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Expression of c-fos gene inhibits proteoglycan synthesis in transfected chondrocyte

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Abstract The effect of expression of c-fos gene on proteoglycan synthesis, one of the important markers of cartilage metabolism, was examined by introducing the c-fos DNA into HCS 2/8 chondrocytes. The [^{35}S]sulfate incorporation into proteoglycan was decreased in the c-fos transfectants expressing exogenous c-fos mRNA, when compared to a control transfectant. A significant increase in transcription of MMP-3 with the suppressed transcription of aggrecan and TIMP-1 were also observed in the c-fos transfectants. Moreover, analysis of the effect of AP-1 proteins on the collagenase and TIMP-1 promoters in gastric carcinoma KKLS cells revealed that c-Fos combined with any of the Jun-related proteins failed to stimulate the TIMP-1 promoter, though collagenase promoter was effectively activated by any Fos/Jun-related protein heterocomplex. These findings indicate that the c-fos expression may govern the cartilage metabolism and hence may play an important role in the pathogenesis of joint destruction in arthritis.

Key words: c-fos; Chondrocyte; Proteoglycan; Matrix metalloproteinase-3; Tissue inhibitor of metalloproteinase-1; Activating protein-1

1. Introduction

The proto-oncogene c-fos is the cellular homologue of the v-fos which was originally obtained from the murine sarcoma virus [1,2]. Overexpression of c-fos in transgenic mice, carrying the exogenous mouse c-fos gene under the control of the metallothionein promoter, deteriorated bone metabolism [3]; interfering with bone formation by inhibiting collagen synthesis in osteoblast [4] and enhancing osteoclast differentiation [5]. In addition, Kuroki et al. [5] reported that expression of c-fos caused the extensive fibroblastic outgrowth of human synovial mesenchymal cells. These findings have been consistent with the observation that the advancing edge of rheumatoid pannus extending into cartilage surface was mainly composed of fibloblast-like synovial cells, and also that the impaired bone formation and enhanced osteoclastic bone resorption have been seen in the rheumatoid juxtaarticular bone [6]. Therefore, the c-fos gene has been considered playing a critical role in the pathogenesis of joint destruction in rheumatoid arthritis (RA). On the other hand, the concerted action of a number of matrix-degrading enzymes produced not only by synovial cells also by chondrocytes themselves has been implied for such a progressive destruction of articular-cartilage matrix.

Proteoglycan, one of the major component of cartilage matrix, could be an important marker of chondrocyte metabolism. Proteoglycan turnover is under control with an intricate balance between synthesis and degradation of the associated molecules. The degradation of proteoglycan is known to be controlled by both matrix metalloproteinases (MMP), especially stromelysin (MMP-3), and tissue inhibitor of metalloproteinase-1 (TIMP-1) [7,8]. It is possible to hypothesize that coordinated control over the expression of both MMP and TIMP is necessary for cartilage homeostasis, and that loss of this control mechanism, leading to an imbalance of degradative proteinases over protective TIMP activities, underlies the pathological tissue destruction in degenerative inflammatory diseases, like arthritis [9]. Since MMP-3 and TIMP-1 DNA contain AP-1-binding site in the upstream region [8,10–12], it is likely that the overexpression of c-fos gene may affect proteoglycan turnover by regulating the MMP-3 and TIMP-1 gene expression in chondrocytes.

These consideration has prompted us to examine the role of c-fos gene in the proteoglycan metabolism of human chondrocyte by transfecting human c-fos DNA into cultured chondrocytes.

2. Materials and methods

2.1. Cell culture

The human chondrosarcoma cell line HCS 2/8 cells (passage no. 35–39) [13] and gastric carcinoma KKLS, a cell line established by Mai et al. (Kanazawa University) [14] were maintained in Dulbecco's modified Eagle's medium (DMEM; Nissui, Japan) containing 10% fetal bovine serum (FBS; HyClone, Logan) at 37°C in a humidified atmosphere of 5% CO_2 in air.

