Autocrine/Paracrine Mechanism of Insulin like Growth Factor-1 secretion, and the Effect of IGF-1 on Proteoglycan Synthesis in Bovine Intervertebral Discs

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Key Words

insulin like growth factor-1, autocrine/paracrine mechanism,
intervertebral disc

Summary: The present study was undertaken to investigate the effect of IGF-1 on proteoglycan (PG) synthesis and the autocrine/paracrine mechanisms involving insulin-like growth factor-1 (IGF-1) in the bovine coccygeal intervertebral disc. IGF-1 stimulated PG synthesis in cultured cells of nucleus pulposus of bovine intervertebral discs in a dose-dependent manner, and the effect was inhibited by an anti-IGF-1 monoclonal antibody. In situ hybridization histochemistry revealed the expression of IGF-1 mRNA in the cultured cells, and IGF-1 production in these cells was demonstrated by radioimmunoassay. IGF-1 receptor in the cultured cells was also immunohistochemically demonstrated. The Scatchard analysis using an ^{125}I-IGF-1 binding assay showed that the cells cultured in monolayer had a single type of IGF-1 receptor, whose affinity and number were estimated to be 7.38 x 10^8/M and 9.27 x 10^4/cell, respectively. These results suggest that IGF-1 stimulates PG synthesis in cells of the nucleus pulposus, and that these cells in culture have an IGF-1 autocrine/paracrine mechanism. The expressions of IGF-1 mRNA and IGF-1 receptor in disc tissue were greater in cells of the nucleus pulposus of fetal bovine intervertebral discs than in those of the adult discs. These findings suggest that the autocrine/paracrine IGF-1 action is more active in cells of young nucleus pulposus than in cells of matured subjects.

INTRODUCTION

The function of the intervertebral disc depends on the composition and integration of its extracellular matrices mainly composed of collagens and proteoglycans (PG), that are synthesized by the cells of the disc. But, little is known about factors to regulate matrix synthesis.

In 1991, Thompson et al. first attempted to stimulate PG synthesis in the intervertebral disc using growth factors and reported that insulin-like growth factor-1 (IGF-1), platelet-derived growth factor, epidermal growth factor and transforming

growth factor-β stimulated PG synthesis in the nucleus pulposus (42). IGF-1, which is produced mainly in the liver in response to the action of growth hormone and is transferred into the serum (37), is known to stimulate the growth of bone and cartilage (36).

Kato et al. extracted a somatomedin-like peptide (cartilage-derived factor) from bovine articular cartilage (20), and D'Ercole et al. detected IGF-1 production in fetal mouse limb bud mesenchymal mass (6,7). The autocrine/paracrine mechanism of IGF-1 action in the bone and cartilage has also been reported in recent years (8,23,25,27,34,38).

However, there has been no report on the local production and receptor of IGF-1 in the intervertebral disc. The possible role of autocrine/paracrine mechanism of IGF-1 for intervertebral disc degeneration also remains unclear. The present study was undertaken to investigate the effect of IGF-1 on PG synthesis and the presence of autocrine/paracrine mechanism of IGF-1 in the bovine intervertebral disc.

METHODS

The nucleus pulposus from 9 intervertebral discs obtained by an aseptic procedure from 3 coccygeal vertebrae of 4-year-old adult bovine were used for cell culture. For in situ hybridization histochemistry and immunohistochemical study, intervertebral discs were taken from 4 other four-year-old adult bovine spine and from 4 seven-month fetal bovine spine. Each tissue sample was immersed in 4% paraformaldehyde in 0.1M PO_4 buffer and subjected to two sessions of 10-second microwave irradiation (26), embedded in paraffin and cut into 3 μm slices. A total of 17 intervertebral discs were used for the experiments.

Cell culture of adult bovine nucleus pulposus

Ham's F12 medium and Dulbecco's modified Eagle's medium (DMEM; Flow Laboratories, Inc., McLean, VA, U.S.A.) were used for cell culture. In both culture systems, the medium was adjusted to pH 7.2 with 20 mmol/l N-2-hydroxyethylpiperazine-N'-2-2-ethane sulfonic acid (HEPES; Dojin Pharmacochemical Institute, Kumamoto, Japan) and sodium bicarbonate. Each medium was supplemented with streptomycin (100 μg/ml), penicillin (100 U/ml) (Meiji Seika, Tokyo, Japan) and 0.5% bovine serum albumin (BSA; Sigma Chemical, St. Louis, MO, U.S.A.). About 0.5g of the central part of the nucleus pulposus was carefully dissected from each adult bovine coccygeal intervertebral disc under magnification, and minced. After treatment with 0.1% collagenase (Wako Pure Chemicals, Tokyo, Japan) at 37°C for 3 hours, the cells were isolated by centrifugation at 1,000 rpm for 10 minutes. The primary culture was continued for 4 weeks using Ham's F12 medium (9,15,39) containing 10% fetal bovine serum (FBS; Bocknek Ltd., Toronto, Canada). The cells were then subcultured in 4-well chamber slides (LabTec, Nunk, Naperville, IL, U.S.A.) and 24-well plates (Corning Inc., Corning, NY, U.S.A.). Each culture was kept in a 5% CO_2 atmosphere at 37°C, and the medium was renewed every 7 days.

