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Transforming Growth Factor-β_1 Responsiveness of Human Articular Chondrocytes *in Vitro*: Normal Versus Osteoarthritis

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The transforming growth factor-β_1 was known as having the most important influence on chondrocytes among various growth factors, being abundant in articular chondrocytes and osteocytes. We performed in vitro monolayer cultures of human articular chondrocytes from normal and osteoarthritic patients and studied the transforming growth factor-β_1 reponsiveness of those chondrocytes. The cell-growth curve indicated that the primary osteoarthritic chondrocyte culture with transforming growth factor-β_1 showed a more rapid growth pattern than normal chondrocytes with or without TGF-β_1 and osteoarthritic chondrocytes without TGF-β_1. The osteoarthritic group showed a sharp decline in growth pattern with subsequent culture. The shape of osteoarthritic chondrocytes was bigger and more bizarre compared to those of normal chondrocytes. With subsequent culture, this change became prominent. The transforming growth factor-β_1 increased the [^{3}H]-TdR uptake in each group. The phenotypes of chondrocytes were more clearly expressed in the normal group. The chondrocytes lost their phenotype (production of collagen type II) following subculture in each group. The transforming growth factor-β_1 could not inhibit or delay the dedifferentiation process (loss of phenotype).

Key Words: Chondrocyte, monolayer culture, transforming growth factor-β_1, osteoarthritis

The proliferation of chondrocytes is important to the repair of chondral defects. The aging process not only results in the reduction of cartilage thickness and cellularity, but also in changes in extracellular matrix composition and chondrocytes. Reduced cellularity of aging cartilage and the production of altered matrix components by cells from older people have been documented and are thought to contribute to the pathogenesis of osteoarthritis, the most common joint disease. The incidence of which increases with age (Abyad and Boyer, 1992; Hamerman, 1993). Loss of chondrocytes may be the result of cell death or failure of the cells to sufficiently replicate and maintain tissue cellularity, as well as homeostasis of the extracellular matrix. These changes could be, at least in part, the result of impaired chondrocyte replication in aging. Cells derived from osteoarthritic cartilage demonstrate an increased proliferation rate (Mankin *et al.* 1971; Bulstra *et al.* 1989), but it is not clear if this reaction is able to compensate

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for the loss of chondrocytes observed at the surface of the damaged tissue.

Mature cartilage is characterized by the absence of blood vessels and nerve fibers. Homeostatic chondrocyte secretory function, DNA synthesis and reponses to traumatic and inflammatory tissue injury can be induced by regulatory factors that are produced by cells in synovial tissue or fluid. An alternative mechanism for the induction of chondrocyte responses is the generation of regulatory factors within cartilage. Chondrocytes have recently been shown to be capable of producing a large series of peptide regulatory factors that affect their growth and secretory function. Among these are three forms of transforming growth factor (TGF)-β, platelet-derived growth factor (PDGF)-AA, basic fibroblast growth factor (bFGF), insulin-like growth factor (IGFs), the cytokines interleukin (IL)-1, IL-6, leukemia inhibitory factor (LIF), oncostatin M (OSM), IL-8, monocyte chemoattractant protein-1 (MCP-1), neuropeptides, and colony-stimulating factors (Nilsson *et al.* 1986; Dingle *et al.* 1990; Guerne *et al.* 1990; Peracchia *et al.* 1991; Lotz *et al.* 1992; Villiger and Lotz, 1992; Villiger *et al.* 1993). Recent studies reported that transforming growth factor (TGF)-β has the most important influence on chondrocytes among various growth factors (Guerne *et al.* 1994; Miura *et al.* 1994; Guerne *et al.* 1995). TGF-β affects chondrogenesis in two ways. One is chondrogenic induction and the other is the enhancement of cartilage formation (Miura *et al.* 1994). Also TGF-β has an inhibitory effect on the IL-1 induced matrix metalloproteinase production that contributed to the catabolic effect in the cartilage (Chandrasekhar and Harvey, 1988). So now TGF-β is applied to the repair of chondral defects in animal models and has been proposed as a new treatment method in osteoarthritis (Hunziker and Rosenberg, 1994, 1996).

Chondrocytes produce two well-characterized structural macromolecules that have been used to define their specific differentiated phenotype, cartilage proteoglycan and type II collagen. When chondrocytes are released from the cartilage matrix and placed in monolayer culture, they stop producing these characteristic molecules in a variety of situations (Schiltz *et al.* 1973; Benya and Shaffer, 1982). Benya and Shaffer (1982) referred to such chondrocytes as "dedifferentiated" only to emphasize the absence of differentiated functions, not to imply regression to an earlier bipotent or multipotent embryonic state.

