [FN A−])} × 100. Both the DMEM/F-12 proliferative conditions and the α-MEM media conditions gave similar responses in the presence of TGF-β1. □ Percentage of A+ containing amplification product; ■ Percentage of A− containing product.

indicated on the figure. For determination of the splicing pattern of the EDB exon, as was seen with collagen type I and type II, there seemed to be a phenotypic change in the cells between days 9 and 12 so that significance was determined separately for the early and late period (Fig. 7B). In the early period (1–9 days), TGF-β1 treatment increased FN EDB exclusion and the level of EDB-containing FN was significantly less than both control levels; at later times (12–42 days), this effect modulated such that cells cultured in αMEM showed a greater exclusion of the EDB exon. This increased exclusion of the FN EDB exon was not characteristic of the known cartilage FN splicing pattern.

Therefore, as in the measures of commitment to the chondrogenic lineage, TGF-β1 treatment causes the most marked effects on FN splicing early in chondrogenesis. However, while exclusion of the EDA exon is consistent with the initiation of chondrogenesis, exclusion of the EDB exon is not, suggesting that at least in ATDC5 cells, TGF-β1 treatment may dissociate effects on chondrogenesis from effects on FN splicing.

Finally, as inclusion of the FN EDA exon has been described to be dependent on interaction with the splicing protein, SRP40, we examined regulation of this factor under parallel conditions to those described above. No dependence of SRP40 expression on TGF-β1 treatment was measured in pre-chondrogenic ATDC5 cells (Han and Hickok, unpublished results).

DISCUSSION

Chondrogenesis is a complex series of events that is characterized by cellular condensation, followed by proliferation and elaboration of a sulfated proteoglycan rich matrix. The cell line ATDC5 serves as an in vitro system to model the stages of chondrogenic differentiation