Effect of IGF-1 on PG synthesis in cultured nucleus cells

The effect of IGF-1 on PG synthesis was determined by analysis of the incorporation of ^{35}S-sulfate of cultured nucleus cells which had become virtually confluent in 24-well plates (2.2-2.4 x 10^5 cells/well). Before the different concentrations of IGF-1 were added, serum components were excluded from the 24-well plates, and the cell layer of each well was washed with phosphate-buffered saline (PBS). The culture medium was changed to DMEM containing graded concentrations of human recombinant IGF-1 (0, 0.1, 1, 10, 100 or 1,000 ng/ml) (Becton-Dickinson Labware, Bedford, MA, U.S.A.) (n=4 each). Cells were labeled for 24 hours at 37°C with 74 kBq/ml ^{35}S-sulfate (1.59 TBq/mg S, ICN Biomedicals, Inc., Costa Mesa, CA, U.S.A.). The incorporation of ^{35}S-sulfate was also determined in cells cultured with

IGF-1 at a concentration of 100 ng/ml in the presence of 10 mg/ml mouse anti-human IGF-1 monoclonal antibody (Cymbus Bioscience Ltd., Southampton, U.K.) (n=4) (24). After labeling, the cells were solubilized with 1 ml of 1 N NaOH. The cell fraction thus obtained was mixed with the medium and neutralized with 1 N HCl. One ml of 50 mM Tris buffer (pH 7.8) with 5 mM $CaCl_2$ and 4,000 U of pronase E (Kaken Pharmaceutical, Tokyo, Japan) were added to each sample and allowed to react at 55°C for 12 hours. Using a PD-10 column (Pharmacia, Uppsala, Sweden), unbound ^{35}S-sulfate was removed from the solution. Thereafter, 2 mg of chondroitin sulfate (Wako Pure Chemicals) and 5% cetylpyridinium chloride (Tokyo Kasei Kogyo, Tokyo, Japan) were added to the solution and allowed to react at 37°C for 2 hours. After centrifugation at 3,000 rpm for 30 minutes, the precipitates were dissolved in 1.2 M NaCl, and the radioactivity (cpm) was measured with a liquid scintillation counter (LSC-903, Aloka, Tokyo, Japan) (15,20,40). One-way analysis of variance (ANOVA) was used for statistical analysis, with $p<0.05$ considered as significant.

Detection of IGF-1 mRNA in bovine intervertebral disc

IGF-1 synthesis was confirmed by detecting IGF-1 mRNA on in situ hybridization histochemistry. After 3 days of culture on 4-well chamber slides, cells were air-dried and fixed in acetone at 4°C for 10 minutes. Tissue sections of adult and fetal bovine intervertebral discs (n=4 each) were dewaxed and rehydrated.

The hybridization procedure was essentially similar to the method reported by Bloch et al. (1). Cultured nucleus cells and tissue sections were treated with proteinase K (Boehringer, Mannheim, Germany) at 37°C for 15 minutes in 50 mM EDTA, 0.1 M Tris-HCl (pH 7.4), 2 mM $CaCl_2$, 10 μg/ml proteinase K). Each section was allowed to react with the ^{35}S-dATP-labeled synthetic oligonucleotide probe, complementary to the 441-476 base sequence of human IGF-1 mRNA (NEN Research Products, Boston, MA, U.S.A.) (6.0-9.0 x 10^8 dpm/ml) in a buffer solution [4-fold sodium chloride-sodium citrate buffer (SSC), 50% deionized formamide, 0.12 M phosphate buffer (pH 7.4), Denhardt's solution (Sigma Chemical), 2.5% yeast tRNA (Boehringer), 10%

dextran sulfate, 50 mM dethiothreitol] at 41°C for 24 hours. Then, the sections were rinsed in 1-fold SSC for 1 hour at 60°C to remove unbound ^{35}S-dATP-labeled oligonucleotide molecules. After dehydrating with alcohol, each section was exposed to Ilford K-5 emulsion (diluted at 1:1 with water) for 2 weeks and subjected to development with D-19 (Eastman Kodak, Rochester, NY, U.S.A.) followed by hematoxylin-eosin staining. Negative controls pretreated with 1 mg/ml ribonuclease A (Cosmo, Tokyo, Japan) were used for judging the binding specificity.