Although a number of studies in the experimental model were reported, the studies about the TGF-β_1 responsiveness of human articular chondrocytes were rarely performed. So we cultured the chondrocytes in vitro from normal and osteoarthritic human cartilage and compared the TGF-β_1 responsiveness of those chondrocytes: distinct profiles in primary chondrocytes versus subcultured chondrocytes; and normal versus osteoarthritic chondrocytes. We also compared the cell morphology between normal and osteoarthritic chondrocytes following subculture in vitro. The changes of the chondrocyte phenotype (dedifferentiation process) were observed with the subsequent subculture of normal and osteoarthritic chondrocytes. We also studied whether this dedifferentiation process can be inhibited by TGF-β_1 or not.

MATERIALS AND METHODS

Chondrocytes isolation and culture

Normal human articular cartilage from the femoral condyle (weight, 200~300 mg) was obtained during notchplasty of anterior cruciate ligament (ACL) reconstruction. Osteoarthritic cartilage (weight, 300~400 mg) was taken from femoral condyles during total knee arthroplasty (TKA). Cells were isolated within two to five hours. Cartilage slices were washed in Dulbecco's modified Eagles's medium (DMEM) and then minced with a scalpel. After mincing, the cartilage slices were washed with DMEM two times, transferred to a digestion buffer containing 10 ml of DMEM, 0.2 ml of collagenase II (1 mg/ml, Sigma, St. Louis, MO, USA) and 0.1 ml of DNase I (0.1 mg/ml, Boehringer Mannheim, Mannheim, Germany), and then incubated in a spinner bottle at room temperature for 16 hours until the fragments were digested. Cells were washed 3 times in DMEM, filtered through nylon mesh (100 um, Spectrum, Houston, Texas, USA), and incubated in a 25 cm^2 culture flask with DMEM, 10% fetal bovine serum (FBS), and antibiotics (penicillin and streptomycin). The medium was changed 2 times a week. At confluency, after 1~2 weeks, cells were trypsinized, divided, and cultured further for three passages. Cells were

divided into four groups: normal chondrocytes without TGF-β_1 (Sigma, St. Louis, MO, USA), normal chondrocytes with TGF-β_1, osteoarthritic chondrocytes without TGF-β_1, and osteoarthritic chondrocytes with TGF-β_1. The concentration of TGF-β_1 was 10 ng/ml. Cells of each 4 groups were cultured further for three passages and evaluated in 3 parameters (cell growth curve, ^{3}H-thymidine uptake studies, and collagen immunofluorescent stain).

Proliferation studies

Cell growth curve: Cells were distributed into 24 well plates and cell counting with a hemocytometer was performed at Day 4 and Day 7 for cell-growth curves in each group. We also observed the shape of chondrocytes in each group.

^{3}H-thymidine uptake studies: Cells were distributed into 96-well plates (5,000/well) in a total volume of 200 ul of DMEM supplemented with L-glutamine, penicillin, streptomycin, 10% FBS and TGF-β_1 (10 ng/ml). At the time points indicated for each set of experiments (Days 2, 3, 4, 5, and 6), the cultures were pulsed with ^{3}H-thymidine ([^{3}H]TdR)(1 uCi/well, Amersham, Buckinghamshire, UK) for 12 hours and the cells that were partially adherent were harvested in two steps. The nonadherent cells were first harvested on a filter paper by a multiwell automated cell harvester (Flow, USA). After washing, the adherent cells were then lysed by adding 100 ul NaOH 0.1 M into each well, and the cell lysates were transferred into liquid scintillation vials together with the corresponding filters. Total radioactivity was quantified by liquid scintillation counting (Beckman, USA). Each experimental study was performed three times. The data were tested by statistical method (ANOVA with repeated measure and paired t-test).

Collagen immunofluorescent stains

Cells were trypsinized, washed, then cultured on the glass chamber for 3~5 days, and then fixed with ethanol. After three more washings with PBS, the slides were first incubated with affinity-purified goat anti-type I or II collagen antibody (Southern Biotechnology Associates, Birmingham, AL, USA), diluted 1/250 in PBS for 1 hour at 37°C, rinsed, and then incubated with fluorescent isothiocyanate(FITC)-tagged conjugate (Capple, Durham, NC, USA) for 1 hour at 37°C. Observations were made under a fluorescent microscope.

RESULTS

Cell growth curve and morphology of cells

The cell growth curve revealed that primary osteoarthritic chondrocyte culture with TGF-β_1 (P_1 OA with TGF) showed a more rapid growth pattern than any other group (Fig. 1~3). TGF-β_1 increased the cell growth rate in all groups. The osteoarthritic group (OA) showed a sharp decline in growth pattern with subsequent subculture. The third-passage culture of osteoarthritic chondrocytes (P_3 OA) had a nearly flat cell-growth curve (Figure 3). The effect of TGF-β_1 declined with subsequent subculture, so in third-passage culture there was no difference of cell growth pattern in each group with or without TGF-β_1.

