

Effect of Transforming Growth Factor- β 1 in Biological Regulation of Primary Chondrocyte

Seyed Ali Khaghani^{1,*}, Morgan C. T. Denyer², Mansour Youseffi³

¹School of Engineering, School of Life Sciences, University of Bradford, Bradford, BD7 1DP, United Kingdom

²School of Life Sciences, University of Bradford, Bradford, BD7 1DP, United Kingdom

³School of Engineering, University of Bradford, Bradford, BD7 1DP, United Kingdom

Abstract The effect of transforming growth factors- β (TGF- β s) in the regulation and control of cell growth is widely studied, but the capability of these cytokines to regulate chondrocyte cell functions such as migration, cell death, proliferation, differentiation and wound repair is not clearly understood. In this work the effect of TGF- β 1 on the biological regulation of chondrocyte was evaluated using a model wound closure assay. The experiments were carried out on primary chondrocyte cells with fibroblast like and chondrocytic phenotypes. The cells were isolated from knee articular cartilage of five day old Sprague-Dawley neonate rats and seeded at low density to obtain a fibroblast like morphology. Chondrocytes with chondrocytic phenotype were derived by seeding at high density. The results revealed that TGF- β 1 slowed down proliferation and migration of cells into a model wound. It was also found that cell attachment, as determined by the detachment time during trypsinization, was greater for cells with a fibroblast like morphology when compared with cells exhibiting chondrocytic morphology. Treatment with TGF- β 1 was found to increase the detachment times of fibroblast like chondrocytes, indicating that TGF- β 1 enhanced cell attachment of this cell type, whilst treatment with TGF- β 1 decreased detachment time for the chondrocytic type chondrocyte cells indicating that TGF- β 1 decreased cell attachment in these cells.

Keywords Chondrocyte cell culture, Wound repair, TGF- β 1, Detachment assay, Cell adhesion

1. Introduction

Cartilage is an avascular tissue[1] with oxygen and nutrients delivered via the synovial fluid[15]. This is believed to contribute to the poor repair characteristic of cartilage [10]. Small lesions in the cartilage can cause severe problems which can affect patient's mobility[19]. In order to understand how cartilage injuries can be treated in vivo[9], chondrocyte behaviour in relation to model wounds[4] in the presence of various cell stimulators[1] such as different growth factors, can be examined in vitro.

TGF- β 1 is a 25kD homodimeric peptide[16] and is widely involved in the biological regulation and cellular activities[22]. The TGF- β superfamily consists of various types of polypeptides effecting up and down-regulation of membrane proteins[24] and consequently inducing cell adhesion, proliferation, differentiation, activation, migration and apoptosis[14]. Stimulation by TGF- β 1 of the synthesis of cartilaginous matrix suggested[12,23] that this type of growth factor may play an important role in cartilage repair by increasing cell proliferation rates, cell migration and cellular adhesion. This may indicate how cartilage repair is

initiated and how this could be accelerated by transforming growth factor-beta1[4]. It has also been reported that chondrocytes can be induced to obtain a chondrocytic morphology by seeding at high cell density[2] and a fibroblastic morphology by culturing in low density after their isolation[18]. This work aims to investigate the effect of TGF- β 1 on cell length, proliferation rate, focal adhesion formation /cell surface attachment, cell motility and formation of the extracellular matrix in both fibroblastic and chondrocytic cartilage cells isolated from knee joint of rats.

2. Materials and Methods

2.1. Cell Culture

Epiphyseal plates were carefully separated from the end of both tibias and femurs derived from 3 days old Sprague-Dawley rats. Tissue was then immersed in 4ml of 0.25% trypsin and 2g per litre EDTA (Sigma, 2007) and stirred for 15 minutes at 37°C. The supernatant was aspirated after 15 minutes and transferred into a 15ml centrifuge tube. Trypsin was deactivated by adding 4 ml of 10% FCS contained DMEM media. The process of digestion by trypsin was repeated three times. The aspirated supernatants and epiphyseal plates were centrifuged at 2000 rpm and the obtained pellet was immersed in 4ml of 0.1% collagenase type-I solution (Sigma, 2007) for 90 minutes. Following treatment

* Corresponding author:

s.khaghani@bradford.ac.uk (Seyed Ali Khaghani)

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with collagenase type -1 solution the supernatant was mixed with 4ml of FCS containing cell culture media and re-centrifuged at 2000 rpm. The supernatant was discarded and the obtained pellet was resuspended with 5 ml chondrocyte culture media, seeded in a 25 cm² tissue culture flask and incubated at 37°C. After 24 hours the epiphyseal plates and non-attached cells were discarded, fresh media was added to the cell culture and incubated at 37°C until 70-80% confluency.