Measurement of IGF-1 secretion

Subcultured cells which had virtually reached a confluent state in 24-well plates were used. The cell layer was washed twice with PBS and incubated in 1 ml of DMEM containing 3% FBS for 24, 48, 72 and 96 hours. Medium samples for IGF-1 determination were obtained from 3 wells each and stored at -20°C until measurement. Each cell layer was treated with trypsin, and the cell number was counted. The concentration of secreted IGF-1 was measured by radioimmunoassay using a Somatomedin-C Eiken II Kit (Eiken Kagaku, Tokyo, Japan) and the concentration of secreted IGF-1 per 1 x 10^5 cultured cells was calculated. The lower detectable limit was 4.1 ng/ml, and cross-reactivity with IGF-2 is less than 0.5%.

Demonstration of IGF-1 receptor

Cultured cells of the nucleus pulposus and tissue sections of intervertebral discs obtained from fetal and adult bovine (n=4 each) were stained by the avidin-biotin-peroxidase complex method (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, U.S.A.) to detect IGF-1 receptor. Cultured nucleus cells and tissue sections were reacted with normal goat serum for 24 hours at room temperature to suppress non-specific staining. A 1:30,000 dilution of rabbit anti-human IGF-1 receptor polyclonal antibody (Promega Co., Madison, WI, U.S.A.) as the primary antibody was added to the sections and then stored at room temperature for 24 hours. After washing, the sections were treated with biotinylated goat anti-rabbit IgG for 15

minutes and with avidin-biotin-peroxidase complex for 30 minutes. For color development, each section was immersed in PBS containing 0.02% diaminobenzidine and 0.005% hydrogen peroxide (Wako Pure Chemicals) for 5 minutes, followed by hematoxylin staining. Negative controls were treated with normal goat serum in place of the primary antibody. The distribution of IGF-1 receptors was determined in relation to the site within the tissue preparation. In the nucleus pulposus and the annulus fibrosus of fetal bovine intervertebral disc, and in the nucleus pulposus and the three equally divided parts of the annulus fibrosus (inner, intermediate and outer layers) of adult bovine intervertebral disc, positive cells were counted at 400x magnification in 5 visual fields each.

Analysis of IGF-1 receptor in cultured nucleus cells

Cultured cells which virtually reached a confluent state in 24-well plates were used. ^{125}I-IGF-1 (0.2 ng/ml, 74 TBq/mmol, Amersham International plc., Buckinghamshire, U.K.) and IGF-1 (0.1, 1, 5, 10, 25, 50, 100, 500 and 1,000 ng/ml) were added to each well and allowed to react at 4°C for 16 hours. After removal of the supernatant, the cell layer was washed 6 times with PBS and dissolved in 1 ml of 1 N NaOH. The radioactivity (cpm) of the solution was measured using a gamma counter (ARC-2000, Aloka). The binding rate was calculated for each well using the ratio of the measured radioactivity to the total amount of ^{125}I-IGF-1 added (30,000 cpm). The binding rate in the presence of IGF-1 at a concentration of 1,000 ng/ml was taken as the non-specific binding rate, and the difference between the overall binding rate and the non-specific binding rate was regarded as the specific binding rate. The number of receptors per cell and their affinity were calculated by the Scatchard analysis.

RESULTS

Effect of IGF-1 on matrix synthesis

PG synthesis in cultured nucleus cells of adult bovine intervertebral disc was stimulated in proportion to the concentration of IGF-1. A significant stimulating effect was found at the concentrations of IGF-1 of 1 ng/ml, reaching the maximum (4.8-fold increase over the basal) at the concentration of 100 ng/ml IGF-1. When anti-IGF-1 monoclonal antibody was added in the presence of IGF-1 (100 ng/ml), PG synthesis was not stimulated (Fig. 1).

Expression of IGF-1 mRNA

IGF-1 mRNA, an index of IGF-1 synthesis, was expressed in the cytoplasm of cultured nucleus cells (Fig. 2). The pattern of expression of IGF-1 mRNA in disc tissue varied according to the degree of maturity of the animal and the site in the disc; the cells of fetal bovine intervertebral disc distinctly expressed IGF-1 mRNA (Figs. 3A and B). However, expression of mRNA was unclear in cells of both the nucleus pulposus and annulus fibrosus of adult bovine intervertebral disc (Figs. 3C and D). There were no specific signals in negative controls.

Secretion of IGF-1 in adult bovine nucleus cells

IGF-1 was first detected in the supernatant of the cultured nucleus cells of adult bovine disc at 48 hours after incubation. Thereafter, the detected IGF-1 concentration increased with time, reaching a level of 92.1 ng/ml/10^5 cells at 96 hours incubation (Fig. 4).

IGF-1 receptor in the intervertebral disc

Cultured nucleus cells of adult bovine intervertebral disc were stained brown, demonstrating the presence of IGF-1 receptor (Fig. 5). The pattern of staining of IGF-1 receptor in the disc tissue varied according to the maturity and the area of the disc.