The morphology of primary normal chondrocytes was polyhedral and small in size (Fig. 4). In second passage culture (P_2), thinner and bigger cells were mixed with cells observed in primary culture (P_1) (Fig. 5). In third passage culture (P_3), bigger cells that were observed in P_2 became predominant (Fig. 6). The morphology of cells with TGF-β_1 in each passage was no difference from that of cells without TGF-β_1. The morphology of osteoarthritic chondrocytes was bigger and more bizzare compared to normal chondrocytes (Fig. 4~6). With subsequent subculture, this change in appearance became prominent. The shape of P_3 normal chondrocytes was similar to that of P_1 osteoarthritic chondrocytes.

Proliferation studies

TGF-β_1 increased the [^{3}H]-TdR uptake, which was sustained with the passage of time as compared to the control group (without TGF-β_1)(Fig. 7~9). But this tendency was statistically significant in the normal P_1, P_2 and Osteoarthritic P1 ($p<0.05$). At Days 2 and 3, the [^{3}H]-TdR uptake was higher or similar in the control group, but after Day 4, the uptake was higher in the TGF-β_1 group. In the primary culture of each group, the osteoarthritic

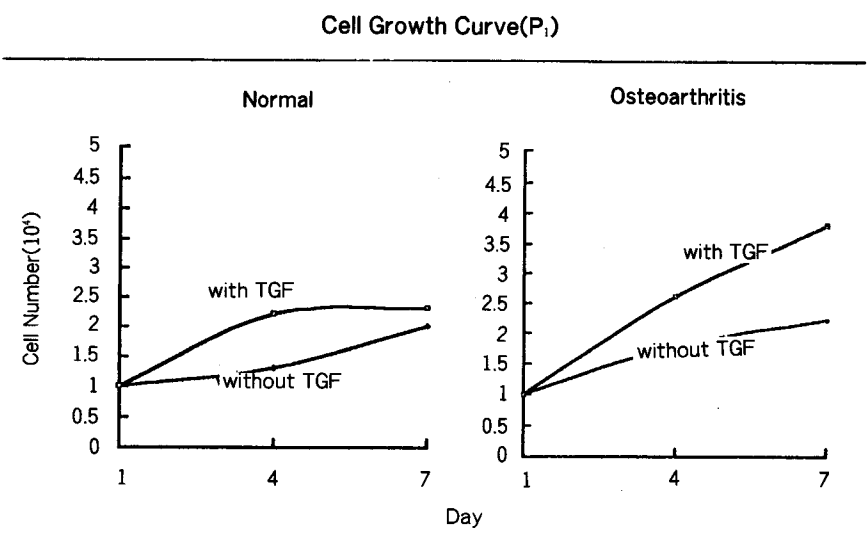

Fig. 1. Cell growth curve of the primary culture (P_1) of human articular chondrocytes. Cell number was counted at Day 4 and day 7 with a hemocytometer in each groups. The concentration of TGF-β_1 (Sigma) was 10 ng/ml.

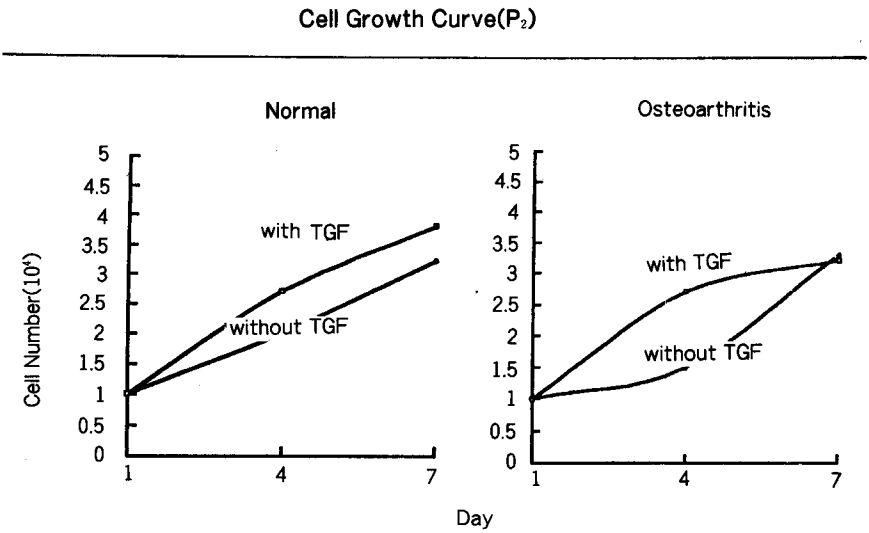

Fig. 2. Cell growth curve of the second passage subculture (P_2) of human articular chondrocytes. Cell number was counted at day 4 and day 7 with a hemocytometer in each group. The concentration of TGF-β_1 (Sigma) was 10 ng/ml.

group showed higher [^{3}H]-TdR uptake than the normal group (Fig. 7). In the normal group, the uptake of the P_2 group was higher than that of the P_1 group (Fig. 8). In the osteoarthritic group, the uptake was sharply decreased with subsequent subculture.