To obtain the chondrocytes with chondrocytic morphology the cells were resuspended at cell density of 250,000 cells/ml in 10ml high glucose DMEM (Dulbecco's modified eagles medium) with 10% FCS supplementation and 0.1mg/ml hyaluronic acid (Sigma, 2007). Five millilitres of cell suspension were transferred into a 25cm² TG grade falcon cell culture flask.

To acquire chondrocytes with a fibroblast like phenotype 1ml of cell suspension was added to 4ml chondrocyte culture media to achieve a cell density of 50,000 cells/ml. Both cultures were incubated at 37°C until 70-80% confluency.

2.2. Cell Proliferation

The effect of TGF- β 1 on proliferation rate of chondrocyte cells with both fibroblast like, and chondrocytic morphologies was assessed. The initial chondrocyte cell density for fibroblast like morphology was 50,000 cell/ml and for chondrocytic cells was 250,000 cell/ml. After 72 hours in culture cells were trypsinized and counted using a haemocytometer and these cell densities were compared with the initial plating densities. The experiment was repeated three times and the mean proliferation rate (\pm SE) were calculated.

2.3. Cell length

From each cell culture two subcultures were provided one was supplemented with a bathing concentration of 10 ng/ml [7] TGF- β 1 (Sigma, 2007). Non-attached cells were removed after 24 hours and the media was subsequently renewed every 48 hours. Time related changes in cell length were determined every two days via the acquisition of photomicrographs and measurement using Image J software (NIH) for 150 control and TGF- β 1 treated cells. The experiment was repeated 3 times and the mean cell lengths at each time point (\pm SE) were calculated.

2.4. Immunocytochemistry

Immunocytochemistry was used to evaluate the effect of TGF- β 1 on antibody localization of collagen type-I, collagen type-II and fibronectin. Monoclonal anti-collagen type-I (mouse IgG1 isotype), monoclonal anti-collagen type-IIA1 (mouse IgG1) and monoclonal anti-fibronectin antibodies (Sigma, UK) were used as primary antibodies and goat anti-mouse IgG, conjugated to Alexa-Fluor 488 SFX Kits (Invitrogen, UK) as secondary antibody.

2.5. Wound Closure Assay

The capacity of cartilage to repair in the presence of

TGF- β 1 was assessed by means of creating a wound via scratching a confluent chondrocyte monolayer. Both control and TGF- β 1 supplemented monolayers were scratched using the tip of a sterilized 3ml fine tip extended transfer pipette with a 1mm tip diameter. The experiments were carried out for both fibroblast like and chondrocytic morphologies.

The scratched areas were imaged in 10 different places by phase contrast microscope at 10x magnification every 2hrs to determine the average width of the wound over a period of 72 hours.

2.6. Cell Adhesion Analysis

Chondrocyte cell surface attachment in both control and TGF- β 1 contained environments was assessed at room temperature using a trypsinization assay. Trypsin is a temperature dependant enzyme which stimulates integrin dependent cell adhesion[20] and digests proteins by cleaving the peptide chains at the carboxyl side of amino acids[21]. This effect causes breakage of cell-cell and cell-extracellular environment adhesion releasing the cells from the culture flask and/or dissociation from each other. The time taken for detachment of the chondrocytes from the surface allows evaluation of the strength of cell attached to the extracellular environment.

In this case primary chondrocyte cells grown in cell culture medium with and without TGF- β 1 supplementation were rinsed 3 times with Hank's Balanced Salt Solution (HBSS) prior to trypsinization and then the cells were removed by adding 2ml of 0.25% Trypsin-EDTA (Sigma, 2007) to the culture flask. As soon as trypsin was added to the culture flask, the cells started to round up. A sequence of 45 images at 10 second intervals was taken during trypsinization using a digital camera connected to the phase contrast microscope and related software. The cell detachment rate was measured by determining the time taken for all chondrocyte cells to round up.

2.7. Statistical Analyses

Data acquired from the experiments in this study were tested for normality and then analysed by t-tests or the non-parametric equivalent tests if required. All means in this study are quoted \pm standard error (SE).

3. Results and Discussion

3.1. Proliferation

The experiments examining the effect of TGF- β 1 on chondrocyte proliferation showed that the proliferation rate of the cells in medium with TGF- β 1 supplementation was noticeably lower than that seen in control cultures (See Table 1). This effect was comparable for both chondrocytic phenotypes and indicates that TGF- β 1 controls the production of chondrocytes by decreasing cell proliferation in vitro. The proliferation rate of chondrocytes without TGF- β 1 supplementation was higher than those cultured with TGF- β 1. The

average cell number after 72 hours in the control cultures of fibroblastic chondrocytes increased to $152,667 \pm 15,147$ cells/ml, whereas treatment with TGF- β 1 for 72 hours resulted in cell numbers increasing to only $94,000 \pm 10,412$ cells/ml. It was also found that TGF- β 1 treatment for 72 hours induced the fibroblastic chondrocytes to take on a much more flattened and spread morphology. This enabled the cells to cover more of the surface without large increases in cell number (See Figures 1, and 2). This change in cell morphology was also seen in the chondrocytic chondrocyte cells, where TGF- β 1 also reduced cell proliferation by approximately 1/3 and promoted a de-differentiation into a more fibroblastic morphology.