2.2. DNA construction

pSV2neo-MT-hc-fos was obtained from pSV2neo, which was inserted with the cadmium-inducible human metallothionein promoter (human MT2a) and human c-fos fragment in *Bam*HI site. The fragment of human c-fos gene was isolated from c-fos pSPT18/19 [15] and mRNA destabilizing sequence [16,17] in its 3′ non-coding region was removed before insertion.

A self-ligation product of the pSV2neo-MT was used as a control. Human MT2a and pSV2neo were the gifts from Dr. T. Tokuhisa, Chiba University School of Medicine, and human c-fos pSPT18/19 was purchased from Japanese Cancer Research Resources Bank (JCRB, CO 054).

2.3. DNA transfection

DNA transfection was carried out by the electroporation method [18]. Following G418 selection, the resistant cells were cloned. Pre-

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sence of the transfected human c-fos gene was examined by polymerase chain reaction (data not shown).

2.4. Northern blot analysis

The total RNA was extracted from cultured cells by the acid guanidinium thiocyanate-phenol-chloroform (AGPC) method [19]. 15 μg of total RNA was denatured, separated by electrophoresis in 1% agarose-formaldehyde gels, transferred to Hybond N^+ nylon membranes (Amersham, Japan) and hybridized with the appropriate probes.

2.5. Hybridization probe

The probes for human c-fos (1.4-kb *Eco*RI-*Acc*I fragment of the human c-fos DNA obtained from the JCRB), metalloproteinase 3 (MMP-3) (1.4-kb *Sac*I-*Xho*I fragment of cDNA), tissue inhibitor of metalloproteinase 1 (TIMP-1) (0.6-kb *Cla*I-*Bam*HI fragment of cDNA) and the 1.2-kb β-actin were labeled with [α-^{32}P]dCTP by nick translation method and hybridized at 42°C in 50% formamide solution. After hybridization, the filter was washed in 0.1% sodium dodecyl sulfate (SDS) and 1×standard saline citrate (SSC) at 42°C. Probe-specific for aggrecan (nucleotides 1096–1120) of cDNA reported by Baldwin et al. [20], 5′-CTCGTGCCAGATCATCACCACACAT-3′ was synthesized with a DNA synthesizer (Applied Biosystems, Foster City, CA) and purified on an acrylamide gel. The oligonucleotide probe was end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase and hybridized in 20% formamide solution (20% formamide, 5×SSPE (0.15 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA), 5% SDS) at 54°C. The blots were washed in 2×SSPE and 1% SDS at 54°C to eliminate partial hybridization and to maintain specific hybridization of the complete 25-bp oligonucleotide.

2.6. Determination of proteoglycan synthesis

Proteoglycan synthesis was monitored by determining the [^{35}S]sulfate incorporation as previously described [21]. After chondrocytes were grown to confluency, they were preincubated in 0.1 ml of the DMEM containing 0.3% FBS for 24 h. The cells were then incubated for 6–12 h with 5 μM of $CdCl_2$ in 0.1 ml of DMEM with 0.3% FBS, and 5 μl of DMEM supplemented with 100 μCi/ml of [^{35}S]sulfate was added 6 h before the end of incubation. The media was then removed and the cell layers were solubilized with 0.5 N NaOH. The media and cell fractions were combined and neutralized with 6 N HCl. Proteoglycan synthesis was determined by measuring incorporation of [^{35}S]sulfate into the mixtures precipitated with cetylpyridinium chloride (CPC) after treatment with Pronase E. Data was expressed as the mean±S.D. Statistical analyses were carried out by Student's *t* test.

2.7. Determination of DNA

DNA content was determined by a standard fluorometric procedure [22].

