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TGF-β1 is the Factor Secreted by Proliferative Chondrocytes to Inhibit Neo-Angiogenesis

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Abstract Growth plate is an avascular tissue, which has been reported to be capable of retarding tumor spread. It is believed that angiogenic inhibitor(s) exist to inhibit the neo-vascularization of tumor, thus impeding the tumor growth. In this study, growth plate chondrocyte-derived TGFβ1 was identified to be anti-angiogenic. It was found that growth plate chondrocytes (GPC) secreted TGFβ1 mostly in latent form as demonstrated by gel filtration and immunoblotting. Enzyme-linked immunosorbent assay (ELISA) was followed to quantify TGFβ1 in GPC conditioned medium (CM), in which 866 pg/ml of TGFβ1 was found. Besides, the angiogenesis inhibitory effect of GPC CM was abolished by the addition of anti-TGFβ1 antibody in the in vitro culture system and the in vivo chick chorioallantoic membrane (CAM) assay. This confirmed the anti-angiogenic properties of chondrocyte-derived TGFβ1. TGFβ1 was expressed predominantly in the proliferative zone of porcine growth plate. This explains the low incidence of tumor invasion across the entire growth plate. Also, this helps to explain the observation that tumor invasion across the physis increases with age as the proliferative zone gradually disappears. J. Cell. Biochem. Suppl. 36:79–88, 2001. © 2001 Wiley-Liss, Inc.

Key words: TGFβ1; growth plate; chondrocytes; endothelial cells; anti-angiogenesis

Physeal retardation to tumor spread is a common clinical phenomenon. Since blood vessels are absent in cartilage, it is thought that avascularity may contribute to the cartilaginous retardation effect [Schajowicz, 1994; Unni, 1996].

Solid tumor growth is well-known angiogenesis dependent. Neovascularization is associated with the increased tumor growth and metastasis. Increasing research work has been done on searching angiogenic inhibitors from different sources [Kuettner et al., 1976; Moses et al., 1992; O'Reilly et al., 1994, 1996, 1997; Gately et al., 1996]. Many anti-angiogenic factors are reported to be isolated from cartilage, including Cartilage-derived Anti-tumor Factor (CATF), Chondrocyte-derived Inhibitor (ChDI), Bovine cartilage-derived Inhibitor (BCDI), Cartilage-derived Inhibitor (CDI), and Elastase inhibitor etc [Kuettner et al., 1976, Roughley et al., 1978; Takigawa et al., 1985, 1987; Murray et al., 1986; Gabrielides and Barreau, 1987; Homandberg et al., 1992; Moses et al., 1992]. However, their properties and molecular sizes are not consistent among one another. There is controversy in this issue.

In our previous study, we demonstrated that porcine growth plate chondrocytes secreted potent anti-angiogenic factor(s) to inhibit the proliferation and migration of endothelial cells in vitro and angiogenesis in vivo [Cheung et al., 2001]. Our results have proven that anti-angiogenic effect dominates in the proliferative zone-rich growth plate chondrocytes. Growth plate has the capability to retard tumor growth, depending on the proliferative activity and differentiative stage of cartilage.

In the present study, we identified and characterized the angiogenic inhibitor(s) present in the growth plate. The growth plate chondrocytes conditioned medium was fractionated by gel filtration (FPLC) and further characterized by SDS-PAGE and Western Blotting. The anti-angiogenic effect of the identified

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protein(s) was then evaluated by adding the blocking antibodies in the in vitro culture system and in vivo chick chorioallantoic membrane (CAM) assay. Immunohistochemistry was also performed to study the distribution of the targeted anti-angiogenic factor(s) in different zones of the growth plate.