Collagen immunofluorescent stains

Collagen type I was more densely stained in

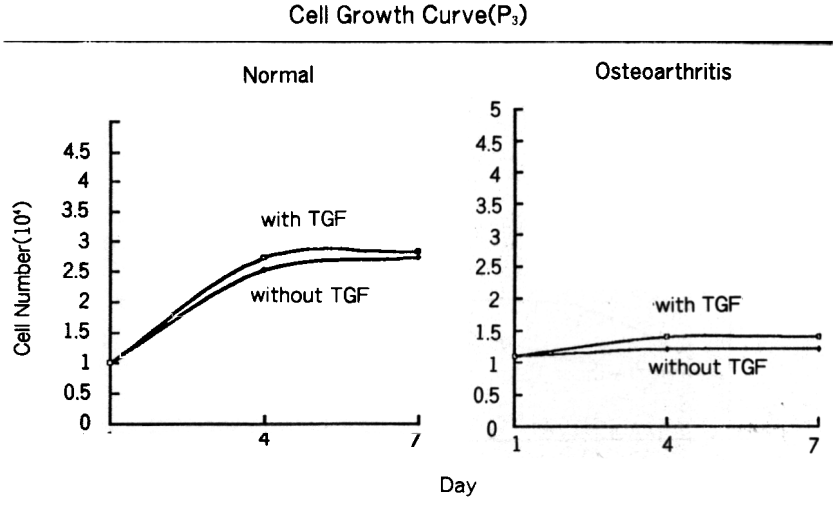

Fig. 3. Cell growth curve of the third passage subculture (P_3) of human articular chondrocytes. Cell number was counted at Day 4 and 7 with a hemocytometer in each groups. The concentration of TGF-β_1(Sigma) was 10 ng/ml.

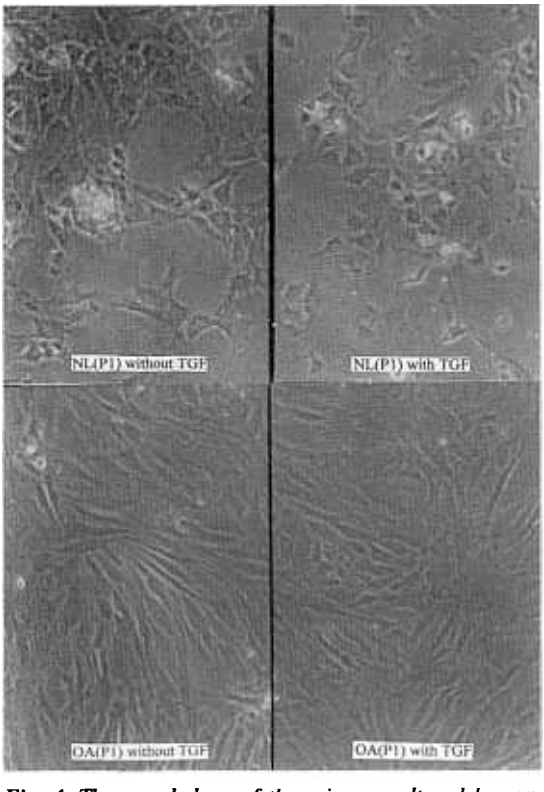

Fig. 4. The morphology of the primary cultured human articular chondrocytes in each group (×400).

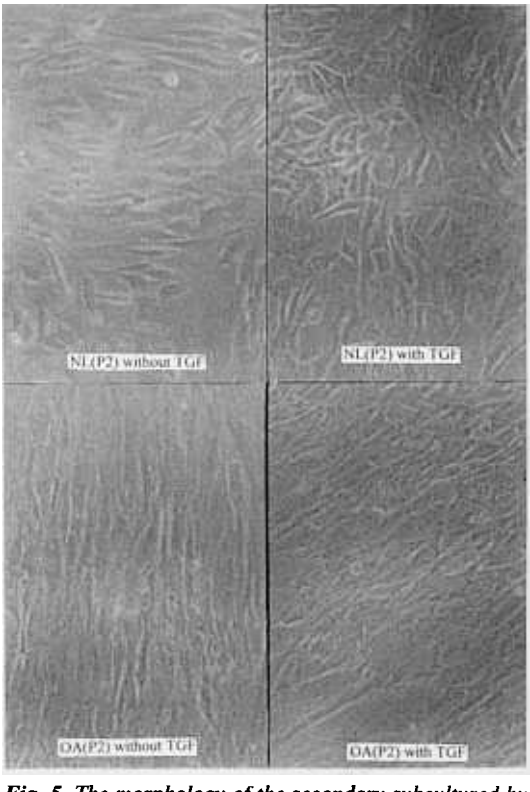

Fig. 5. The morphology of the secondary subcultured human articular chondrocytes in each group(×400).