2.8. Relative hydrodynamic sizes of proteoglycan

Cells were seeded at a density of 1×10^5 cells/35-mm plastic culture dish and cultured for 10 days in DMEM containing 10% FBS. They were then incubated for 12 h in the presence or absence of 5 μM of $CdCl_2$ in 1 ml of DMEM containing 10% FBS. 10 μl of DMEM supplemented with 100 μCi of [S^{35}]sulfate was added 6 h before the end of incubation. The medium was kept frozen at −70°C until analyzed. The cell layers were overlaid with 1.0 ml of 50 mM Tris–HCl (pH 8.0) buffer containing 4 M guanidine HCl, 1 mM iodoacetic acid, 1 mM phenylmethylsulfonyl fluoride, 0.1 M 6-amino-*n*-caproic acid, 20 mM EDTA, and 1 mg/ml benzamidine HCl. The culture dishes were then put on a shaker for 24 h at 4°C. After the clarification by centrifugation (4000×*g* for 15 min), the cell extracts were stored at −70°C until analyzed. Aliquots (0.5 ml) of the medium were mixed with 0.5-ml aliquots of the respective cell extracts and 1.0 ml of 8 M guanidine in water. The mixtures were applied to a Sepharose CL-2B column (1.6×96 cm) equilibrated in 4 M guanidine HCl, 50 mM Tris–HCl buffer, pH 8.0, with protease inhibitors.

2.9. Analysis of the TIMP-1 and collagenase promoter activity

Construction of expression plasmids and promoter plasmids have been described previously [12,23]. Briefly, expression plasmids for c-Jun, JunB, JunD, c-Fos, Fra-1 and Fra-2 were constructed by inserting the coding regions of human c-jun, jun B, jun D, c-fos, fra-1 and fra-2 genes, respectively, into cloning sites of the pSG5 vector. Promoter plasmids, collagenase promoter-CAT and TIMP-1 promoter-CAT, were constructed by fusing the collagenase and TIMP-1 promoters, respectively, to the chloramphenicol acetyltransferase (CAT) structural gene. For transfection, KKLS cells were seeded at 2×10^5 cells/40-mm dish, and cultured for 16 h in DMEM containing 5% FBS. Promoter CAT plasmids (2–10 μg) were co-transfected with or without 1 μg of the expression plasmids by calcium phosphate coprecipitation. After a 36-h incubation, the cells were harvested and CAT activity was determined [24]. Radioactive spots were quantified with a Bioimage analyzer BAS 2000 (Fuji Film)

3. Results and discussion

The time course of c-fos mRNA expression after induction by cadmium chloride ($CdCl_2$) was examined by Northern blot analysis (Fig. 1). The expression of c-fos mRNA was induced in pSV2neo-MT-hc-fos transfectants (f2, f5 and f17) after induction by 5×10^{-6} M cadmium. The message pattern showed the two bands with different sizes which were considered the c-fos messages, since these bands were consistently confirmed at the same locations in Northern blots using another human c-fos probe (data not shown). Interestingly, Rüther et al. previously reported when human MT2a promoter and c-fos together with a part of the polyoma virus early region 3′ were introduced into embryonal carcinoma cells, similar pattern of c-fos mRNA was observed in some transfectants, which was found to contain more than 100 copies of the exogenous c-fos DNA [25]. Thus, it is possible that the pattern of c-fos mRNA in f2, f5 and f17 may be due to, at least in part, the high copy numbers of exogenous c-fos gene constructs. The band in HCS 2/8 and C5 transfectant at 12 h after the cadmium induction may represent the endogenous c-fos mRNA. Zhu et al. have reported that the expression of c-fos mRNA was increased in HCS 2/8 cells as the cells grown over confluency [26]. In the sparse and subconfluent phases of culture, this band could not be found either in HCS 2/8 or C5 transfectant at 12 h after the cadmium induction.