MATERIALS AND METHODS

Porcine Growth Plate Chondrocytes (GPC) Culture

Animal ethics was approved by Animal Research Ethics Committee, C.U.H.K. Chondrocytes were isolated from pigs of 4 and 5 weeks of age (about 10 kg) by the method from Lee et al. [1996]. After the animal was euthanized by over dosage of pentobarbital, the rib cage was aseptically dissected free from soft tissue. The growth plate cartilage was excised at osteochondral junctions of all the ribs and placed in modified F-12 medium (magnesium-free, 0.5 mM $CaCl_2$) (Sigma, St. Louis, MO). After weighing, the cartilage was cut into 0.1 mm^3 pieces and digested with 0.1% trypsin (type III; Sigma) for 30 min at 37°C to break down the core and link protein in the cartilage matrix. Then, the tissue was digested by 0.1% hyaluronidase (type I-S; Sigma) for 1 h to cleave the proteoglycan aggregate. Overnight digestion in 0.075% collagenase (type IIA; Sigma) in a shaking 37°C water bath was applied to break down the collagen fibrils. One mililiter of each enzyme solution was used for every 20 mg of cartilage. After centrifugation (for 4 min at 600 *g*), the cells were then filtered through a glass wool filter and washed twice with the modified F-12 medium to remove the digested matrix debris. Cells were resuspended in fresh modified F-12 medium with 10% fetal bovine serum (FBS; GibcoBRL, Grand Island, NY) and cell number was determined by trypan blue exclusion test. Chondrocytes (10^6) were seeded into a 75 cm^2 culture flask and incubated at 37°C, 5% CO_2, 95% humidity. Medium was changed every three days.

When GPC grew to 80% confluent, the chondrocytes were washed with PBS. Eight mililiter serum-free DMEM was added into the 75 cm^2 culture flask and incubated for 24 h. The conditioned medium (CM) was collected and centrifuged at 1,000 *g* for 10 min to remove cell debris. The conditioned medium was aliquoted into eppendorfs and stored at −20°C until it was used for analysis.

Endothelial Cell (ECV304) Culture

Human ECV304 endothelial cell line (ATCC, Manassas, VA), which was a spontaneously transformed immortal endothelial cell line established from the vein of an apparently normal human umbilical cord, was used for biological assays in this study. These cells were maintained in culture medium DMEM with 5% heat-inactivated FBS. Medium was changed every other day. For the biological assays, cells were plated in 96-well plates at a density of 10,000 cells/well and seeded for overnight before adding the samples being tested.

Fast Protein Liquid Chromatography (FPLC)

Gel filtration was used to screen the proteins of the GPC CM. Superdex 200 HR 10/30 (Pharmacia Biotech, Piscataway, NJ) with molecular weight range 10,000–600,000 was used to separate GPC conditioned medium. The mobile phase was 50 mM sodium phosphate buffer with 0.15 M NaCl (pH 7.0). Protein standard containing blue dextran, BSA, transferrin, ribonuclease A and trypsin inhibitor was used to calibrate the system. 200 μl of GPC CM was run for 80 minutes. Fractions were collected every 5 minutes. Wavelength of 280 nm was set to detect the collected fractions. Molecular weights of the eluted proteins were determined using the standard curve.

Fractions of each FPLC peak were pooled together and concentrated using Centriprep-3 (Amicon, Beverly, MA) with a molecular weight cut-off of 3,000 daltons. The concentrated retenate of each peak was mixed with equal volume of DMEM-10% FBS and sterilized by filtering through a 0.22 μm membrane (Millipore, Bedford, MA). The mixture was used to culture human endothelial cells (ECV304) in 96-well plates (10,000 cells/well) at 37°C for 48 h. The proliferation of endothelial cells was determined by thymidine incorporation assay.

Thymidine Incorporation Assay

Thymidine incorporation assay was used to measure the proliferation of cells. Medium in the wells of 96-well plate was aspirated. 100 μl freshly prepared [^{3}H]thymidine (Amersham, Piscataway, NJ) containing medium was added into wells being assayed (0.5 μCi/well). The plate was incubated for 4 h to label endothelial

cells. After removing the medium with excess thymidine, the wells were rinsed with 200 µl PBS twice to remove trace of ^{3}H containing medium. The cells were lysed by 500 µl of 0.25 N NaOH. The lysate was collected in test tubes. To neutralize the alkalinity, 500 µl of HCl (0.25N) was added. 400 µl Hepes with 2.5 mg/ml Bovine Serum Albumin (BSA) was added to the lysate, followed by the addition of 10 N perchloric acid to precipitate the thymidine labeled DNA. The lysates were kept at 4°C for 1 h and then centrifuged at 15,000 *g* at 4°C for 30 min to spin down the precipitate. The supernatant was decanted. The pellets were resuspended in 500 µl 0.25 N NaOH and then transferred to counting vials. Four milliliter of scintillation fluid (Wallac Scintillation products, Turku, Finland) was added to each vial. The radioactivity of the acid-insoluble DNA content was measured by a Beckman liquid scintillation spectrometer (LS3801).