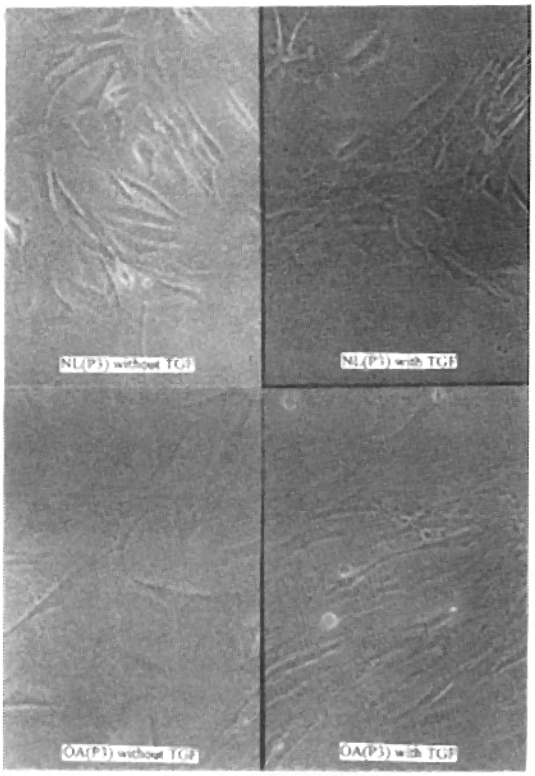

Fig. 6. The morphology of the tertiary subcultured human articular chondrocytes in each group (×400).

osteoarthritic chondrocytes than in normal chondrocytes (Fig. 10, 11). With subsequent subculture, collagen type I was more densely stained in each group. Collagen type II was most densely stained in the primary culture of normal chondrocytes and with subsequent subculture, collagen type II was more and more weakly stained (Fig. 10, 11). So we could observe that the chondrocytes lost their characteristic phenotype (production of collagen type II) and changed into the fibroblast-like characteristics (production of collagen type I) with subsequent subculture. We could not observe the difference of the density of collagen type II immunostain between cultures without TGF-β_1 and with TGF-β_1 at the same passage-culture of each group. So TGF-β_1 could not inhibit the loss of chondrocyte phenotype (dedifferentiation).

DISCUSSION

Biologic resurfacing of the damaged joints is an area of great interest and clinical promise because of the limited potential of damaged articular cartilage for healing (Meachim and Roberts, 1971; Convery

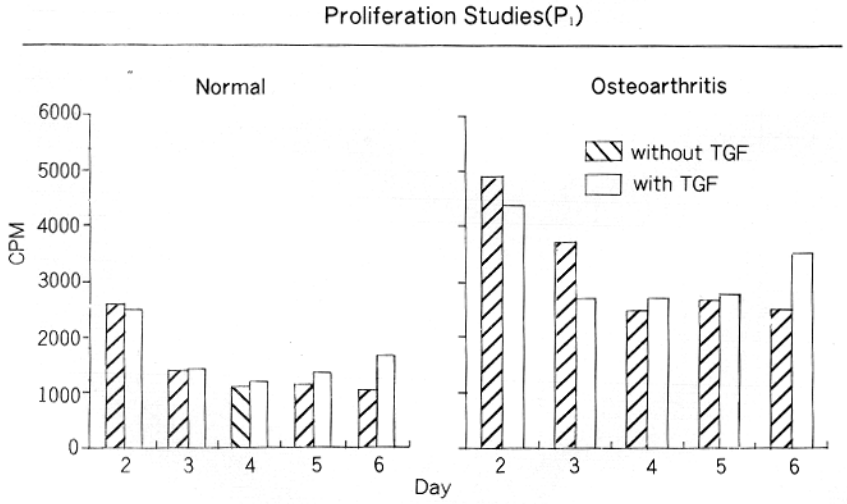

Fig. 7. Effects of TGF-β_1(10 ng/ml) on the proliferation of human articular chondrocytes in the primary culture (P1). Cell cultures(5000 /well) were stimulated with TGF-β_1. Cells were pulsed with [^{3}H]TdR(1 uCi/well) during the last 12 hours of the culture at the indicated time. Results were shown as mean cpm of triplicate determinations. They showed a statistically significant difference between each group ($p<0.05$). The difference occurred at Day 4 ($p<0.05$).