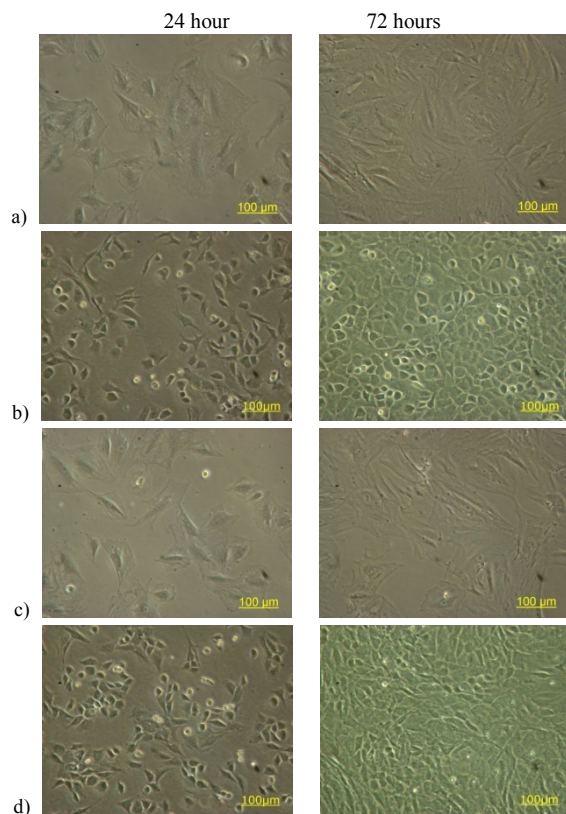


Figure 1. Chondrocyte cells cultured in monolayer without TGF- β 1 supplementation: a) fibroblastic, and b) chondrocytic phenotype. Chondrocyte cells with 10 ng/ml TGF- β 1 supplementation c) fibroblastic, and d) chondrocytic morphology

Table 1. Proliferation of chondrocytic and fibroblast like chondrocyte with and without TGF- β 1 supplementation

Fibroblastic morphology	Initial cell number (Plotting density)	Mean cell number after 72hrs
Control	50,000	$152,667 \pm 15,147$
TGF- β -1	50,000	$94,000 \pm 10,412$
Chondrocytic morphology	Initial cell number (Plotting density)	Mean cell number after 72hrs
Control	252,000	$890,000 \pm 4,362$
TGF- β -1	252,000	$518,667 \pm 4,832$

Mean cell numbers/ml \pm SE

The results of statistical analyses using t-tests supported the view that TGF- β 1 significantly ($p < 0.05$) decreased fi-

broblastic and chondrocytic chondrocyte cell proliferation after 72 hours in culture when compared with controls.

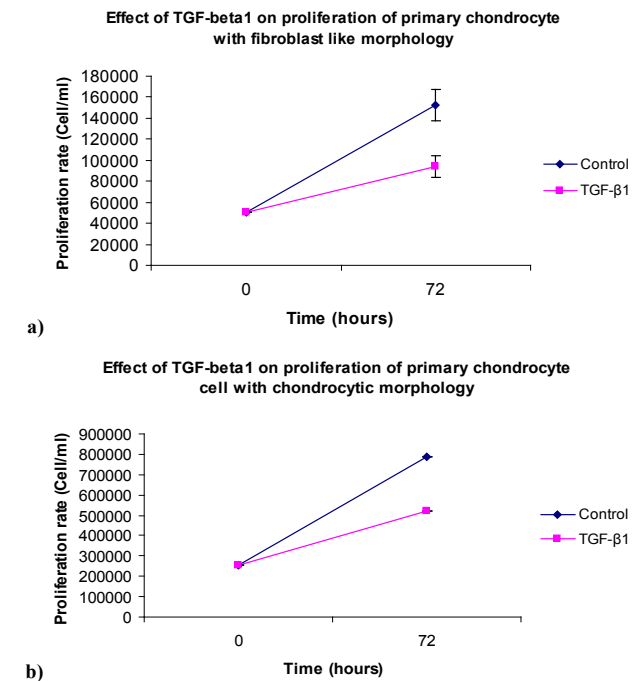


Figure 2. Effect of TGF- β 1 on the proliferation of primary chondrocyte monolayer: (a) with fibroblast like morphology, and (b) with chondrocytic morphology (mean cell numbers \pm SE)