The staining of the nucleus cells of fetal bovine intervertebral disc tissue were most strongly positive and the mean percentage of positive cells was 76.7% (Fig. 6A and Table 1). However, IGF-1 receptor staining was weaker in the annulus fibrosus, and the positive cells was 39.1% of all the cells (Fig. 6B and Table 1). In the adult bovine intervertebral disc, the positive cells for IGF-1 receptor were sporadic (Fig. 6C), and staining of annulus fibrosus cells was weak (Fig. 6D). In the adult intervertebral disc, the mean percentage of the receptor positive cells in relation to the area of the disc was 40.2% for the nucleus pulposus, 33.7% for the inner layer, and approximately 10% for the intermediate and outer layers of the annulus fibrosus (Table 1). In the negative control discs, no immunoreactivity was present.

Binding of ^{125}I-IGF-1 to nucleus cells

Unlabeled IGF-1 inhibited the binding of ^{125}I-IGF-1 to cultured nucleus cells. The specific binding rate decreased as the concentration of unlabeled IGF-1 increased, and was inhibited to 50% at a total IGF-1 concentration of approximately 5 ng/ml (Fig. 7A). The Scatchard plots showed that adult bovine nucleus cells possessed only a single type of IGF-1 receptor with an affinity of 7.38×10^8/M and the number of 9.27×10^4/cell (Fig. 7B).

DISCUSSION

IGF-1 is a peptide which is known to activate matrix metabolism, particularly PG synthesis, and to proliferate various cells including chondrocytes (10,13,17,21,24,28,41,42,44,47). Thompson et al. reported that PG synthesis in cultured nucleus pulposus tissue of adult canine intervertebral disc is increased in the presence of IGF-1 (42). The present result shows that PG synthesis in cultured cells of the nucleus pulposus of adult bovine intervertebral disc is increased in proportion to

the concentration of IGF-1 and this suggests that IGF-1 is a possible regulating factor for PG synthesis in nucleus cells.

IGF-1 in the serum exists as an inactive complex with IGF-binding protein (IGFBP) (37). Although it has been implied that positively charged IGF-1 can penetrate into the cartilage matrix, which is abundant in negatively charged PG (22), recent evidence suggests that the amount of IGF-1-IGFBP complexes which can penetrate through the cartilage matrix and reach the cells is very small (35), and it is unclear whether or not IGF-1 in the serum can reach the avascular nucleus pulposus of the intervertebral disc via the vertebral end-plate. It is also known that IGF-1, which is essential for the growth of long bones, is not derived from serum but is produced in the growth plate (16,23,27,32).

In 1987, Han et al. succeeded for the first time in detecting IGF-1 mRNA in human fetal tissue through in situ hybridization histochemistry, demonstrating its presence in many tissues such as heart, thymus, skeletal muscle, skin, pancreas, spleen, kidney, rib, stomach and small intestine (11). IGF-1 mRNA expressed in chondrocytes was demonstrated in the epiphyseal plate of rat tibia by Nilsson et al. in 1990 (27). It has been confirmed that IGF-1 secretion by cultured cells such as human fibroblasts, porcine aortic smooth muscle cells and rabbit articular chondrocytes (2,3,4,8). The present study demonstrated that cultured nucleus cells of adult bovine intervertebral disc express IGF-1 mRNA and secrete IGF-1. This finding suggests that adult nucleus cells in vivo have the potential to produce IGF-1.

The presence of IGF-1 receptor has been found in cultures of chondrocytes of fetal chickens (14), rat adipocytes, liver-cells (14) and chondrocytes (33), rabbit articular chondrocytes (29), bovine articular chondrocytes (45,48), and human circulating mononuclear cells (31), and fibroblasts (5,30). The present study showed that cultured bovine nucleus cells had only one type of IGF-1 receptor, and that its affinity was close to the level calculated for cultured chondrocytes (33,45,48). The number of IGF-1 receptors per nucleus cell, however, was judged to be higher than that of the cultured articular chondrocytes (45,48). It remains to be clarified whether this

difference is attributable to the type of cell or culture condition. Thus, nucleus cells of the intervertebral disc proved to have the autocrine/paracrine mechanism of IGF-1 in culture, suggesting the involvement of IGF-1 in the regulation of PG synthesis. The autocrine/paracrine mechanism of IGF-1 is considered to be significant especially for the cells in the intervertebral discs which is the largest avascular tissue. It should be determined whether IGFBPs that modulate the biological effects of IGF-1 are also produced by the cells in intervertebral disc.

From the evidence of IGF-1 mRNA and IGF-1 receptor obtained in histological sections of the intervertebral disc, it is obvious that the autocrine/paracrine mechanism of IGF-1 is also present in the intervertebral disc tissue; IGF-1 mRNA is obviously present in cells of the nucleus pulposus and annulus fibrosus of fetal bovine intervertebral discs. However, IGF-1 mRNA was scarcely detected in adult bovine intervertebral discs. IGF-1 receptors were present in both fetal and adult bovine intervertebral discs, and the pattern of IGF-1 receptor distribution virtually corresponded to the degree of mRNA expression, and was most active in young nucleus pulposus cells. Namely, it appears that the autocrine/paracrine mechanism of IGF-1 is stronger in young cells, and declines with age, and that it is also more active in the nucleus pulposus compared to the annulus fibrosus.