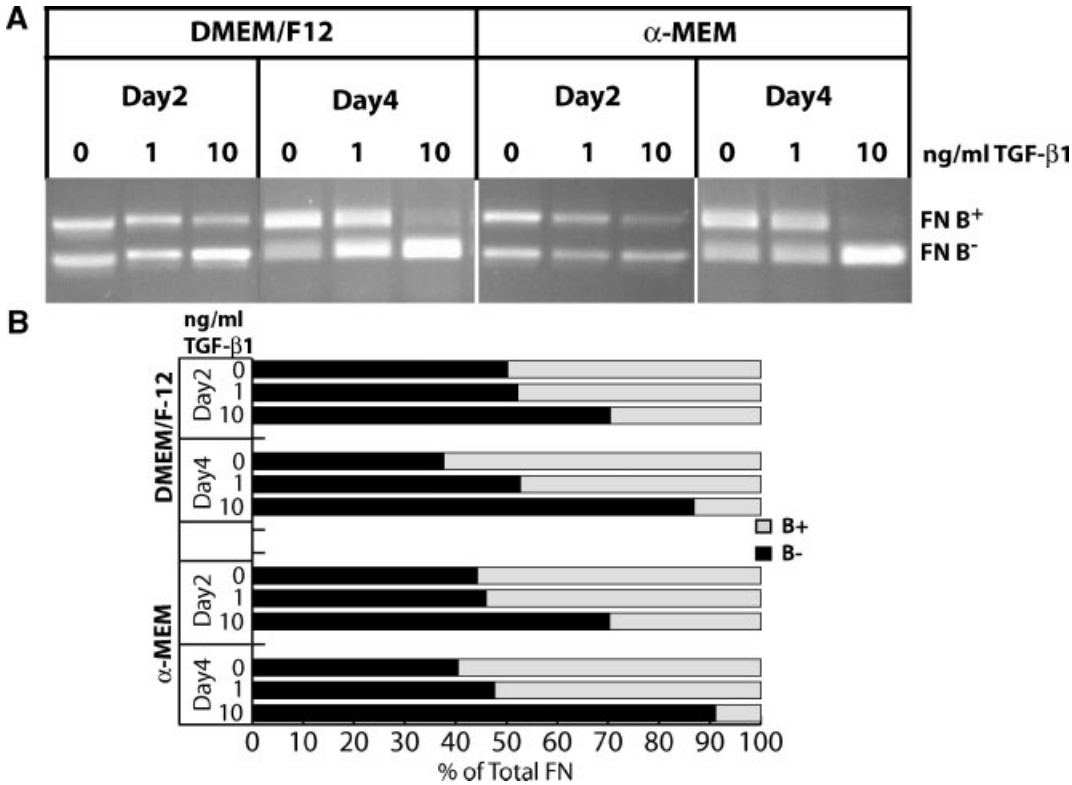

Fig. 5. TGF-β1 treatment favors exclusion of the FN extra domain B (EDB) exon and is dose-responsive. ATDC5 cells were maintained in αMEM or DMEM/F-12 containing 0, 1, or 10 ng/ml TGF-β1 for 2 or 4 days. RNA was collected and amplified by RT-PCR. **A**: Representative gel for effects of TGF-β1 on FN EDB inclusion. Inclusion of the exon results in a longer product, designated as B+; similarly the exclusion of the EDB exons results in a shorter project labeled or B−. **B**: Densitometry of the above gels. Amounts for each of the splice products are expressed as a percentage of the total, that is, FN B+ = {[FN B+]/([FN B+] + [FN B−])} × 100. The two different media resulted in similar responses to TGF-β1 treatment. □ Percentage of B+ containing amplification product. ■ Percentage of B− containing product.

and maturation, with defined stimuli being necessary for the progression from one stage to the next [Atsumi et al., 1990; Shukunami et al., 1996; Siebler et al., 2002]. While the phenotypic effects of TGF-β1 on ATDC5 cells combined with insulin are known [Kawai et al., 1999; Palmer et al., 2000], this is the first report of the effects of TGF-β1 on FN splicing during the transition from the mesenchymal precursor to committed chondrogenic cell. By characterizing ATDC5 cells in the absence of insulin, we have maintained the cells in their pre-chondrogenic state and asked to what extent treatment with TGF-β1 will drive them down the differentiation pathway. TGF-β1 accelerated early chondrogenesis as evidenced by increased Alcian blue staining and decreased expression of collagen type I. Because the presence of the many factors in serum could influence ATDC5 differentiation, we also performed mRNA expression analysis over the first 4 days of culture in the presence and absence of serum. For all analyses and culture conditions, trends observed in serum-free medium paralleled those observed in serum-containing medium. In some cases, the serum-free cultures seemed to very slightly favor (<10%–15%) a more dedifferentiated pattern (Han and Hickok, unpublished results). During the later culture period (>12 days), a time that has been associated with the post-condensation, pre-hypertrophic stage [Atsumi et al., 1990], TGF-β1 treatment appeared to no longer facilitate the maintenance of the chondrogenic phenotype, based on the markers of chondrogenesis. However, higher relative levels of FN B+ mRNA were present in these same cultures, suggesting attainment of a more chondrocytic splicing pattern and underlining the complexity of the many events that integrate to signal chondrocytic maturation.

TGF-β has been used under many circumstances with mesenchymal pre-cursors to induce their differentiation into the chondrogenic pathway [Cassiede et al., 1996; Worster et al.,