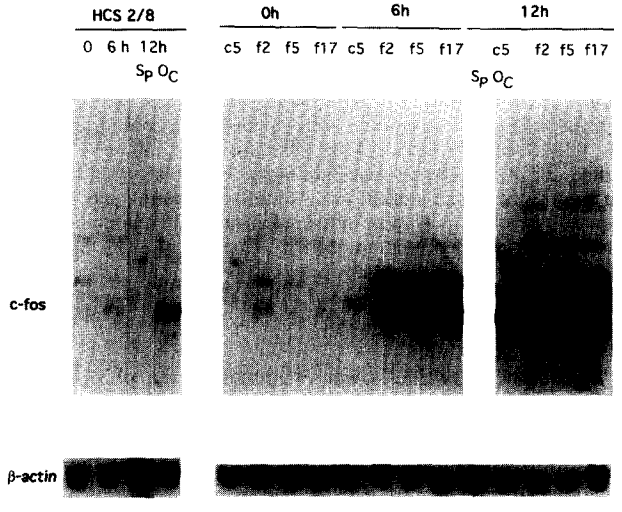

Fig. 1. Time course of c-fos mRNA expression after cadmium induction. 15 μg of total RNA was extracted from untransfected HCS 2/8 cells, pSV2neo-MT transfectant (c5) and pSV2neo-MT-hc-fos transfectants (f2, f5 and f17) at the times indicated in the figure as described in section 2. Equivalent amounts of total RNA/well were assessed by monitoring the β-actin mRNA. SP, in the sparse and OC, overconfluent phases of culture.