Immunodetection of Transforming Growth Factor β1 in GPC CM

Immunodetection was done to confirm that the protein(s) with anti-angiogenic activity was TGFβ1-related. Before loading into polyacrylamide gel, GPC conditioned medium was acid-activated to release active TGFβ1. To 0.5 ml conditioned medium, 0.1 ml 1 N HCl was added and mixed well. It was incubated at room temperature for 10 min and neutralized by adding 0.1 ml 1.2 N NaOH/0.5 M Hepes.

SDS-PAGE was performed with 10% polyacrylamide gels. Samples (non-activated and activated GPC CM) were reduced with mercaptoethanol (Sigma) before application. The gel was immediately subjected to Western Blotting. The proteins on the gels were blotted to a nitrocellulose membrane which was immunodetected with anti-TGFβ1 antibodies (1:1000, R&D Systems, MN). After the incubation with secondary antibody (Chick IgG, 1:1000, Chemicon, Temecula, CA) and Streptavidin/biotinylated–HRP complex solution (DAKO, Glostrup, Denmark), the signal was detected by ECL detection Kit (Amersham).

Assay of Transforming Growth Factor β1 by Enzyme-Linked Immunosorbent Assay (ELISA)

Human TGFβ1 was quantified in GPC conditioned medium after acid activation using a Quantikine® TGFβ1 ELISA Kit (R&D Systems), according to the manufacturer's instructions. Activation of GPC CM was performed as described above.

In Vitro Antibody Neutralization Assay

Antibody neutralization assay was conducted by depleting TGFβ1 from GPC conditioned medium before addition to the endothelial cells (ECV304). Briefly, anti-TGFβ1 antibody (R&D Systems) was added to the GPC CM with the final concentration of 0, 0.1562, 0.3125, and 0.625 µg/ml. The GPC CM with antibody was mixed with DMEM-10% FBS at 1:1 ratio. The mixture was used to culture the endothelial cells (ECV304) in 96-well plates (10,000 cells/well) at 37°C for 48 h. The proliferation of endothelial cells was determined by thymidine incorporation assay.

In Vivo Antibody Neutralization Assay

Antibody neutralization assay was conducted by depleting TGFβ1 from GPC conditioned medium before addition to the in vivo model–chick CAM assay. Fertilized eggs obtained from local Kadoorie Farm and Botanic Garden were incubated at 37°C, 60–70% humidity for 5 days. At Day 5, a small window was made at the air sac of the eggs. The shell membrane was peeled off to expose the CAM of chick embryo. Two sterile 6-mm diameter circular filter paper discs soaked with total 60 µl different conditioned medium were applied onto the surfaces of the growing CAMs. The windows were sealed with 3M Tegaderm and the eggs were incubated for another 48 h. At Day 7, the filter paper discs were removed and the growth pattern of blood vessels around the filter papers was assessed.

Immunohistochemistry

Immunohistochemistry was performed to study the distribution of TGFβ1 in growth plate sections. The goat brain was used as the positive because the TGFβ1 antibody was purified from bovine brain. All paraffin sections were dewaxed by two changes of xylene and rehydrated through a graded series of aqueous ethanol solution. To quench the endogenous peroxidase, sections were treated with 3% hydrogen peroxide solution for 10 minutes. After washing in phosphate buffered saline (pH 7.4), sections were digested briefly with trypsin (0.25 µg/ml) and hyaluronidase (5 mg/ml, Sigma) at 37°C for 5 and 7 min, respectively. Then, the nonspecific binding sites on the sections were blocked by 20% goat serum

(Sigma) in 0.5% bovine serum albumin (Sigma) in phosphate buffered saline for 20 minutes. Then sections were incubated with chick anti-human TGFβ1 primary antibody (1:800, R&D Systems) diluted in 0.5% bovine serum albumin in phosphate buffered saline at 4°C overnight in a humid chamber.

After being washed three times with phosphate buffered saline, sections were incubated with the biotinylated secondary antibody, goat anti-chick Immunoglobulin Type G (1:800, Chemicon) for 30 min at room temperature. After being washed with phosphate buffered saline, sections were incubated with streptavidin–biotin–peroxidase complex (1:100) included in the DAKO Duet kit for 30 min at room temperature. After washing with phosphate buffered saline, sections were reacted with diaminobenzidine solution containing hydrogen peroxide (DAB Chromogen Kit, DAKO) in phosphate buffered saline. The reaction was stopped by washing with phosphate buffered saline and the sections were counterstained with Mayer's hematoxylin for 1 min before permanent organic mounting by DPX Mountant (Fluka, Milwaukee, WI). For negative control, 0.5% bovine serum albumin in phosphate buffered saline was used instead of the primary antibody.