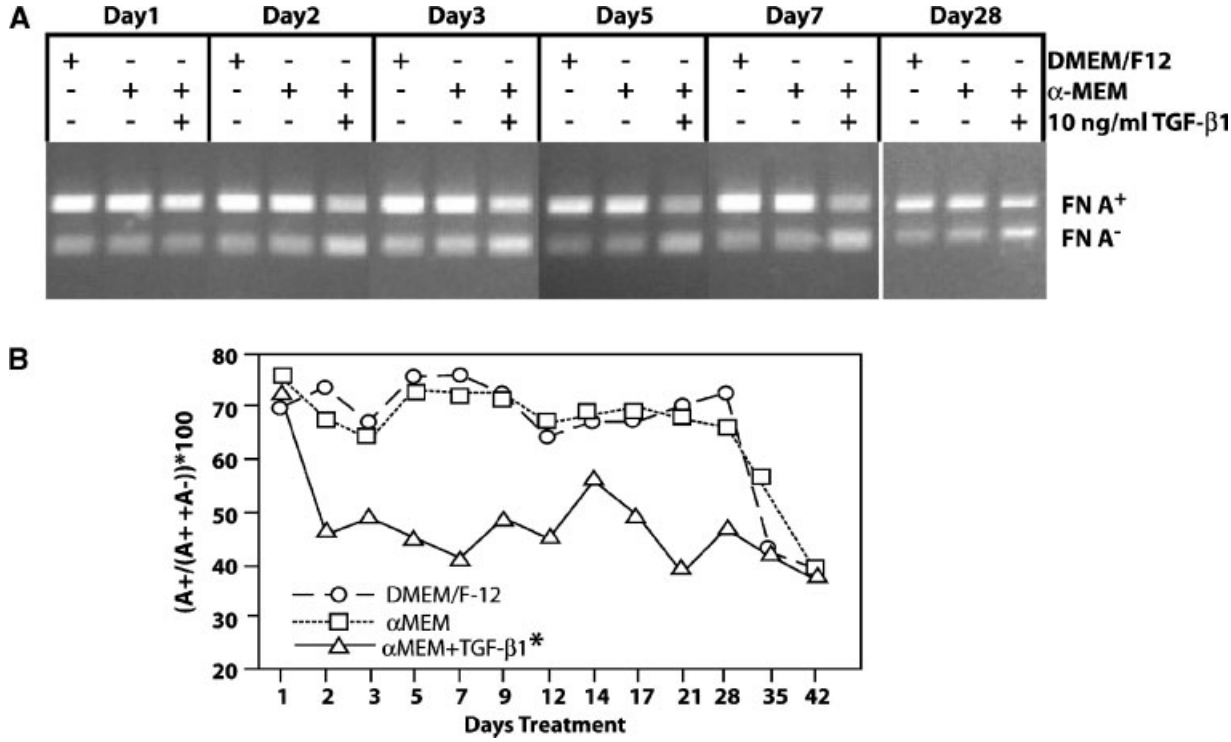

Fig. 6. 10 ng/ml TGF-β1 causes a time-dependent exclusion of the FN extra domain A (EDA) exon. ATDC5 cells were maintained in DMEM/F-12, αMEM, or αMEM + 10 ng/ml TGF-β1 at times out to 42 days. **A**: Representative gel for effects of TGF-β1 on inclusion of the FN EDA exon at 1, 2, 3, 5, 7, and 28 days is shown. **B**: Densitometry of the complete time course gels. Amounts of the splice products are expressed as a percentage of the total, (i.e., FN A+ = {[FN A+]/([FN A+] + [FN A−])} × 100. Results for inclusion of the A exon are shown in the left graph; results for A− are the reciprocal of those for A+. Statistical analysis showed that TGF-β1 treatment significantly decreased EDA inclusion, thereby significantly increasing EDA exclusion (*). (○) DMEM/F-12; (□) αMEM; (△) αMEM + 10 ng/ml TGF-β1.

2001; Nakayama et al., 2003; Tuli et al., 2003]. These primary mesenchymal cells are capable of sustaining their differentiation and maturation program solely with the stimuli supplied by the high-density cultures and the medium conditions. Our studies suggest that while TGF-β1 is capable of facilitating the initial stages of chondrogenic differentiation, it is incapable of stimulating further differentiation and may, in fact, be inhibitory toward terminal differentiation.

This programmed differentiation of the chondroprogenitor cells is accompanied by a stage-specific expression of FN isoforms. Again, TGF-β1 has been shown in multiple systems to influence FN splicing [Balza et al., 1988; Magnuson et al., 1991; Wang et al., 1991; Borsi et al., 1992; Zhang et al., 1995; Inoue et al., 1998, 1999]. This influence has been to favor inclusion of the EDA exon, and when measured, the EDB exon. In our system, TGF-β1 treatment favored exclusion of both the EDA and EDB exons. While others have reported that TGF-β treatment modestly increased EDA inclusion in articular chondrocytes, with no significant alteration in the extent of EDB inclusion [Zhang et al., 1995], some evidence suggests that the ability of TGF-β to alter FN splicing is dependent on cell growth state and/or cell architecture. Increased cell density favored a decline in EDA inclusion in fibroblasts; TGF-β treatment had little effect on EDA inclusion in low-density cultures but increased inclusion in high-density cultures [Inoue et al., 1998]. In polarized airway epithelial cells, the response to TGF-β treatment depended on the nature of the stimulation, with only apical stimulation resulting in increased EDA inclusion [Wang et al., 1991].