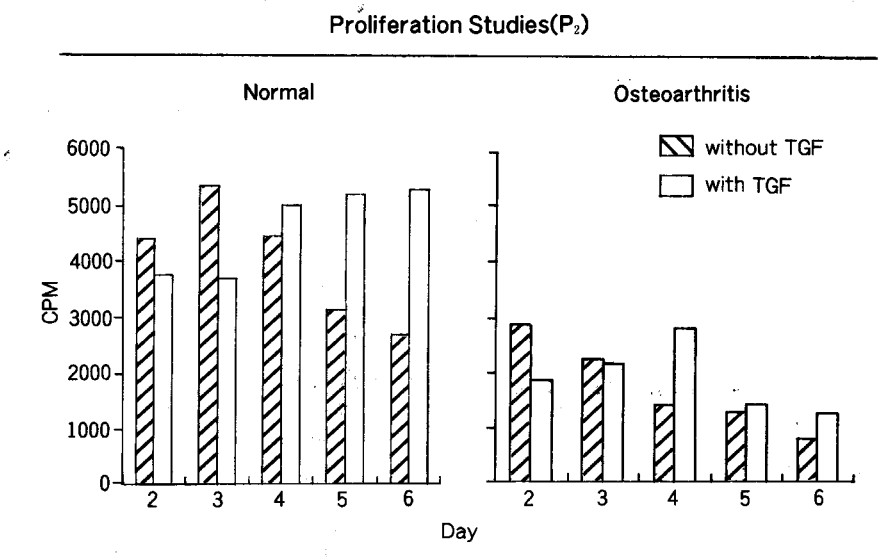

Fig. 8. Effects of TGF-β_1(10 ng/ml) on the proliferation of human articular chondrocytes in the secondary subculture (P_2). Cell cultures (5000/well) were stimulated with TGF-β_1. Cells were pulsed with [^{3}H]TdR(1 uCi/well) during the last 12 hours of the culture at the indicated time. Results were shown as mean cpm of triplicate determinations. The normal group showed a statistically significant difference ($p<0.05$) between each group, but the osteoarthritic group did not ($p=0.07$). The difference occurred at Day 4 ($p<0.05$).

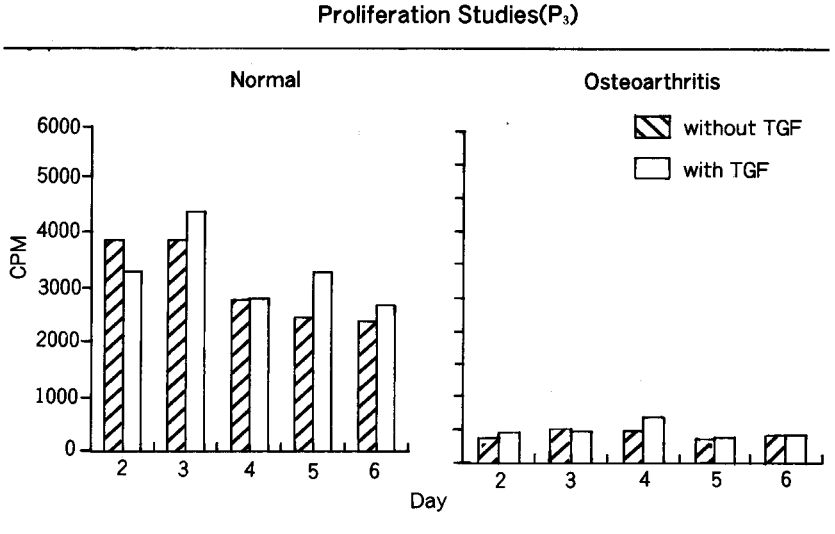

Fig. 9. Effects of TGF-β_1(10 ng/ml) on the proliferation of human articular chondrocytes in the third subculture (P_3). Cell cultures (5000 /well) were stimulated with TGF-β_1. Cells were pulsed with [^{3}H]TdR(1 uCi/well) during the last 12 hours of the culture at the indicated time. Results were shown as mean cpm of triplicate determinations. They didn't show any statistically significant difference between each group ($p>0.05$).

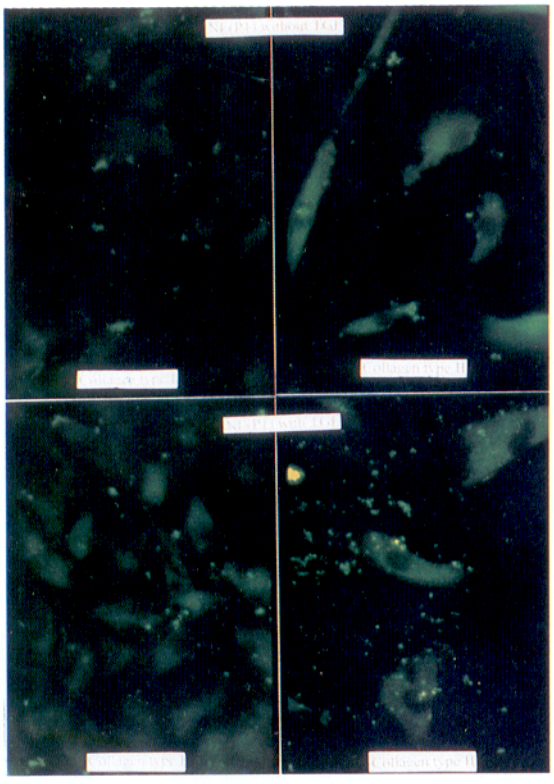

Fig. 10. Collagen I and II expression in the primary culture of normal chondrocytes(NL P1). The immunofluorescent staining was performed with a goat anti-collagen type I or II antibody.