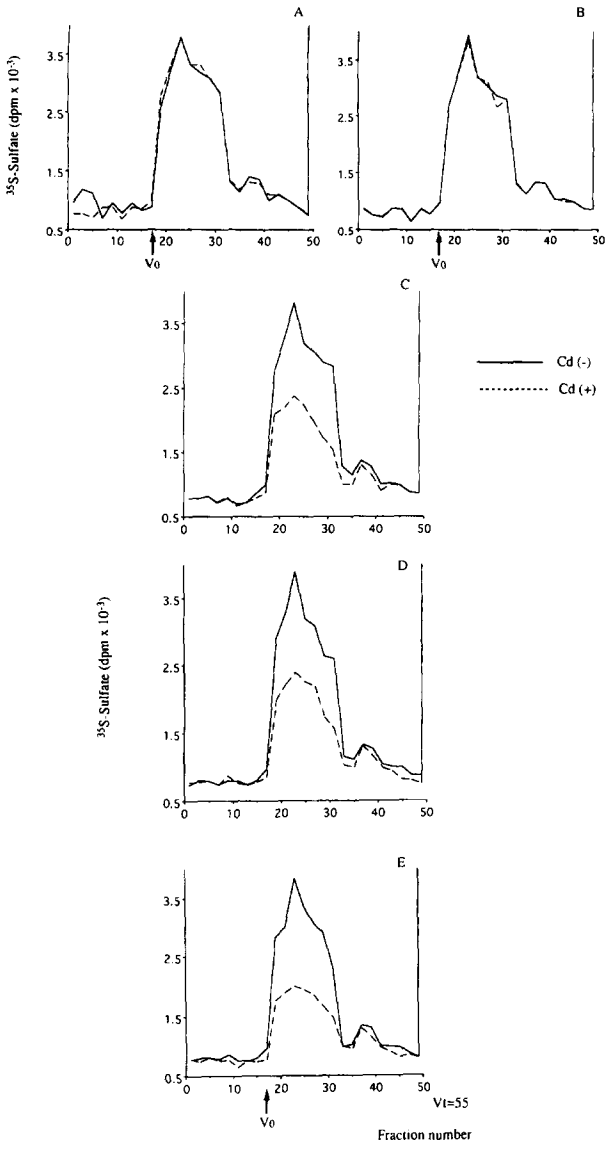

Fig. 2. Sepharose CL-2B chromatography of proteoglycans from cultures with (broken line) or without (solid line) cadmium induction. Cells were seeded and exposed to [^{35}S]sulfate as described in section 2. Portions (1 ml) of mixtures of the cell extract fraction and the medium from cultures were applied on a column of Sepharose CL-2B equilibrated in 4 M guanidine HCl, 50 mM Tris-HCl buffer, pH 8.0, with protease inhibitors. Aliquots (0.3 ml) of each fraction (3 ml) were mixed with 0.3 ml of ethanol and 10 ml of Aquasol. Radioactivity was measured in a LKB scintillation counter. Free unincorporated radioactivity eluted at V_t. V_0 represented the void volume. (A) Untransfected HCS 2/8 cells; (B) pSV2neo-MT transfectant (c5); (C, D and E) pSV2neo-MT-hc-fos transfectants f2, f5 and f17, respectively.

Proteoglycan synthesis was estimated in the pSV2neo-MT-hc-fos transfectants, pSV2neo-MT transfectant and also in untransfected HCS 2/8 cells by measuring incorporation of [^{35}S]sulfate into proteoglycan precipitated with CPC after pronase digestion. The rate of [^{35}S]sulfate incorporation into proteoglycan was significantly decreased after the cadmium induction in f2, f5 and f17 transfectants, while normal amount of proteoglycan was synthesized in C5 transfectant, which did not express exogenous c-fos mRNA (Table 1). Next, we analyzed the hydrodynamic sizes of ^{35}S-labeled proteoglycans by gel chromatography on Sepharose CL-2B. The elution profiles are shown in Fig. 2. Chondrocytes produce two proteoglycan population with different sizes: a large proteoglycan, aggrecan, characteristically found in the cartilage matrix and small proteoglycans found in both non-chondrogenic and chondrogenic cells. [^{35}S]sulfate incorporation into the large proteoglycan was significantly decreased after the cadmium induction in f2, f5 and f17 transfectants, while no significant change could be seen in C5 transfectant. The reduction of [^{35}S]sulfate incorporation in the large proteoglycan was greater than that in the small proteoglycan. These changes were coincident with the expression of exogenous c-fos mRNA. Though c-fos may have various effects on the regulation of proteoglycan metabolism, there are two major reasons to cause the observed decrement of the proteoglycan synthesis in the pSV2neo-MT-hc-fos transfectants; either the repression of aggrecan gene transcription by c-Fos or the digestion of the proteoglycan by MMP, especially by stromelysin (MMP-3) produced excessively in the pSV2neo-MT-hc-fos transfectants, since MMP-3 gene also contain AP-1 sites in its upstream promoter position.

Changes in expression of aggrecan mRNA were analyzed by Northern blot hybridization (Fig. 3B). Expression of aggrecan mRNA started to decrease 6 h after cadmium induction in f2, f5 and f17 transfectants but not in c5 or HCS 2/8 cells. Furthermore, in order to assess the influence of the degradation activities of MMP over the decrement of aggrecan synthesis in pSV2neo-MT-hc-fos transfectants, expression of MMP-3 and TIMP-1 mRNA were monitored by Northern blot analysis (Fig. 3A). The level of MMP-3 mRNA in f2, f5 and f17 transfectants was increased 6 h after cadmium induction. On the other hand, the level of TIMP-1 mRNA appeared to decrease significantly in f2, f5 and f17 transfectants at 6 h after the cadmium induction.