3.2. Cell Length

The lengths of the both fibroblastic and chondrocytic chondrocytes was influenced by treatment with TGF- β 1 (See Figure 3). Cell lengths for fibroblast like chondrocytes cultured in TGF- β 1 contained media were $41 \pm 0.27 \mu\text{m}$ after 24 hours, $64 \pm 0.28 \mu\text{m}$ after 48 hours and $75 \pm 0.46 \mu\text{m}$ after 72 hours, respectively, and for cells without TGF- β 1 supplementation were $40 \pm 0.7 \mu\text{m}$ on day one, $48 \pm 0.14 \mu\text{m}$ on day two and $54 \pm 0.26 \mu\text{m}$ on day three, respectively.

In contrast with fibroblastic chondrocytes TGF- β 1 had a slightly converse effect on length of cells with chondrocytic phenotype. The average lengths of the chondrocytes in the medium without TGF- β 1 supplementation were $22 \pm 0.07 \mu\text{m}$ on day one, $25 \pm 0.06 \mu\text{m}$ on day two and $26 \pm 0.18 \mu\text{m}$ on day three, respectively and $19 \pm 0.22 \mu\text{m}$ on day one, $16 \pm 0.32 \mu\text{m}$ on day two and $21 \pm 0.34 \mu\text{m}$ on day three in the TGF- β 1 contained environment.

Statistical analyses of cell length in relation to time in culture indicated that TGF- β 1 significantly ($p < 0.05$), increased cell length at the 48 hour and 72 hour time point for the fibroblastic chondrocytes. However, in the case of chondrocytic chondrocytes, TGF- β 1 induced a significant decrease in cell length ($p < 0.05$) at the 48 hour time point, but no overall increase in cell length after 72 hours in culture. It is highly likely that the decrease in cell length seen at the 48 hour time point in the chondrocytic type cells may be associated with cell division at that time.

These results indicate that TGF- β 1 induced a significant increase in fibroblastic cell spreading possibly associated

with increased extracellular matrix protein secretion; (See Figure 3). In comparison, TGF- β 1 had little effect on chondrocytic cell length after 72 hours in culture, however the cells did change their morphology, becoming more elongated and more fibroblastic.

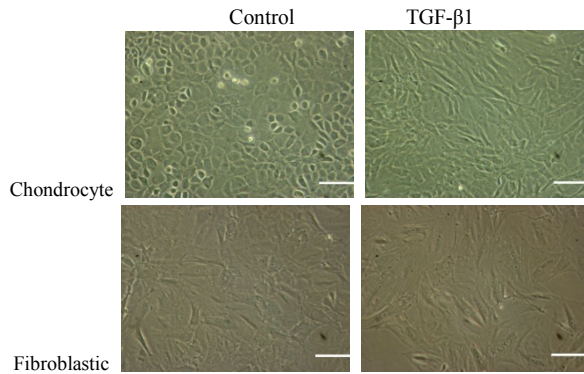


Figure 3. Cultured chondrocyte cells after 72 hours: (left) without, and (right) with TGF- β 1 supplementation (Scale bar=100 μ m)

Table 2. Change in relation to the increase in cell length for chondrocytic and fibroblastic chondrocytes

Fibroblastic CHC			
Hours	12	36	72
Control	40,01 \pm 0,07	48,27 \pm 0,14	54,69 \pm 0.26
TGF- β 1	41,40 \pm 0.27	64,64 \pm 0,28	74,95 \pm 0.46

Chondrocytic CHC			
Hours	12	36	72
Control	22,48 \pm 0.07	24,15 \pm 0.06	22,83 \pm 0.18
TGF- β 1	22,40 \pm 0.22	20,77 \pm 0.32	23,11 \pm 0.34

Mean cell length (μ m) \pm SE

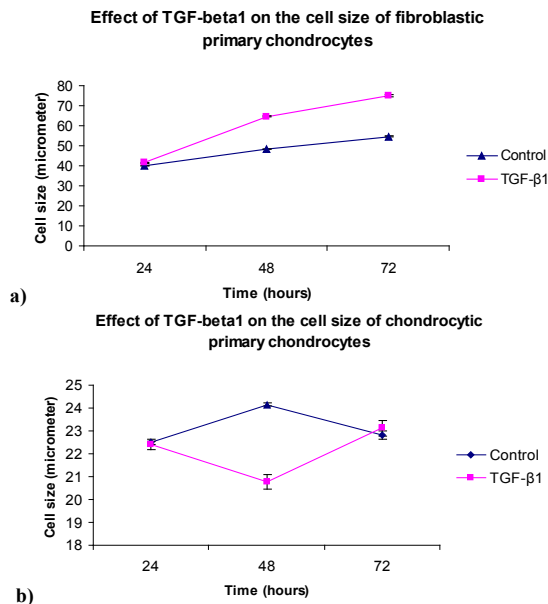


Figure 4. Cell length of primary chondrocyte monolayers vs time: (a) Fibroblastic, and (b) Chondrocytic. The error bars represent SEM of measured cell length