Image Analysis

The results of immunohistochemistry were analyzed with Metamorph 4.5 image analysis software (Universal Imaging System). The analysis was performed using Hue-Saturation-Intensity (HSI) system to detect the interested colour. The average optical density (OD) of the proliferative zone and the hypertrophic zone were compared, where OD=$\log_{10}$(1/transmittance). Stronger signal gave a higher average OD.

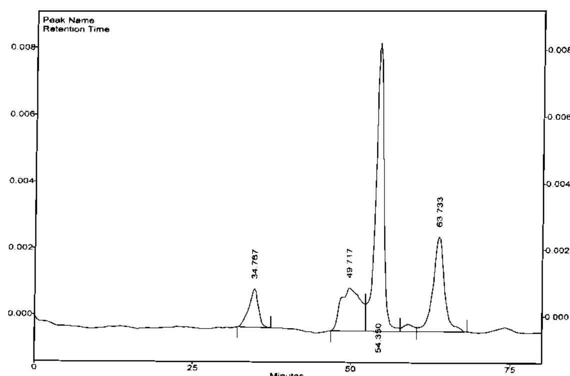


Fig. 1. Chromatograph showing the protein profile of growth plate chondrocytes conditioned medium (GPC CM). Four peaks were shown at the retention time of 34.8, 49.7, 64.4, and 63.7 minutes. With the reference of standard curve, their molecular weights were respectively 107 k, 12 k, 6 k, and 1.5 k.

Statistical Analysis

All results were expressed as the arithmetic mean ± standard deviation (SD). Data from each experimental group was compared with those from the corresponding control group using Student's t-test. Normally, $P < 0.05$ was regarded as significantly different unless otherwise specified.

RESULTS

Gel Filtration

The growth plate chondrocytes conditioned medium was separated by molecular sizes. The FPLC chromatograph of GPC CM demonstrated that there were four main peaks. Their retention time were respectively 34.8, 49.7, 54.4, and 63.7 minutes (Fig. 1). Referring to the standard curve, the molecular weights of the four peaks were respectively around 107 k, 11.7 k, 5.9 k, and 1.5 k.

The peak fractions of different peaks were collected and their anti-angiogenic effects on the proliferation of endothelial cells were tested. Results demonstrated that peak 1 fractions and peak 2 fractions exerted around 9% and 12.5% inhibition on the proliferation of endothelial cells significantly ($P = 0.004$ for peak 1; $P < 0.001$ for peak 2) (Fig. 2). In other words, fractions of 107 kDa and 11.7 kDa had the inhibitory activity.

Immunodetection of TGFβ1

The proteins in the SDS-PAGE were blotted to a nitrocellulose membrane and probed with anti-TGFβ1 antibodies. After the ECL detection, it was found that for non-activated growth plate chondrocytes conditioned medium, a major band at 70 kDa and a minor band at 140 kDa were detected (Fig. 3a).

After acid activation of the growth plate conditioned medium, an additional band at the position of 25 kDa, in addition to the bands at 70 kDa and 140 kDa, was observed (Fig. 3b).

Quantitation of Transforming Growth Factor β1 by Enzyme-Linked Immunosorbent Assay (ELISA)

To quantify the amount of active TGFβ1 in GPC conditioned medium, ELISA was performed. Results showed that in non-activated conditioned medium, the concentration of active TGFβ1 was 206 pg/ml only. After acid-activation, the concentration of activated TGFβ1 increased to 866pg/ml. In other words, only 24% TGFβ1 existed in active form in growth plate chondrocytes conditioned medium.

In Vitro Antibody Neutralization Assay

Results shown in Figure 4a revealed that when antibodies-free GPC CM mixture was used to culture ECV304, there was a statistically significant 9.4% inhibition of endothelial cells proliferation ($P < 0.001$).