The finding that transfection with self-ligated pSV2neo-MT

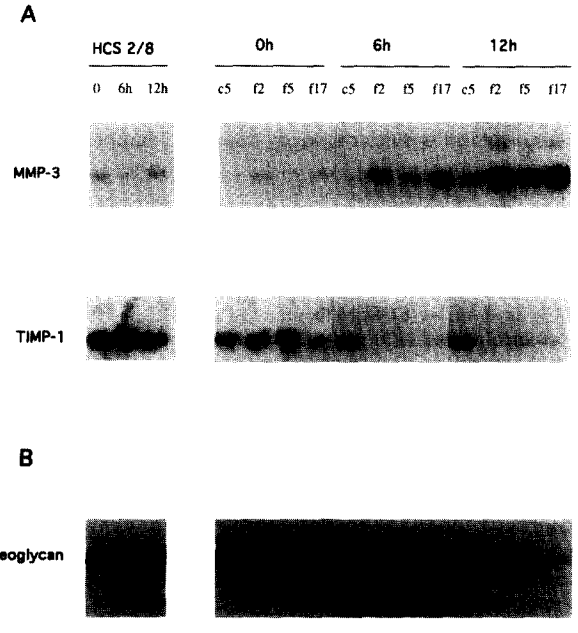

Fig. 3. Northern blot analysis of MMP-3, TIMP-1 and proteoglycan mRNA after cadmium induction. The same filters as in Fig. 1 were used for blotting with MMP-3, TIMP-1 (A) and proteoglycan (aggrecan) (B) probes. HCS 2/8, untransfected HCS 2/8 cells in the confluent phase, c5, pSV2neo-MT transfectant in the confluent phase; f2, f5 and f17, pSV2neo-MT-hc-fos transfectants.

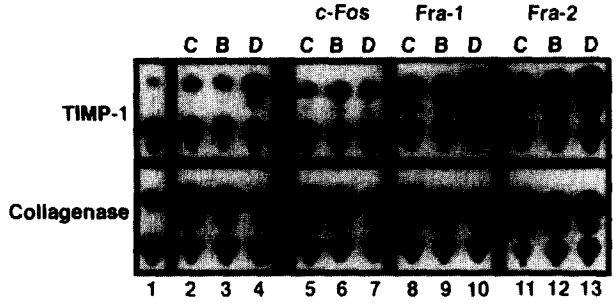

Fig. 4. Effect of AP-1 on the collagenase and TIMP-1 promoter genes. Promoter activity was analyzed by transient transfection assay using KKLS cells. Cells were co-transfected with 1 μg each of c-Jun, JunB, JunD, c-Fos, Fra-1 and Fra-2 expression plasmids, and 2–10 μg each of promoter plasmids, collagenase promoter-CAT and TIMP-1 promoter-CAT. The cells were harvested, and CAT activity was analyzed 36 h after the transfections. Lane 1, transfected with promoter plasmids; co-transfected with promoter plasmids and; c-Jun expression plasmid (lane 2, C); JunB expression plasmid (lane 3, B); JunD expression plasmid (lane 4, D); c-Jun and c-Fos expression plasmids (lane 5); JunB and c-Fos expression plasmids (lane 6); JunD and c-Fos expression plasmids (lane 7); c-Jun and Fra-1 expression plasmids (lane 8); JunB and Fra-1 expression plasmids (lane 9); JunD and Fra-1 expression plasmids (lane 10); c-Jun and Fra-2 expression plasmids (lane 11); JunB and Fra-2 expression plasmids (lane 12); JunD and Fra-2 expression plasmids (lane 13). Each experiment was performed in triplicate, and representative results are shown.

vector affected neither proteoglycan synthesis nor the transcription of aggrecan, MMP-3 and TIMP-1 genes, indicates that SV40 enhancer and MT sequences are irrelevant with the inhibition of proteoglycan synthesis. It is, therefore, supposed that aggrecan synthesis in HCS 2/8 chondrocytes is under the control of transacting c-fos gene, as shown by decreased transcription of aggrecan and TIMP-1, and by increased transcription of MMP-3.