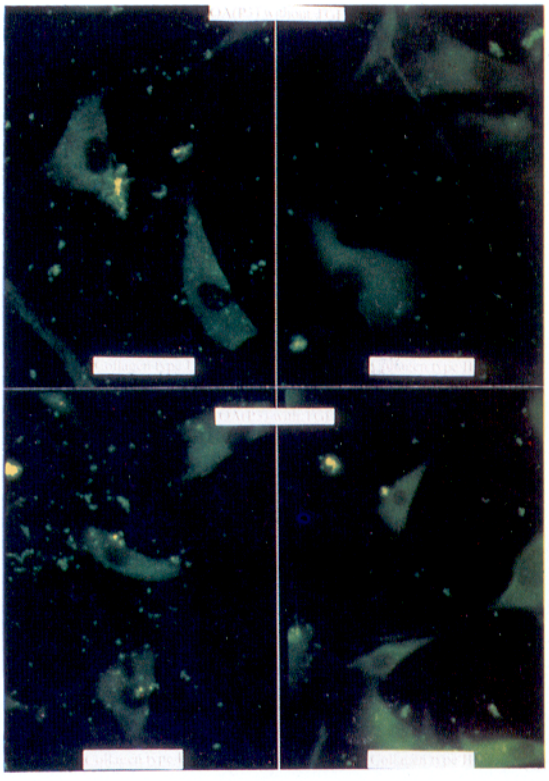

Fig. 11. Collagen I and II expression in the third passage subculture of osteoarthritic chondrocytes(OA P3). The immunofluorescent staining was performed with a goat anticollagen type I or II antibody.

et al. 1972; Furukawa *et al.* 1980; Mankin, 1982). Clinically, spongiolization (Ficat *et al.* 1984), joint debridement and abrasion of subchondral bone (Friedman *et al.* 1984; Johnson, 1986; Ewing, 1990), perichondral grafts (Homminga *et al.* 1990), osteochondral grafts (Meyers *et al.* 1989; Gross *et al.* 1992), and carbon fiber matrix insertion (Muckle and Minns, 1990; Brittberg *et al.* 1994a) have been used, but the results were not satisfactory. The main disadvantage of the above mentioned treatment modalities is the regeneration of the chondral defects with fibrous cartilage producing collagen type I instead of hyalin cartilage. Recently, Brittberg *et al.* (1994b) performed autologous chondrocyte transplantation with a periosteal patch and reported their successful results. The advantage of their method was that the chondral defects were regenerated with hyalin cartilage. Hunziker and Rosenberg (1994, 1996) reported that the induction of cartilage repair by TGB-β_1 in an experimental model was obtained. So interest in the articular chondrocytes and TGF-β_1 was increased.

Transforming growth factor-beta (TGF-β) is a multifunctional protein that has the potential to influence cell differentiation, proliferation, and synthesis of extracellular matrix components, depending on the cell and tissue type being studied (Roberts *et al.* 1981). It is abundant in articular cartilage chondrocytes and osteocytes (Morales *et al.* 1990; Frazer *et al.* 1991). These facts indicate that TGF-β is involved in connective tissue morphogenesis, including chondrogenesis. The effects of TGF-β on chondrogenesis or cartilage matrix production have generally been stimulatory. TGF-β has been variably reported

to stimulate cartilage-specific matrix components such as proteoglycan and Type II collagen. Several investigators have reported such stimulation using cartilage organ culture or chondrocyte cultures (Sandell *et al.* 1989; Redini *et al.* 1991), whereas others have reported inhibition (Skantze *et al.* 1985; Roiser *et al.* 1988; Rosen *et al.* 1988; Inoue *et al.* 1989). Guerne *et al.* (1994, 1995) studied the growth factor responsiveness of human articular chondrocytes and said that TGF-β was the most potent mitogen among various growth factors, and after skeletal maturity there was a profound decline in the levels of DNA synthesis and cell replication in response to TGF-β. So in order to use TGF-β clinically, the basic data of human chondrocyte physiology in response to TGF-β was needed. Carrington *et al.* (1988) measured tissue concentrations of TGF-β between 2.5 and 49 ng/ml in rat bone matrix, and predicted that local concentrations of TGF-β are probably much higher. Miura *et al.* (1994) selected 10ng/ml TGF-β_1 for enhancing chondrogenesis. Guerne *et al.* (1994) reviewed the complete response profile of growth factor in human articular chondrocytes. They found the concentration of fetal bovine serum (FBS) and TGF-β_1 was directly correlated with proliferation of chondrocytes until 20% FBS and 10ng/ml TGF-β_1. So we performed these studies with 10% FBS and 10 ng/ml TGF-β_1.