When anti-TGFβ1 antibodies were added to the culture, the inhibition of endothelial cells proliferation was decreased. The proliferation of endothelial cells became comparable to that of the control when the concentration of antibodies increased to 0.3125 μg/ml. Further increase in antibodies concentration to 0.625 μg/ml could not further reduce the effect of growth plate chondrocytes conditioned medium on endothelial cells. In the meantime, antibody controls were also done by culturing ECV304 in DMEM containing 5% FBS and supplemented with different concentration of anti-TGFβ1 antibodies. In the absence of GPC CM, anti-TGFβ1 antibodies has no significant effect on the proliferation of endothelial cells (Fig. 4b).

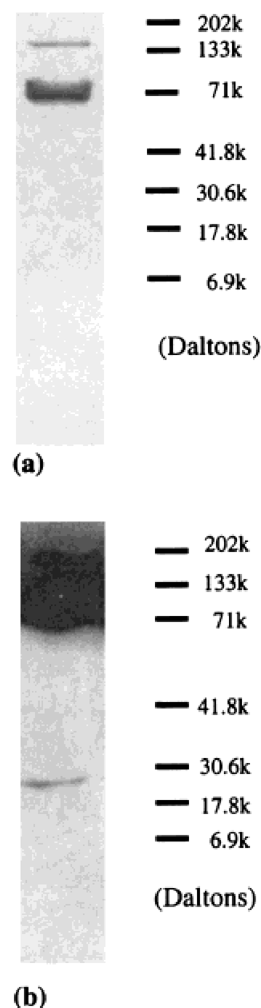


Fig. 3. Immunodetection of growth plate chondrocytes conditioned medium (GCPCM) using anti-TGFβ1 antibody. The non-activated GPC CM was shown in (**a**) while activated GPC cm with additional 25 kDa band in (**b**).

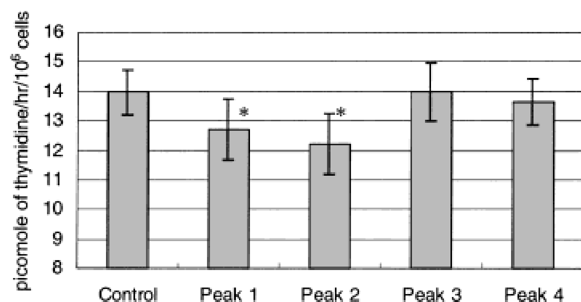


Fig. 2. Effect of FPLC peak fractions of GPC CM on the proliferation rate of endothelial cells (ECV304).FPLC fractions of GPC CM were used to culture ECV304 for 48 h and the cell proliferation rate was measured by thymidine uptake assay. * showed statistically significant difference for peak 1 ($P = 0.004$) and peak 2 fractions ($P < 0.001$). 9.1% and 12.5% decrease of cell proliferation rate was found respectively for peak 1 and peak 2 fractions. No significant difference was found for peak 3 ($P = 0.949$) and peak 4 fractions ($P = 0.367$). *Error bar = 1SD*

In Vivo Antibody Neutralization Assay

GPC CM supplemented with 0.3125 μg/ml anti-TGFβ1 antibodies was applied into the CAM in vivo. After 48-h incubation, the CAM morphology, including the number, density, and distribution of blood vessels, was very similar to the control (DMEM). Under the filter paper discs, the capillaries remained very smooth and continuous and did not show any abnormality (Fig. 5).

Immunohistochemistry

Results showed that TGFβ1 was mainly distributed in the proliferative zone of growth