Based on our data, TGF-β1 treatment of ATDC5 cells is able to accelerate early assumption of the chondrogenic phenotype and loss of the EDA and EDB exons. Loss of the EDA exon is compatible with known events in chondrogenesis; loss of the EDB exon is not. Loss of

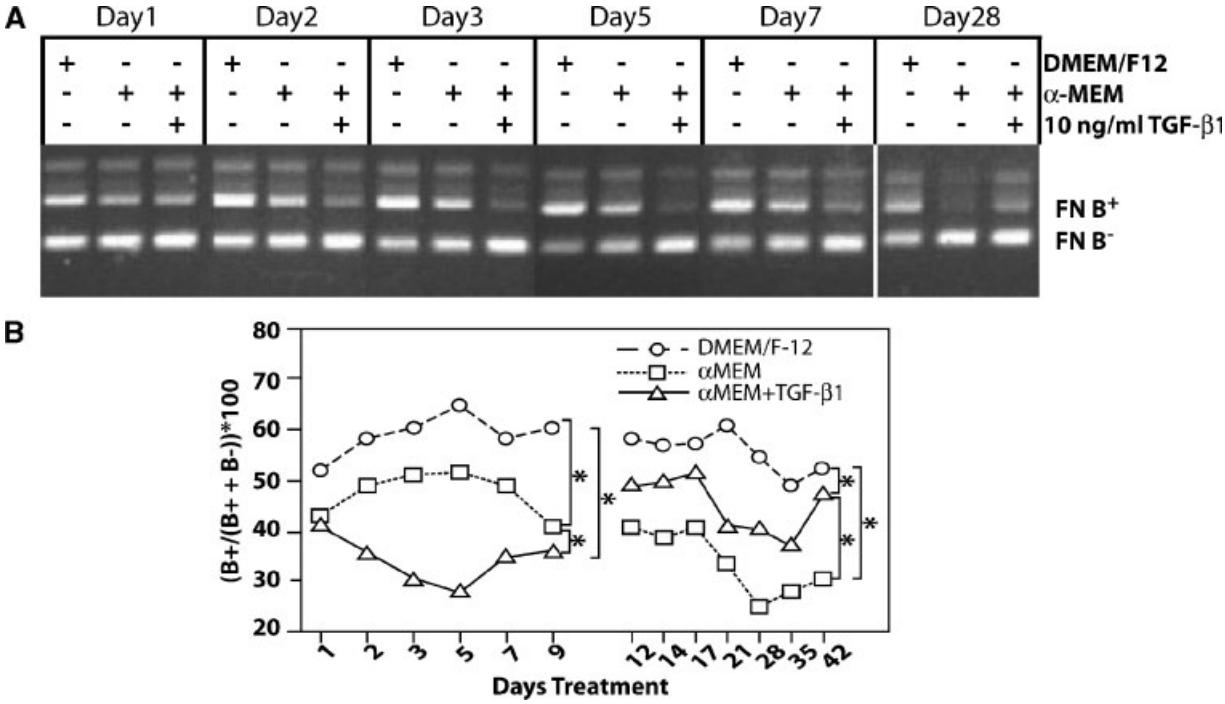

Fig. 7. 10 ng/ml TGF-β1 causes a time-dependent exclusion of the FN extra domain B (EDB) exon. ATDC5 cells were maintained in DMEM/F-12, αMEM, or αMEM + 10 ng/ml TGF-β1 at times out to 42 days. **A**: Representative gel for effects of TGF-β1 on inclusion of the FN EDB exon at 1, 2, 3, 5, 7, and 28 days is shown. **B**: Densitometry of the complete time course gel. Amounts for each of the splice products are expressed as a percentage of the total, that is, FN B+ = {[FN B+]/([FN B+] + [FN B−])} × 100. Results for inclusion of the B exon are shown in the left graph; results for B− are the reciprocal of B+. As in Figures 2 and 3, the data split into an early and late time course encompassing days 1–9 and 12–42, respectively. During the early period, statistical analysis showed that TGF-β1 treatment significantly decreased EDB inclusion, thereby significantly increasing EDB exclusion (*). During the later period, TGF-β1 treatment resulted in a splicing pattern intermediate between that observed with DMEM/F-12 and α-MEM and the amounts produced under these conditions were significantly different from each other for each condition(*). (○) DMEM/F-12; (□) αMEM; (△) αMEM + 10 ng/ml TGF-β1.

the two exons would favor a more rounded phenotype [White et al., 2003], an event necessary for progression of chondrogenesis [Loty et al., 1995] and could therefore be a key event in the early effects of TGF-β1 on ATDC5 maturation [Benya and Padilla, 1993]. We are currently investigating the roles of these isoforms in more detail.