Recently, several lines of evidence suggest that c-Fos, the product of the c-fos, together with c-Jun protein or its related proteins (JunB or JunD) represents the transcription factor AP-1 (activating protein-1). These may act as intracellular messengers that convert short-term signals generated by extracellular stimuli into long-term changes in cell phenotype by regulating the expression of downstream genes that possess AP-1-binding site [11]. The AP-1 sequences were found in the promoters of many genes, including human collagenase, MMP-3 and TIMP-1. Although it is well-documented that collagenase and MMP-3 were induced through the AP-1 pathway, controversy exists concerning its effect on TIMP-1 [27–29]. In order to assess the effect of AP-1 proteins on the expression of collagenase and TIMP-1, the promoter CAT plasmids, collagenase-CAT or TIMP-1-CAT, were transfected into recipient KKLS cells with or without the expression plasmids for c-Jun, JunB, JunD, c-Fos, Fra-1 or Fra-2. Then, the CAT activity was analyzed to monitor collagenase and TIMP-1 promoters activity (Fig. 4). This particular cell line was used here because activation by AP-1 proteins can be readily observed in these cells due to their low basal promoter activity as described previously [12]. The collagenase promoter was clearly activated by Jun-related proteins as well as Fos/Jun-related protein heterocomplex. On the other hand, c-Fos combined with any of the Jun-related proteins failed to stimulate the TIMP-1 promoter, although this promoter was also activated by Fra-1 or Fra-2/Jun-related protein heterocomplex. Furthermore, c-Fos inhibited the activation of TIMP-1 promoter by JunD.

In this study, we have demonstrated that introduction of exogenous c-fos DNA in HCS 2/8 chondrocytes resulted in the decreased endogenous transcription of proteoglycan and TIMP-1, and the increased transcription of MMP-3. It was also shown that c-Fos-regulated TIMP-1 promoter negatively in a transient transfection assay with KKLS cells while other AP-1 proteins were positive regulators. This is the first demonstration of the repressive effect of c-Fos on TIMP-1 promoter, although its inducing effect on collagenase and MMP-3 promoters has been shown previously [23]. Thus, the c-Fos may cause a similar effect on TIMP-1 and MMP-3 promoters in HCS 2/8 chondrocytes. This possibility is now under investigation.

These results taken together may indicate that the c-fos gene plays an important role not only in the cartilage homeostasis also in the pathogenesis of cartilage destruction in patients with arthritis.

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Table 1
Proteoglycan synthesis in transfectants

Incubation time	Transfectant	Exogenous c-fos mRNA	[^{35}S] Sulfate incorporation (dpm 10^{-3}/μg DNA)		% of control
			Cadmium induction		
			(−)	(+)	
6 h	HCS 2/8	(−)	9.77 ± 0.08	8.75 ± 1.00	89.6 ± 10.2
	c5	(−)	10.47 ± 3.38	10.71 ± 1.02	102.3 ± 9.7
	f2	(+)	8.51 ± 0.72	7.78 ± 0.52	91.4 ± 6.1
	f5	(+)	10.88 ± 10.8	10.74 ± 0.88	98.7 ± 8.1
	f17	(+)	9.58 ± 0.10	8.09 ± 0.68	84.4 ± 7.1
12 h	HCS 2/8	(−)	9.98 ± 0.13	9.85 ± 0.96	98.7 ± 9.6
	c5	(−)	11.07 ± 0.32	10.37 ± 1.32	93.7 ± 11.9
	f2	(+)	9.78 ± 0.68	4.31 ± 0.38*	44.1 ± 3.9
	f5	(+)	11.08 ± 0.37	5.35 ± 1.00*	48.3 ± 9.0
	f17	(+)	10.70 ± 0.62	3.89 ± 0.75*	36.4 ± 7.0

The cells were incubated for 6–12 h with 5 μM/ml of $CdCl_2$ in 0.1 ml of 0.3% FCS/DME medium. The cultures were labeled with [^{35}S]sulfate (0.5 μCi) for the last 6 h before the end of incubation. Each value represents the mean ± S.D. for six dishes. HCS 2/8, HCS 2/8 chondrocyte; c, control pSV2neo-MT transfectant; f, pSV2neo-MT-hc-fos transfectant. *$P < 0.01$ by the Student's t-test as compared with cadmium induction (−) in each group.

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