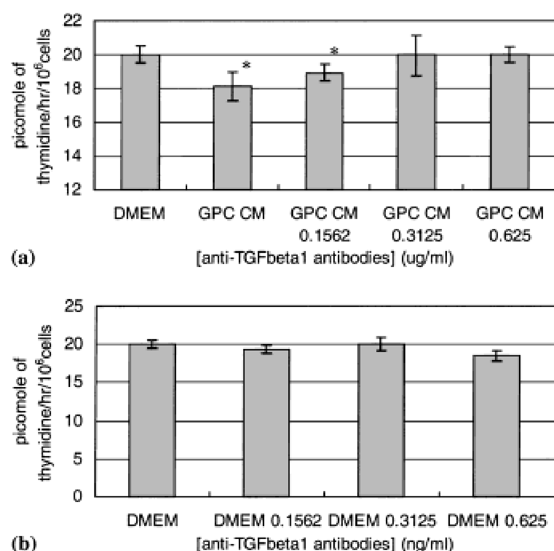


Fig. 4. Effect of anti-TGFβ1 antibodies on the anti-angiogenic activity of GPC CM in vitro. Anti-TGFβ1 antibodies were added into GPC CM and used to culture ECV304 for 48 h and the cell proliferation rate was measured by thymidine uptake assay. **(a)** * showed statistically significant difference for GPC CM ($P < 0.001$) and GPC CM 0.1562 ($P = 0.002$). 9.4% and 5.4% decrease of cell proliferation rate was found respectively for GPC CM and GPC CM 0.1562 compared with control (DMEM). No significant difference was found for GPC CM 0.3125 ($P = 0.898$) and GPC CM 0.625 ($P = 0.850$). *Error bar = 1SD.* **(b)** showed antibodies control in which different concentration of antibodies were added into DMEM to culture endothelial cells. The antibodies in DMEM did not demonstrate statistically significant effect on endothelial cell proliferation rate. *Error bar = 1SD*

plate cartilage (Fig. 6). Positive signal was also detected in the hypertrophic and resting zone but the intensity was much lower when compared with that at the proliferative zone. The average OD at the proliferative zone was 0.19 ± 0.06 while that at the hypertrophic zone was 0.17 ± 0.04. The signal at the proliferative zone was stronger than that at the hypertrophic zone.

DISCUSSION

In the present study, we have identified the proteins with anti-angiogenic activity in growth plate chondrocytes conditioned medium (GPC CM). Our findings demonstrated that TGFβ1 was the anti-angiogenic factor in growth plate, which contributed to the physeal retardation effect to tumor spread. Our previous study has confirmed that growth plate chondrocytes secreted potent anti-angiogenic factor(s) to inhibit angiogenesis in vitro and in vivo. In this study, we analyzed the GPC CM using gel filtration. Results demonstrated that the protein profile of GPC CM consisted of 4 peaks and the 107 kDa and 11.7 kDa peak fractions showed inhibitory activity. From the information of the molecular sizes, latent transforming growth factor β1 (TGFβ1) may be one of the candidates, which has been well reported to be around 100 kDa [Bonewald et al., 1991; Boyan et al., 1994; Dallas et al., 1994]. Furthermore, TGFβ1 was rich in growth plate [Thorp et al., 1992; Jingushi et al., 1995] and was capable of inhibiting endothelial cell proliferation and tube formation [Baird and Durkin, 1986; Frater-Schroder et al., 1986; Muller et al., 1987]. We therefore suspected TGFβ1 to be the anti-angiogenic factor in growth plate.

To confirm whether GPC CM consisted of TGFβ1, immunodetection using anti-TGFβ1 antibody was performed. Two bands at 70 kDa and 140 kDa were shown, which was believed to be latent TGFβ1. Latent TGFβ1 complex actually consists of mature homodimer TGFβ1 (25 kDa) associated with latency-associated peptide (LAP, 75–80 kDa), which in turns is linked by a disulfide bond to a latent TGFβ binding protein (LTBP, 190 kDa) [Kanzaki et al., 1990; Dallas et al., 1994]. In the present study, under reducing condition, all the disulphide linkages were broken. As a result, relatively large amount of noncleaved propeptide monomer containing TGFβ1 monomer should be present in the reduced GPC CM, with the relative molecular weight of around 55–60 kDa [Bonewald et al., 1991]. Our results, however, demonstrated the band with 70 kDa. This discrepancy might be attributed to two reasons. Firstly, the higher molecular weight might be due to the extensively glycosylated proregion (LAP), which has been reported by McMahon et al. [1996]. Secondly, the protein marker provided the information of apparent molecular weights only. Some deviation might be resulted. Besides, the presence of a minor band at 140 kDa in Western Blotting was due to the re-formation of some reduced latent TGFβ1 molecules. The intramolecular disulfide bonds reformed in some latent TGFβ1 dimers.