The differences between fetal and adult discs are supposed to come from the cell heterogeity. The fact is of importance that notochordal cells appear in a process of differentiation and growth of nucleus pulposus. It has been reported that the autocrine/paracrine mechanism of IGF-1 is involved in the differentiation and growth of tissues. Using a culture system of mouse myoblasts, Tollefsen et al. found that the expression of mRNA of creatine kinase, characteristic of myocytes, occurs after the IGF-1 mRNA reached the maximum amount. They concluded that autocrine secretion of IGF-1 is necessary for differentiation of myocytes (43). Trippel et al. also reported that the IGF-1 binding rate is higher in the proliferative layer of the growth plate of long bones than in the hypertrophic layer (46). These findings are consistent with the manifestation of IGF-1 autocrine/paracrine mechanism in the process of differentiation

and growth of the nucleus pulposus of the intervertebral disc. In the present study, although the expression of IGF-1 receptors was demonstrated in about 40% of cells of the nucleus pulposus of adult bovine intervertebral disc, the expression of IGF-1 mRNA in these cells remains unclear. The IGF-1 autocrine/paracrine mechanism may be practically suspended in the intervertebral disc in healthy adult bovine. However, considering the fact that cells of adult nucleus pulposus in culture system were abundant in IGF-1 mRNA and IGF-1 receptor, it is possible that the cells which had potential to express the autocrine/paracrine action of IGF-1 might predominate other static cells in culture condition. The evidence that local production of IGF-1 occurs in the tissue repair process (12,18,19,49), and that IGF-1 mRNA signal intensity in osteoarthritic cartilage is significantry higher than in anatomically normal cartilage (25), the IGF-1 autocrine/paracrine mechanism in the adult nucleus pulposus might represent in an attempt to repair the matrices. Further investigation is required to determine the relationship between the modulation of the IGF-1 autocrine/paracrine mechanism and the effect for degeneration or repair of the intervertebral disc.

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FIGURE LEGENDS

Fig. 1. Effect of IGF-1 on PG synthesis in cultured cells of the nucleus pulposus of adult bovine intervertebral disc

The PG synthesis was stimulated by IGF-1, and this enhancement was inhibited by anti-IGF-1 monoclonal antibody. The bars denote standard deviations.

Fig. 2. IGF-1 mRNA in cultured nucleus cells of adult bovine intervertebral disc

In situ hybridization histochemistry in cultured cells indicating the expression of IGF-1 mRNA in the cytoplasm (bar=10μm, x1,000).

Fig. 3. IGF-1 mRNA in fetal (A and B) and adult bovine intervertebral disc (C and D) tissues

A: Significant signals of IGF-1 mRNA were seen in the fetal nucleus pulposus (bar=10μm, x 1,000). B: Expression of IGF-1 mRNA in annulus fibrosus is weaker than that in the fetal nucleus pulposus (bar=10μm, x 1,000). C and D: IGF-1 mRNA signals are very slight or negative in the nucleus pulposus (C) and in the intermediate layer of the annulus fibrosus (D) of adult bovine intervertebral disc (bar=10μm, x 1,000).

Fig. 4. Chronological changes in IGF-1 secretion from cultured nucleus cells of adult bovine disc

IGF-1 secretion into the culture supernatant increased with time of incubation after the exchange of medium. The bars denote standard deviations.

Fig. 5. IGF-1 receptors in cultured adult bovine nucleus cells shown by immunohistochemical study

IGF-1 receptors were stained brown in a mesh-like pattern on the protoplasm of the cultured cells (bar=10μm, x 1,000).

Fig. 6. IGF-1 receptors in fetal and adult bovine intervertebral disc tissues

A: Fetal bovine nucleus pulposus. Nucleus cells present in an insular pattern are stained strongly (bar=10μm, x 1,000). B: Fetal bovine annulus fibrosus. The sporadically distributed cells are stained more weakly than the fetal nucleus cells (bar=10μm, x 1,000). C: Adult bovine nucleus pulposus. Receptors are scattered in cells forming a cluster (bar=10μm, x 1,000). D: Intermediate layer of the adult bovine annulus fibrosus. Few receptors are present (bar=10μm, x 1,000).

Fig. 7. The specific binding of ^{125}I-IGF-1 to cultured adult bovine nucleus cells and the Scatchard analysis of the binding

A: ^{125}I-IGF-1 specific binding rate decreased as the concentration of unlabeled IGF-1 increased. B: Scatchard plot showing that cultured nucleus cells of adult bovine intervertebral disc have only a single type of IGF-1 receptor. The affinity (Ka) was 7.38×10^8/M, and the number of receptor sites was 9.27×10^4/cell.