3.3. Immunostaining

Immunocytochemistry of monolayer cultures of chondrocytes revealed that the primary chondrocytes isolated

from knee joint of neonate Sprague-Dawley rats form different type of morphologies. Immunofluorescence micrograph of chondrocytes stained for type-I collagen, type-II collagen and fibronectin showed that the chondrocytes with 10 ng/ml TGF- β 1 supplementation obtain fibroblast like morphology while at the same density of chondrocytes cultured in environment without TGF- β 1 exhibit a rounded shape (Figure 5). The type-II collagen and fibronectin spread around the cell membrane of chondrocytes without TGF- β 1 supplementation, whereas this protein was mainly distributed between cell membrane and extracellular matrix in presence of TGF- β 1. There was no significant difference between both cultures stained for collagen type-I (Figure 5).

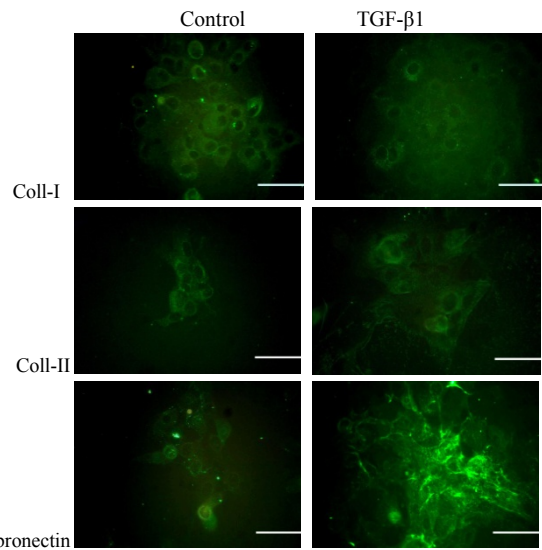


Figure 5. Immunofluorescence micrographs of primary chondrocytes stained collagen type-I, collagen type-II and fibronectin without, and collagen type-I, and collagen type-II (right) with TGF- β 1 (Scale bar=100 μ m)

3.4. Wound repair

The results for the *in vitro* monolayer wound repair assay of primary chondrocyte with fibroblastic and chondrocytic phenotypes showed that the process of healing for a mechanically created wound model in the presence of TGF- β 1 was much slower than control cultures (Figures 6-8). Addition of TGF- β 1 to fibroblastic chondrocytes caused 14.1 % increase in wound size after 24 hours, and 9.8% increase in wound size after 72 hours as compared to control which showed 52.2% wound closure after 24 hours and 96.6% wound closure after 72 hours, respectively.

Statistical analyses indicated that wound widths in response to TGF- β 1 treatment in fibroblastic chondrocytes cultures remained unchanged after 72 hours in culture ($p=0.94$). However, in controls cultures of fibroblastic chondrocytes wound size decreased significantly after 72 in culture ($p<0.05$).

In contrast, control culture of chondrocytes with a chondrocytic phenotype showed ~ 28.3% wound closure after 24 hours and 79.1% after 72hours. Addition of TGF- β 1 caused 8.4% wound closure after 24 hours and 71.2% after 72 hours (see Figure 6).

This suggests that TGF- β 1 down regulates cell proliferation, adhesion and cell migration which are essential in wound repair. In all experiments the wound width increased during the first 6 hours after creating the wound. Statistical analyses of wound closure in chondrocytic cell cultures indicated that although TGF- β 1 seemed to decrease wound closure this decrease in wound closure as determined by comparing wound width after 72 hours was not significant ($p=0.133$).