Enzyme-linked immunosorbent assay (ELISA) was followed to further confirm the presence of TGFβ1 in GPC CM and the level of TGFβ1 in GPC CM was determined. Results demonstrated that GPC CM contained 866 pg/ml TGFβ1, including the active TGFβ1 released

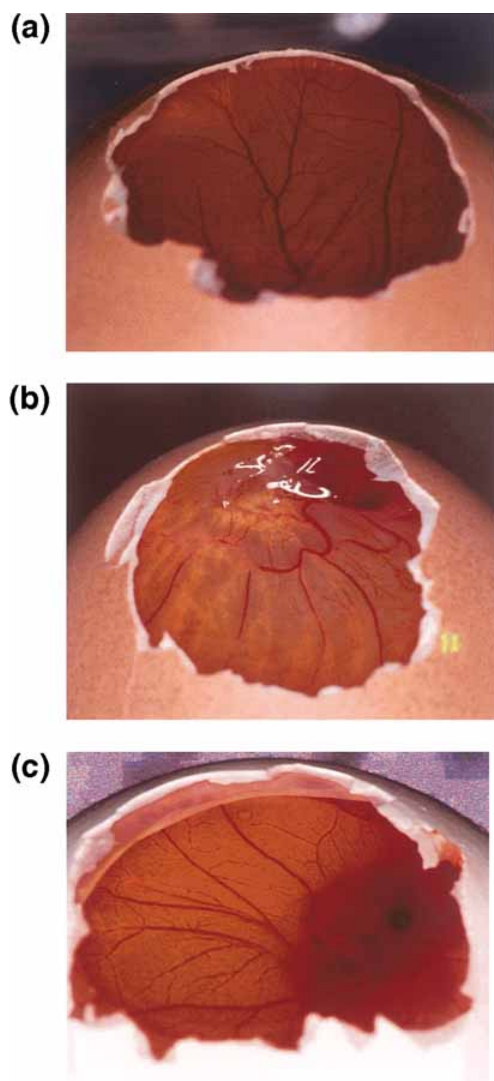
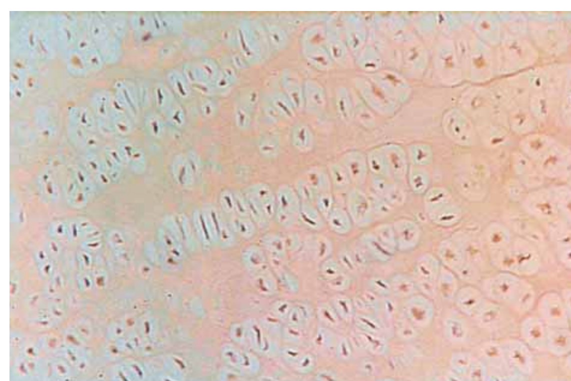


Fig. 5. Effect of anti-TGFβ1 antibodies on the anti-angiogenic activity of GPC CM in vivo. Angiogenic response of the CAM at Day 7 after 48-hour incubation with (**a**) DMEM (**b**) GPC CM (**c**) GPC CM supplemented with anti-TGFβ1 antibody. Highly disrupted and discontinuous blood vessels were observed in (b). But smooth and straight blood vessels were observed in (c) similar to the control using DMEM (a). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

from latency by mild acid treatment. Previous study has reported that 100 pg/ml TGFβ could significantly inhibit the bFGF-induced endothelial cell proliferation [Baird and Durkin, 1986]. It is also known that high concentration of TGFβ1 inhibits the endothelial cell proliferation and invasion significantly [Baird and Durkin, 1986; Frater-Schroder et al., 1986; Muller et al., 1987]. These previous reports agreed with our results.



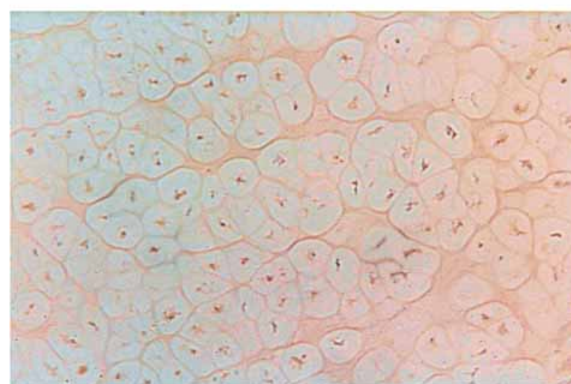


Fig. 6. Immunodetection of TGFβ1 in porcine growth plate section **(a)** Positive signal was mainly distributed in the proliferative zone of growth plate cartilage. **(b)** The signal was also detected in the hypertrophic zone but the intensity was much lower when compared with that of the proliferative zones. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

From the results, we revealed that growth plate chondrocytes secreted TGFβ1 in latent form rather than in active form. Active form of TGFβ1 appeared at the position of 25 kDa in SDS-PAGE only after acid activation. The result of ELISA also showed that only 24% TGFβ1 existed in active form in the GPC conditioned medium. This confirmed that most of the TGFβ1 in growth plate was in latent form. This was in agreement with the previous findings. It has been reported that chondrocytes expressed TGFβ1 with 90% in latent form [Villiger and Lotz, 1992; Farquharson et al., 1996; D'Angelo and Pacifici, 1997]. Other cell types also secreted latent TGFβ1 of around 100 kDa [Bonewald et al., 1991; Boyan et al., 1994; Dallas et al., 1994].