Table 1. ***Immunohistochemical evidence of IGF-1 receptor on bovine intervertebral discs***

	location	% positive cells (mean±SD)	immunoreactivity
fetal bovine	**nucleus pulposus** (n=20)	76.7±14.9	+++
	annulus fibrosus (n=20)	39.1±29.2	++
adult bovine	**nucleus pulposus** (n=20)	40.2±14.9	++
	inner annulus fibrosus (n=20)	33.7±19.0	++
	intermediate annulus fibrosus (n=20)	9.4±9.6	+
	outer annulus fibrosus (n=20)	10.2±10.1	+

+++ strong, ++ moderate, + faint

Fig.1

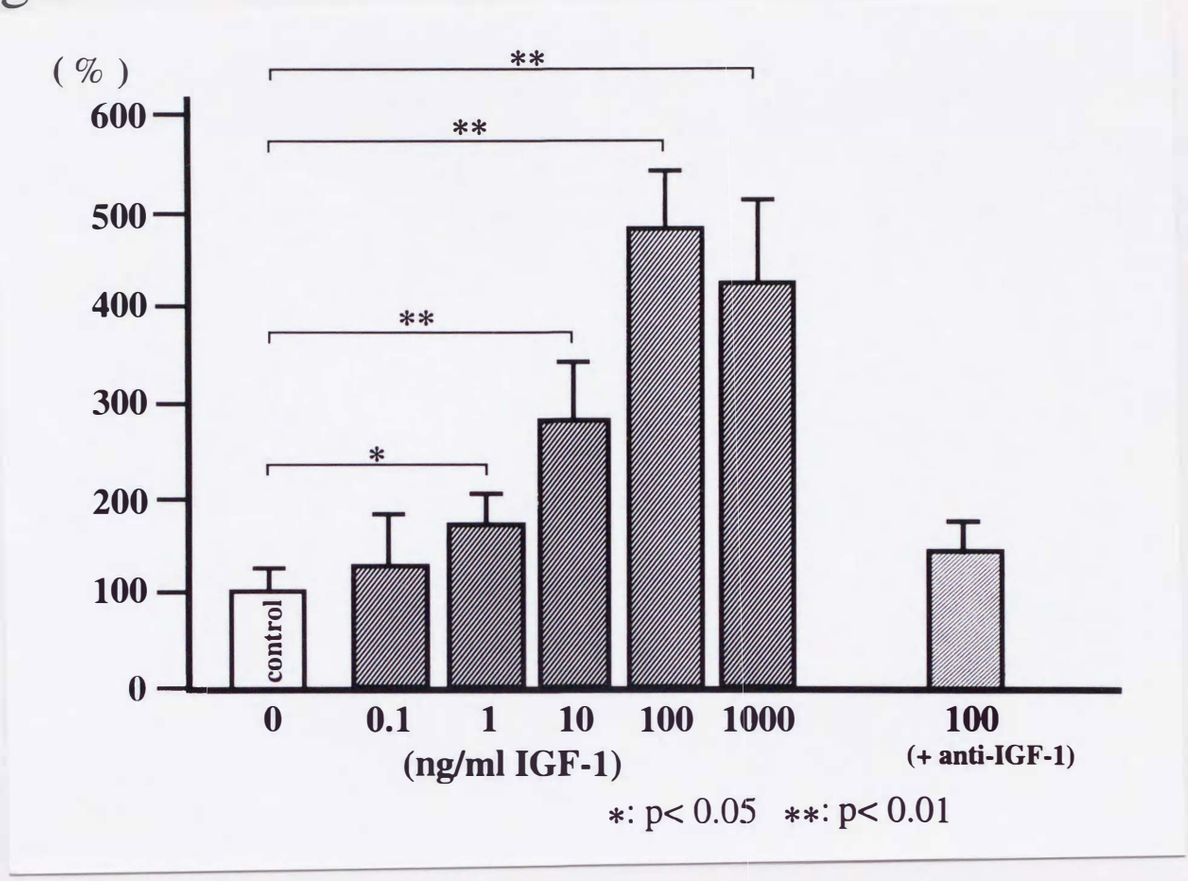
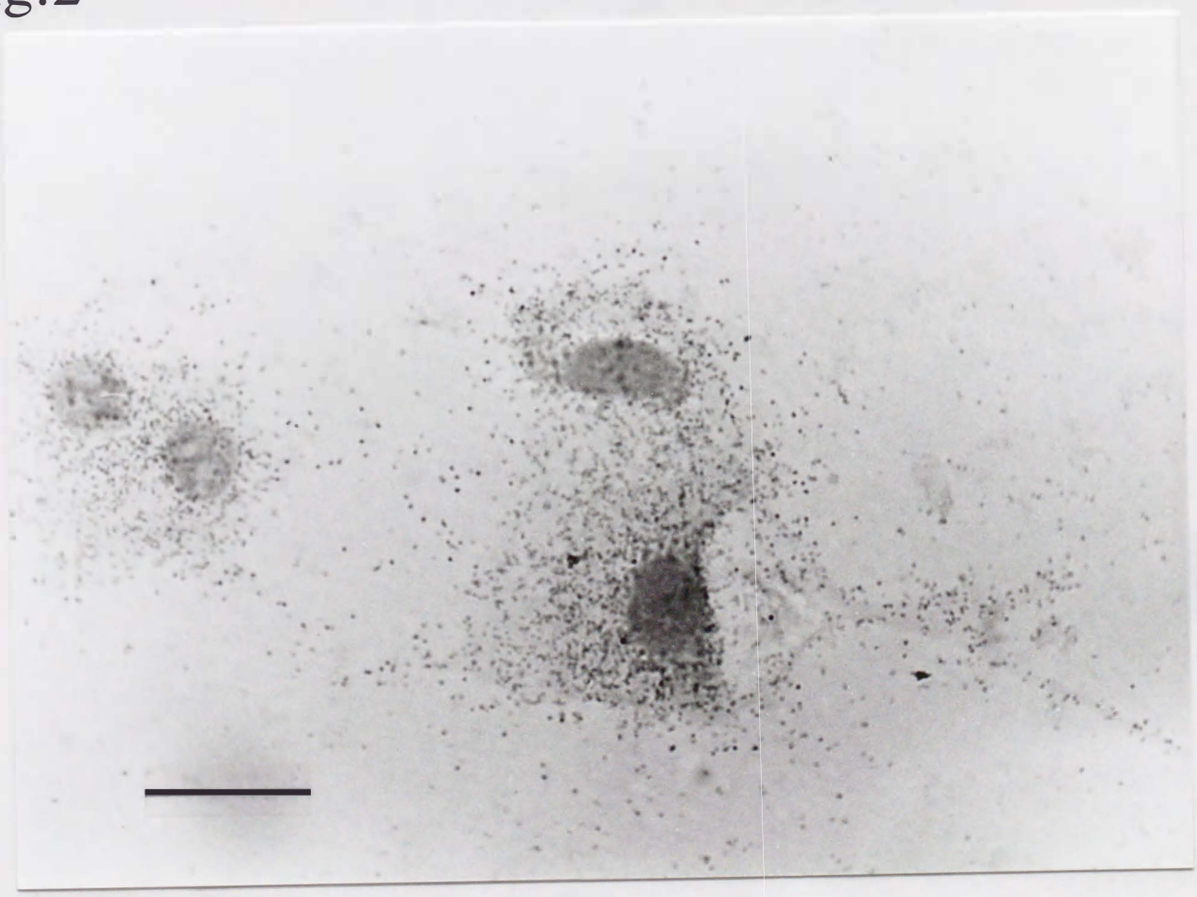