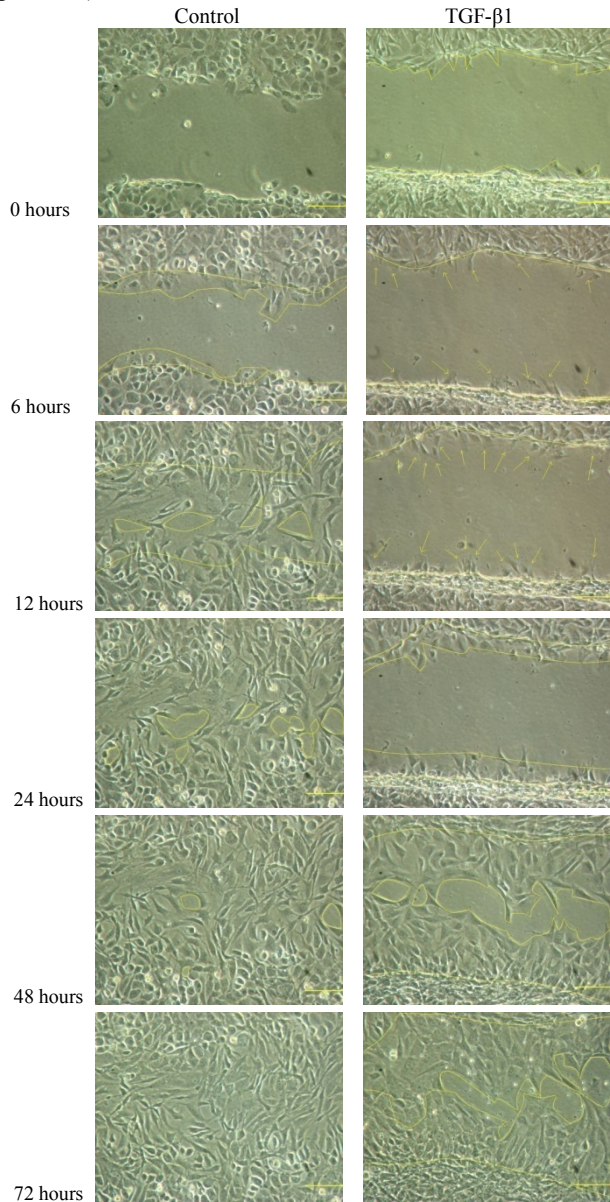


Figure 6. An evaluation of wound closure for primary chondrocytes with chondrocytic phenotype with and without TGF- β 1 supplementation (Scale bar=100 μ m)

In both the fibroblastic cultures and chondrocytic chondrocyte cultures, TGF- β 1 seemed to induce a further wound enlargement. This may indicate that TGF- β 1 may play a part in inducing a stress response in chondrocytic cells similar to that described by Lin and Helmke (2009) and a production of nitric oxide, consequently induction in cell death[13] as described by Im and Shin (2002). This may play a part in the

down regulation of chondrocyte mitogenesis.

Interaction of cells with ligands expressed by neighboring cells, and cell-cell signaling plays an important role in cell proliferation[11]. In fact, cell-cell communication can stimulate cell proliferation[5], the chondrocytes monolayer consisting of fibroblast like cells demonstrated an increase in wound size during 72 hours wound closure (See Figures 7 and 8). This may be related to TGF- β 1 increasing the secretion of ECM molecules at the wound edge, which functions by capturing cells via the integrin ECM interactions at this edge, thus preventing migration into the wound site. This could go some way towards explaining the poor repair capacity of cartilage *in vivo*. *In vivo* such a mechanism may be overridden in therapeutic procedures by filling a wound site with large numbers chondrocytes which would presumably enable the deposition of ECM across the wound surface enabling cell migration and proliferation into the wound. Such a system would certainly explain the partial success seen in autologous chondrocyte implantation techniques.

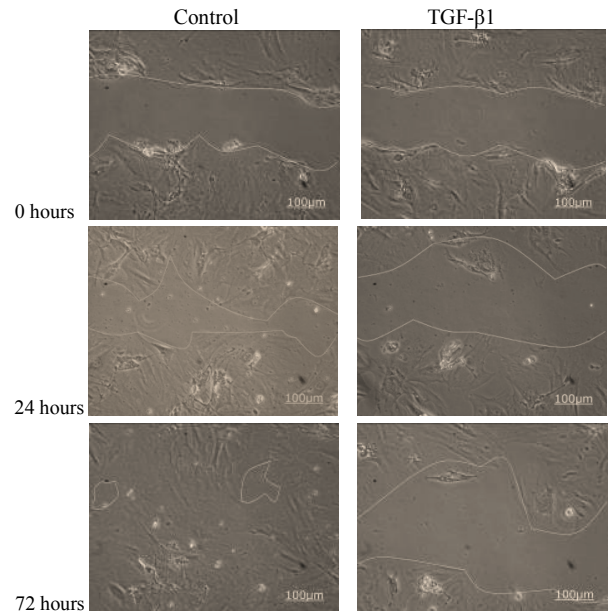
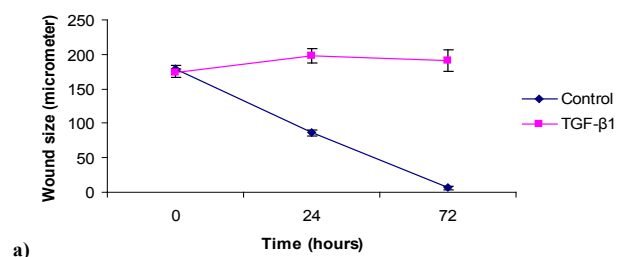
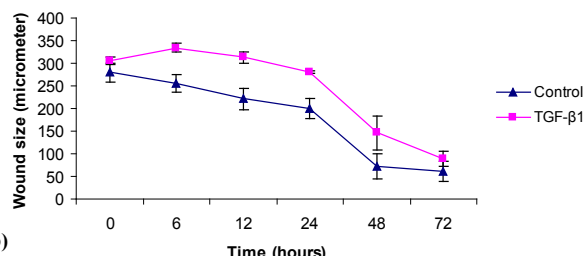