ACKNOWLEDGMENTS

We thank Dr. Terry Freeman for help with densitometry of Alcian blue-stained plates. This work was supported by NIH grants AR45181 (N.J.H.), AR44360 (C.J.W.), AR39740 (C.J.W. and N.J.H.), AR46821 (P.A.N.), DE-13310 (C.S.A.), DE-10875 (C.S.A.), and DE-05748 (C.S.A.). We also acknowledge sponsorship of this work by the Department of the Army, Award no. DAMD17-03-1-0713 (N.J.H. and C.S.A.). The US Army Medical Research Acquisition Activity, 820 Chandler Street, Fort Detrick MD 21702-5014 is the awarding and administering acquisition office. The content of the information in this poster does not necessarily reflect the position or the policy of the Government, and no official endorsement should be inferred.

REFERENCES

Asahina I, Sampath TK, Hauschka PV. 1996. Human osteogenic protein-1 induces chondroblastic, osteoblastic, and/or adipocytic differentiation of clonal murine target cells. Exp Cell Res 222:38–47.

Atsumi T, Miwa Y, Kimata K, Ikawa Y. 1990. A chondrogenic cell line derived from a differentiating culture of AT805 teratocarcinoma cells. Cell Diff Develop 30:109–116.

Balza E, Borsi L, Allemanni G, Zardi L. 1988. Transforming growth factor beta regulates the levels of different fibronectin isoforms in normal human cultured fibroblasts. FEBS Lett 228:42–44.

Bennett VD, Pallante KM, Adams SL. 1991. The splicing pattern of fibronectin mRNA changes during chondrogenesis resulting in an unusual form of the mRNA in cartilage. J Biol Chem 266:5918–5924.

Benya PD, Padilla SR. 1993. Dihydrocytochalasin B enhances transforming growth factor-beta-induced reexpression of the differentiated chondrocyte phenotype without stimulation of collagen synthesis. Exp Cell Res 204:268–277.

Borsi L, Balza E, Allemanni G, Zardi L. 1992. Differential expression of the fibronectin isoform containing the ED-B oncofetal domain in normal human fibroblast cell lines originating from different tissues. Exp Cell Res 199: 98–105.

Burger A, Wagner C, Viedt C, Reis B, Hug F, Hansch GM. 1998. Fibronectin synthesis by human tubular epithelial cells in culture: Effects of PDGF and TGF-beta on synthesis and splicing. Kidney Int 54:407–415.

Burton-Wurster N, Lust G, Macleod JN. 1997. Cartilage fibronectin isoforms: In search of functions for a special population of matrix glycoproteins. Matrix Biol 15:441–454.

Cassiede P, Dennis JE, Ma F, Caplan AI. 1996. Osteochondrogenic potential of marrow mesenchymal progenitor cells exposed to TGF-beta 1 or PDGF-BB as assayed in vivo and in vitro. J Bone Min Res 11:1264–1273.

Chen W, Culp LA. 1998. Adhesion to fibronectin's EDB domain induces tyrosine phosphorylation of focal adhesion proteins in Balb/c 3T3 cells. Clin Exp Metastasis 16:30–42.

Colman-Lerner A, Fischman ML, Lanuza GM, Bissell DM, Kornblihtt AR, Baranao JL. 1999. Evidence for a role of the alternatively spliced ED-I sequence of fibronectin during ovarian follicular development. Endocrinology 140:2541–2548.

ffrench-Constant C. 1995. Alternative splicing of fibronectin—many different proteins but few different functions. Exp Cell Res 221:261–271.

Fukuda T, Yoshida N, Kataoka H, Manabe R, Mizuno-Horikawa Y, Sato M, Kuriyama K, Yasui N, Sekiguchi K. 2002. Mice lacking the EDB segment of fibronectin develop normally but exhibit reduced cell growth and fibronectin matrix assembly in vitro. Cancer Res 62: 5603–5610.

Gehris AL, Brandli DW, Lewis SD, Bennett VD. 1996a. The exon encoding the fibronectin type III-9 repeat is constitutively included in the mRNA from chick limb mesenchyme and cartilage. Biochimica et Biophysica Acta 1311:5–12.