(%)
600
500
400
300
200
100
0
control
**
**
**
*
0
0.1
1
10
100
1000
100
(ng/ml IGF-1)
(+ anti-IGF-1)
*: p< 0.05 **: p< 0.01

Fig.2



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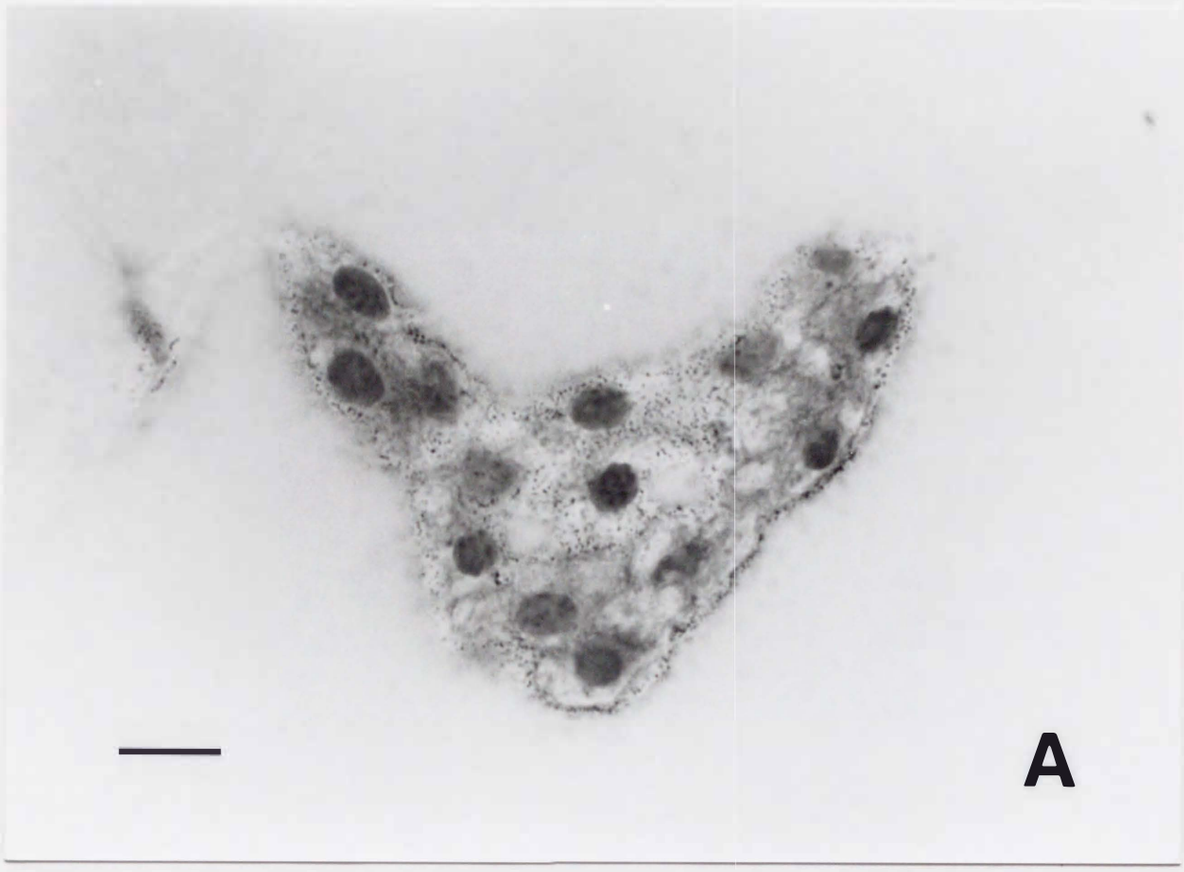