Figure 7. A comparison in wound closure of chondrocytes with fibroblast like morphology with and without TGF- β 1 addition

Figure 8 is the graph of wound closure vs. time for the fibroblastic and chondrocytic chondrocytes which shows different cell proliferation rate and cell migration for control culture and treated with TGF- β 1. TGF- β 1 inhibited wound closure in the fibroblastic monolayer.

Effect of TGF-beta1 on wound closure of chondrocyte with fibroblast like morphology

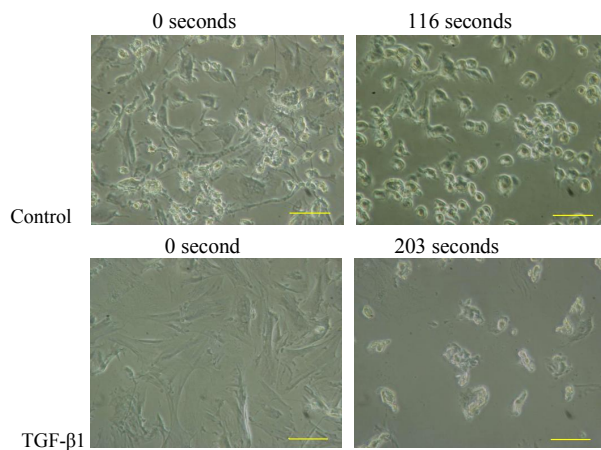


Effect of TGF-beta1 on wound closure of chondrocyte with chondrocytic morphology**Figure 8.** Wound closure assay for primary chondrocyte with: (a) fibroblast like morphology, and (b) chondrocytic morphology. The error bars represent SEM of wound size measurements during the experiment

One important effect of TGF- β 1 on chondrocytes *in vitro* was the rapid differentiation into fibroblast like cells (See Figures 3, 6, and 7). This may be linked to increased synthesis of extracellular matrix and extended fibres causing the cells to occupy larger area which prohibited cell migration and consequently reduction of cell proliferation.

3.5. Trypsinization Assay

Trypsinization of chondrocytes monolayers revealed that the detachment time (See Table 3) from a solid surface for chondrocytes with fibroblast like morphology with TGF- β 1 supplementation was longer than those cultured in medium without TGF- β 1. However, this time for cells with chondrocytic morphology and TGF- β 1 supplementation was relatively short when compared to the cells without TGF- β 1 supplementation (See Table 3).

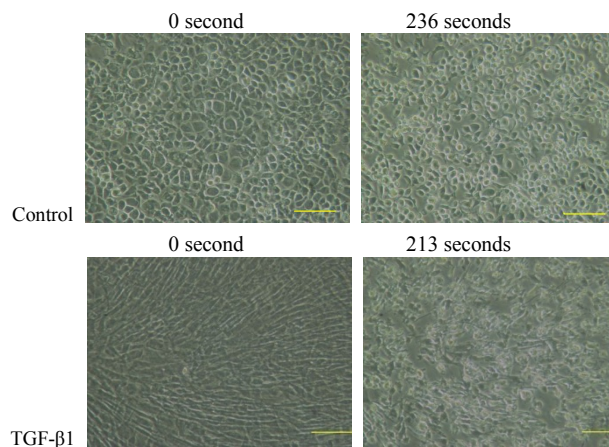
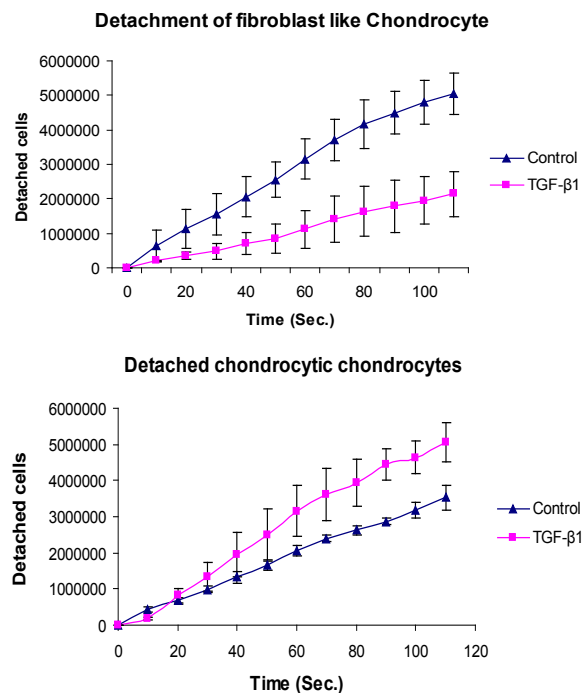
**Figure 9.** Detachment of primary chondrocyte cells with fibroblast like morphology from solid surface by trypsinization (Scale bar=100 μ m)