The morphology of human articular chondrocyte in primary monolayer culture was small and polygonal, but with subsequent subculture they were changed into larger, flattened and amoeboid-like shapes. The morphology of osteoarthritic chondrocytes in primary culture was similar to that of P_3 subculture in the normal group. It is probably the result from *in vivo* senescence or dedifferentiation of chondrocytes. The osteoarthrirtic chondrocyte had a more bizzare, larger, flattened and amoeboid-like appearance with subsequent subculture. Secondary culture of normal chondrocytes showed that the polygonal cells were mixed with flattened and amoeboid-like cells. We thought that this change of cell morphology could be related to the phenotypic change of chondrocytes (dedifferentiation). However, Von der Mark *et al.* (1977) said that flattened, fibroblast-like cells synthesized type I collagen, whereas round or polygonal chondrocytes generally synthesized type II collagen, but there was no strict correlation between cell morphology and phenotype. There was no difference in cell morphology between each group with or without TGF-β_1 at the same passage of culture. TGF-β_1 didn't influence the change of the cell morphology.

The cell growth curve showed that the primary culture of osteoarthritic chondrocytes with TGF-β_1 had a sharper curve than any other group. It meant that the primary-culture osteoarthritic chondrocytes have a more rapid proliferation rate. Mankin *et al.* (1971) and Bulstra *et al.* (1989) said that cells derived from osteoarthritic cartilage demonstrated an increased proliferation rate. In all groups except the P_3 subculture of osteoarthritic chondrocytes, TGF-β_1 increased the cell growth rate. Other studies have reported similar results (Guerne *et al.* 1994; Guerne *et al.* 1995). In normal chondrocytes, the secondary subculture showed a sharper cell-growth curve than primary culture. This was probably due to the period of *in vitro* enviornmental adaptation. In osteoarthritic chondrocytes, the cell growth curve was abruptly flattened in the third subculture. It meant that during subculture the chondrocytes progressed into senescence and this process was accentuated in the osteoarthritic chondrocytes.

Cell proliferation was studied with [^{3}H]-thymidine uptake because [^{3}H]-thymidine was incorporated into DNA during mitosis. Proliferation studies in each group showed that the uptake at Day 2 and 3 was similar or less in cells with TGF-β_1, but after Day 3 it was higher in cells with TGF-β_1. Cell proliferation studies with [^{3}H]-thymidine uptake were concordant with the cell-growth curve. So we could reason that the effect by TGF-β_1 was slow in onset and became apparent after 3 days. TGF-β_1 increased the uptake, so it functioned as a mitogen. Similar results were found in other literature (Iwamoto *et al.* 1989; Galera *et al.* 1992; Guerne *et al.* 1994; Miura *et al.* 1994).

When chondrocytes were kept in monolayer culture, they eventually lost their cartilage phenotype (Von der Mark *et al.* 1977; Benya and Shaffer, 1982). Within 4 passages, or approximately 1 month in culture, they synthesized the genetically different type I collagen instead of cartilage collagen (type II collagen). So we performed the immunofluorescent stain with collagen type I and II. Collagen type I was densely stained in the P_3 subculture of the

osteoarthritic group, and collagen type II was densely stained in the P_1 culture of the normal group. In the normal group, collagen type II was weakly stained and collagen type I was strongly stained with further passage of the subculture. We found this tendency in the osteoarthritic group, but the intensity of stain was weak in collagen type II and strong in collagen type I. These results showed that the dedifferentiation process was started *in vivo* with the aging process or osteoarthritic change. Collagen I and II expression in freshly isolated and dedifferentiated chondrocytes was observed by Guerne *et al.* (1994). They said that in primary culture the cells expressing type I collagen were less than 2% of the population and that after three passages the cells synthesizing type II collagen were less than 2%. But we could not analyze quantatitively the intensity of immunostain. The above-mentioned tendency of immunostain in normal and osteoarthritic groups was likewise observed in the cells with TGF-β_1. So we thought that TGF-β_1 was unable to prevent or delay the dedifferentiation process of chondrocytes in culture. Generally TGF-β_1 was considered the most potent stimulator of collagen synthesis, exerting its action at the gene transcription level (Rossi *et al.* 1988; Sandell *et al.* 1989). However, several reports demonstrated that the effect of TGF-β_1 on collagen in chondrocytes appeared dependent on whether the cells were in a differentiated state or were losing their specific phenotype (Skantze *et al.* 1985; Rosen *et al.* 1988; Horton *et al.* 1989; Rosier *et al.* 1989; Galera *et al.* 1992). Therefore, it may be possible that TGF-β_1 acts to stimulate collagen and proteoglycan syntheses of articular chondrocytes when an early reparative process occurs in injured cartilage. But the properties of the new matrix formed in normal or osteoarthritic situations are still to be investigated.

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