Gehris AL, Oberlender SA, Shepley KJ, Tuan RS, Bennett VD. 1996b. Fibronectin mRNA alternative splicing is temporally and spatially regulated during chondrogenesis in vivo and in vitro. Dev Dyn 206:219–230.

Guan JL, Trevithick JE, Hynes RO. 1990. Retroviral expression of alternatively spliced forms of rat fibronectin. J Cell Biol 110:833–847.

Hashimoto-Uoshima M, Yan YZ, Schneider G, Aukhil I. 1997. The alternatively spliced domains EIIIB and EIIIA of human fibronectin affect cell adhesion and spreading. J Cell Sci 110:2271–2280.

Hickok NJ, Haas AR, Tuan RS. 1998. Regulation of chondrocyte differentiation and maturation. Microsc Res Tech 43:174–190.

Hocevar BA, Brown TL, Howe PH. 1999. TGF-beta induces fibronectin synthesis through a c-Jun N-terminal kinase-dependent, Smad4-independent pathway. EMBO J 18: 1345–1356.

Inoue T, Nabeshima K, Shimao Y, Kataoka H, Koono M. 1998. Cell density-dependent regulation of fibronectin splicing at the EDA region in fibroblasts: Cell density also modulates the responses of fibroblasts to TGF-beta and cancer cell-conditioned medium. Cancer Lett 129: 45–54.

Inoue T, Nabeshima K, Shimao Y, Koono M. 1999. Hepatocyte growth Factor/Scatter factor (HGF/SF) is a regulator of fibronectin splicing in MDCK cells: Comparison between the effects of HGF/SF and TGF-beta1 on fibronectin splicing at the EDA region. Biochem Biophys Res Commun 260:225–231.

Kawai J, Akiyama H, Shigeno C, Ito H, Konishi J, Nakamura T. 1999. Effects of transforming growth factor-beta signaling on chondrogenesis in mouse chondrogenic EC cells, ATDC5. Eur J Cell Biol 78:707–714.

Kornblihtt AR, Umezawa K, Vibe-Pedersen K, Baralle FE. 1985. Primary structure of human fibronectin: Differential splicing may generate at least 10 polypeptides from a single gene. EMBO J 4:1755–1759.

Kuo BA, Uporova TM, Liang H, Bennett VD, Tuan RS, Norton PA. 2002. Alternative splicing during chondrogenesis: Modulation of fibronectin exon EIIIA splicing by SR proteins. J Cell Biochem 86:45–55.

Loty S, Forest N, Boulekbache H, Sautier J-M. 1995. Cytochalasin D induces changes in cell shape and promotes in vitro chondrogenesis: A morphological study. Biol Cell 83:149–161.

Lust G, Burton-Wurster N, Leipold H, Steinmeyer J, Todhunter R. 1991. Modulation of fibronectin and proteoglycan synthesis by chondrocytes. Scand J Rheumatol Suppl 27:58–59.

MacLeod JN, Burton-Wurster N, Gu DN, Lust G. 1996. Fibronectin mRNA splice variant in articular cartilage lacks bases encoding the V, III-15, and I-10 protein segments. J Biol Chem 271:18954–18960.

Magnuson VL, Young M, Schattenberg DG, Mancini MA, Chen DL, Steffensen B, Klebe RJ. 1991. The alternative splicing of fibronectin pre-mRNA is altered during aging and in response to growth factors. J Biol Chem 266: 14654–14662.

Manabe R, Ohe N, Maeda T, Fukuda T, Sekiguchi K. 1997. Modulation of cell-adhesive activity of fibronectin by the alternatively spliced EDA segment. J Cell Biol 139:295–307.

Manabe R, Ohe N, Sekiguchi K. 1999. Alternatively spliced EDA segment regulates fibronectin-dependent cell cycle progression and mitogenic signal transduction. J Biol Chem 274:5919–5924.

Muro AF, Chauhan AK, Gajovic S, Iaconcig A, Porro F, Stanta G, Baralle FE. 2003. Regulated splicing of the fibronectin EDA exon is essential for proper skin wound healing and normal lifespan. J Cell Biol 162:149–160.

Nakayama N, Duryea D, Manoukian R, Chow G, Han CY. 2003. Macroscopic cartilage formation with embryonic stem-cell-derived mesodermal progenitor cells. J Cell Sci 116:2015–2028.