From Table 3 and Figures 9 and 10 it can be seen that control culture of fibroblast like cell started to detach after approximately 63 seconds and completely detachment achieved after 116 seconds, whereas the same cells in presence of TGF- β 1 started to detach after approximately 93 seconds and achieved completely detachment after about 203 seconds.

In contrast the control culture of chondrocytic cells started detaching after about 85 seconds and showed complete detachment after 263 seconds, whereas the chondrocytes in

TGF- β 1 contained medium started to detach after about 96 seconds and after approximately 213 seconds fully detachment was achieved.

Although the surface of the culture flask was covered completely by cells, the population of fibroblast like chondrocytes was very low as compared to the cells with chondrocytic morphology.

**Figure 10.** Detachment of primary chondrocyte cells with chondrocytic morphology from solid surface by trypsinization (Scale bar=100 μ m)**Figure 11.** Detachment of the primary chondrocytes: (above) with fibroblast like morphology, and (below) with chondrocytic morphology, with and without TGF- β 1

The ECM molecules are easily digestible, but the digestion time depends on initial distribution and concentration of proteins. In the case of fibroblast like morphology, excessive production of ECM proteins in the presence of TGF- β 1 (Figures 3, 6 and 7) may cause an increase in detachment time.

Detachment times for both fibroblastic and chondrocytic cells (See Table 3 and Figure 11) showed that TGF- β 1 in-

creases adhesion of chondrocytes with fibroblast like morphology to a solid surfaces, but decreased adhesion of chondrocytic chondrocytes to a solid surface in relation to untreated cells.

Table 3. Detachment time in minutes for chondrocytes with fibroblastic morphology (top) and chondrocytic morphology (below)

Fibroblast like cells	
Experiment	Detachment time
Control	116 seconds
TGFβ1	203 seconds
Difference	87 seconds
Chondrocytic cells	
Experiment	Detachment time
Control	263 seconds
TGFβ1	213 seconds
Difference	50 seconds

4. Conclusions

Primary chondrocyte cells acquired a fibroblast like morphology after the first subculture in the 2-dimensional cell culture system. TGF-β1 caused down regulation of chondrocyte proliferation and up-regulation of cell death.

TGF-β1 caused increase in cell length of chondrocyte with fibroblastic phenotype but not with chondrocytic chondrocyte.

TGF-β1 had a negative effect in wound closure process of chondrocytes *in vitro* suggesting that the inactive TGF-β1 down regulates cell proliferation and cell migration which are essential for tissue repair.

The healing process of scratched chondrocytes monolayer started after ca. 6 hours after creating the wound model. This confirms the stress responsive nature of the cell and delay in the cell activation, particularly the down regulation of chondrocyte mitogenesis[3].

TGF-β1 therefore plays a very important role in rapid formation of fibroblast like chondrocyte cells *in vitro*. This could be linked to an up-regulation in the production of extracellular matrix and extended collagen type-I fibres causing the cells to flatten and occupy a larger area which prohibits cells migration and consequently reduces cell proliferation[6].

Results of this study also suggest that TGF-β1 stimulates chondrocyte-ECM adhesion but that this depends on the cell phenotype. Chondrocytes with a fibroblast like morphology cultured in medium with TGF-β1 supplementation displayed a greater degree of cell/surface attachment than did those those cultured in medium without TGF-β1. In contrast the degree of cell surface attachment was decreased in cells with chondrocytic morphology when cultured in TGF-β1 contained media. It is believed that TGF-β1 is involved in chondrocyte cell-ECM adhesion suggesting that TGF-β1 may cause synthesis of different type of CAMs, which may influence cell migration and proliferation in the wound site. However, the negative effect of this cytokine in wound repair of cartilage suggests the hypothesis that the inhibition or

activation of TGF-β1 may induce cartilage repair[8]

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