To determine whether chondrocyte-derived TGFβ1 was responsible for inhibiting angiogen-

esis, in vitro and in vivo neutralization assays using antibody against TGFβ1 were conducted. The addition of anti-TGFβ1 antibody to the GPC conditioned medium blocked the anti-angiogenic activity in vitro. This provides solid evidence that the angiogenic inhibitor secreted by growth plate chondrocytes is TGFβ1. Besides, our observation of in vivo neutralization assay was also consistent with the in vitro findings. GPC CM supplemented with anti-TGFβ1 antibody did not inhibit blood vessels formation. Our results were in agreement with previous studies that chondrocyte-induced inhibition of tube formation was partly abrogated by anti-TGFβ antibody [Tada et al., 1994]. Pepper et al. [1991] has also reported that addition of anti-TGFβ antibodies to the cocultures reduces the inhibition of chondrocytes on sprout formation [Pepper et al., 1991]. These results of CAM assay not only verify the in vitro results, but also prove that chondrocyte-derived TGFβ1 is a potent anti-angiogenic factor in vivo.

Since growth plate chondrocytes secrete TGFβ1 in inactive form, it is believed that chondrocyte-derived latent TGFβ1 should be activated by endothelial proteases. Proteolytic activity is critical for migration and invasion of endothelial cells into the neighbouring tissue, e.g. plasminogen activator (PA) system [Bickneel et al., 1997]. It has been observed that latent TGFβ is activated in cocultures of endothelial cells and pericytes or smooth muscle cells and that this depends on close heterocellular proximity or cell-to-cell contact [Nunes et al., 1996; Gleizes et al., 1997]. In our study, endothelial cells and growth plate chondrocytes may cooperate by providing sufficient levels of different components necessary to this mechanism. This detailed co-culture activation mechanism, however, is still unknown. Besides, acidic microenvironment and proteolysis by thrombospondin secreted by endothelial cells are possible ways to activate latent TGFβ1 [Bickneel et al., 1997].

Since TGFβ1 was proven to be the anti-angiogenic factor in growth plate, we next investigated the distribution of TGFβ1 in growth plate. Results of immunohistochemistry demonstrated that TGFβ1 was dominated in the proliferative zone. Although positive signal was also detected in the hypertrophic and resting zone, the intensity was relatively lower. Image analysis also showed a stronger signal in the proliferative zone. Similar results have been reported that strong intracellular staining was observed in proliferative chondrocytes [Jingushi et al., 1995]. This immunohistochemistry result was consistent with our previous findings that the anti-angiogenic effect dominated in the proliferative zone-rich growth plate chondrocytes. This explains why tumors seldom invade across the whole growth plate since the proliferative zone expresses anti-angiogenic factors to cut off the nutrient supply for tumor growth. The different zonal distribution of TGFβ1 reflects that the retardation ability of growth plate on tumor is a sum of all the inhibitory activities in each zone. The relative retardation effect also depends on the balance of angiogenic stimulation of tumors and angiogenic inhibition of growth plate. This also explains the higher incidence of tumor invasion across the growth plate with age. As the growth plate closes, the proliferative and the resting zones progressively disappear. Dodds et al. [1994] reported that TGFβ expression was lost with the progression to calcifying cartilage. In addition, VEGF was reported to be present in hypertrophic zone [Gerber et al., 1999; Horner et al., 1999; Carlevaro et al., 2000]. Therefore, tumors can invade through the growth plate easily without the retardation contributed by the angiogenic inhibitors. Therefore, zonal effect of the growth plate has to been taken into account when interpreting its anti-tumor effect.

This study cannot exclude the possibility that other angiogenic inhibitor(s) may exist to retard the tumor spread but the results suggest that the anti-angiogenic effect is at least partly mediated by TGFβ1. In summary, our findings suggest that growth plate chondrocyte-derived TGFβ1 plays an important role in inhibiting angiogenesis. This may contribute to the physeal retardation of tumor spread.

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