Norton PA, Hynes RO. 1987. Alternative splicing of chicken fibronectin in embryos and in normal and transformed cells. Mol Cell Biol 7:4297–4307.

Palmer G, Guicheux J, Bonjour JP, Caverzasio J. 2000. Transforming growth factor-beta stimulates inorganic phosphate transport and expression of the type III phosphate transporter Glvr-1 in chondrogenic ATDC5 cells. Endocrinology 141:2236–2243.

Paul JI, Schwarzbauer JE, Tamkun JW, Hynes RO. 1986. Cell-type-specific fibronectin subunits generated by alternative splicing. J Biol Chem 261:12258–12265.

Peters JH, Hynes RO. 1996. Fibronectin isoform distribution in the mouse. I. The alternatively spliced EIIIB, EIIIA, and V segments show widespread codistribution in the developing mouse embryo. Cell Adhes Commun 4:103–125.

Schwarzbauer JE, Tamkun JW, Lemischka IR, Hynes RO. 1983. Three different fibronectin mRNAs arise by alternative splicing within the coding region. Cell 35:421–431.

Schwarzbauer JE, Patel RS, Fonda D, Hynes RO. 1987. Multiple sites of alternative splicing of the rat fibronectin gene transcript. EMBO J 6:2573–2580.

Shukunami C, Shigeno C, Atsumi T, Ishizeki K, Suzuki F, Hiraki Y. 1996. Chondrogenic differentiation of clonal mouse embryonic cell line ATDC5 in vitro: Differentiation-dependent gene expression of parathyroid hormone (PTH)/PTH-related peptide receptor. J Cell Biol 133: 457–468.

Shukunami C, Ohta Y, Sakuda M, Hiraki Y. 1998. Sequential progression of the differentiation program by bone morphogenetic protein-2 in chondrogenic cell line ATDC5. Exp Cell Res 241:1–11.

Siebler T, Robson H, Shalet SM, Williams GR. 2002. Dexamethasone inhibits and thyroid hormone promotes differentiation of mouse chondrogenic ATDC5 cells. Bone 31:457–464.

Tan MH, Sun Z, Opitz SL, Schmidt TE, Peters JH, George EL. 2004. Deletion of the alternatively spliced fibronectin EIIIA domain in mice reduces atherosclerosis. Blood 104: 11–18.

Tuli R, Tuli S, Nandi S, Huang X, Manner PA, Hozack WJ, Danielson KG, Hall DJ, Tuan RS. 2003. Transforming growth factor-beta-mediated chondrogenesis of human mesenchymal progenitor cells involves N-cadherin and mitogen-activated protein kinase and Wnt signaling cross-talk. J Biol Chem 278:41227–41236.

Viedt C, Burger A, Hansch GM. 1995. Fibronectin synthesis in tubular epithelial cells: Up-regulation of the EDA splice variant by transforming growth factor beta. Kidney Int 48:1810–1817.

Wang A, Cohen DS, Palmer E, Sheppard D. 1991. Polarized regulation of fibronectin secretion and alternative splicing by transforming growth factor. J Biol Chem 266: 15598–15601.

White DG, Hershey HP, Moss JJ, Daniels H, Tuan RS, Bennett VD. 2003. Functional analysis of fibronectin isoforms in chondrogenesis: Full-length recombinant mesenchymal fibronectin reduces spreading and promotes condensation and chondrogenesis of limb mesenchymal cells. Differentiation 71:251–261.

Worster AA, Brower-Toland BD, Fortier LA, Bent SJ, Williams J, Nixon AJ. 2001. Chondrocytic differentiation of mesenchymal stem cells sequentially exposed to transforming growth factor-beta1 in monolayer and insulin-like growth factor-I in a three-dimensional matrix. J Orthop Res 19:738–749.

Wu LN, Genge BR, Ishikawa Y, Wuthier RE. 1992. Modulation of cultured chicken growth plate chondrocytes by transforming growth factor-beta 1 and basic fibroblast growth factor. J Cell Biochem 49:181–198.

Zhang DW, Burton-Wurster N, Lust G. 1995. Alternative splicing of ED-A and ED-B sequences of fibronectin premRNA differs in chondrocytes from different cartilaginous tissues and can be modulated by biological factors. J Biol Chem 270:1817–1822.