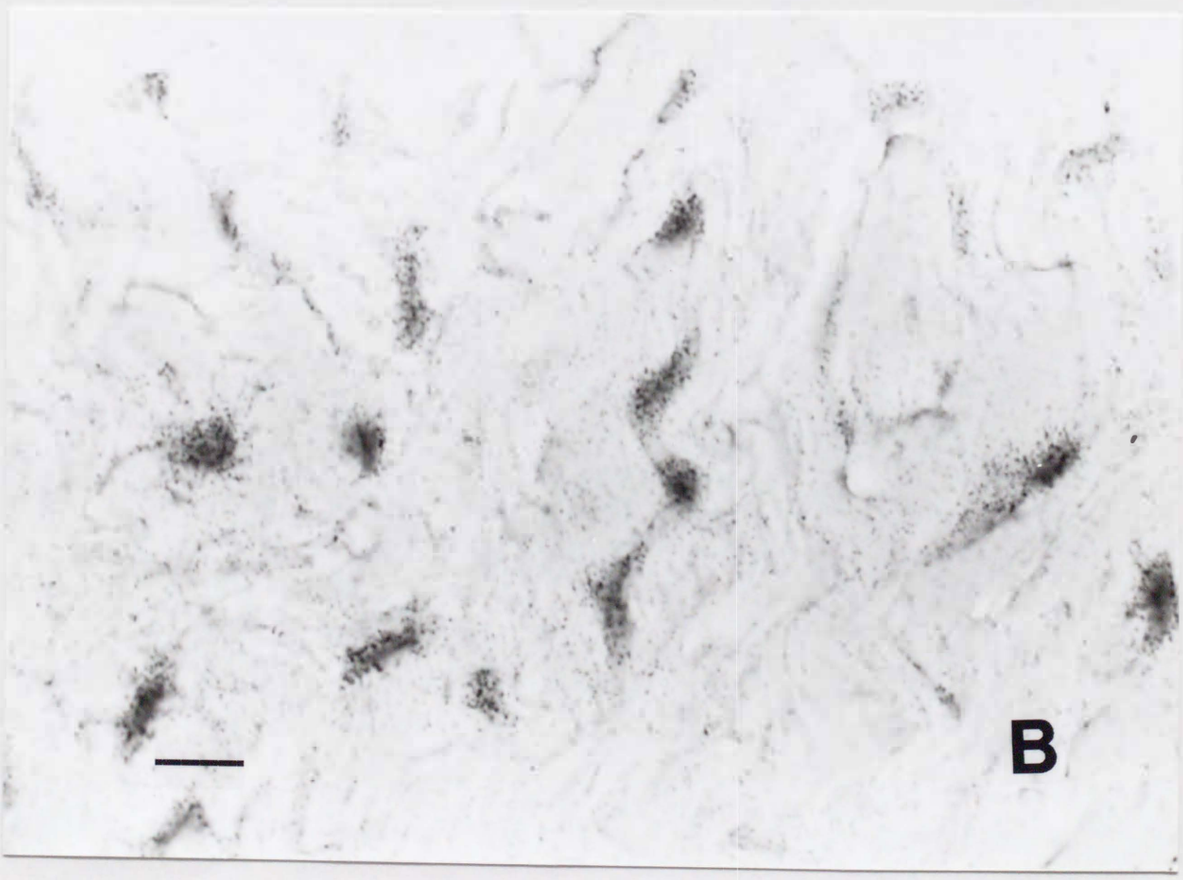
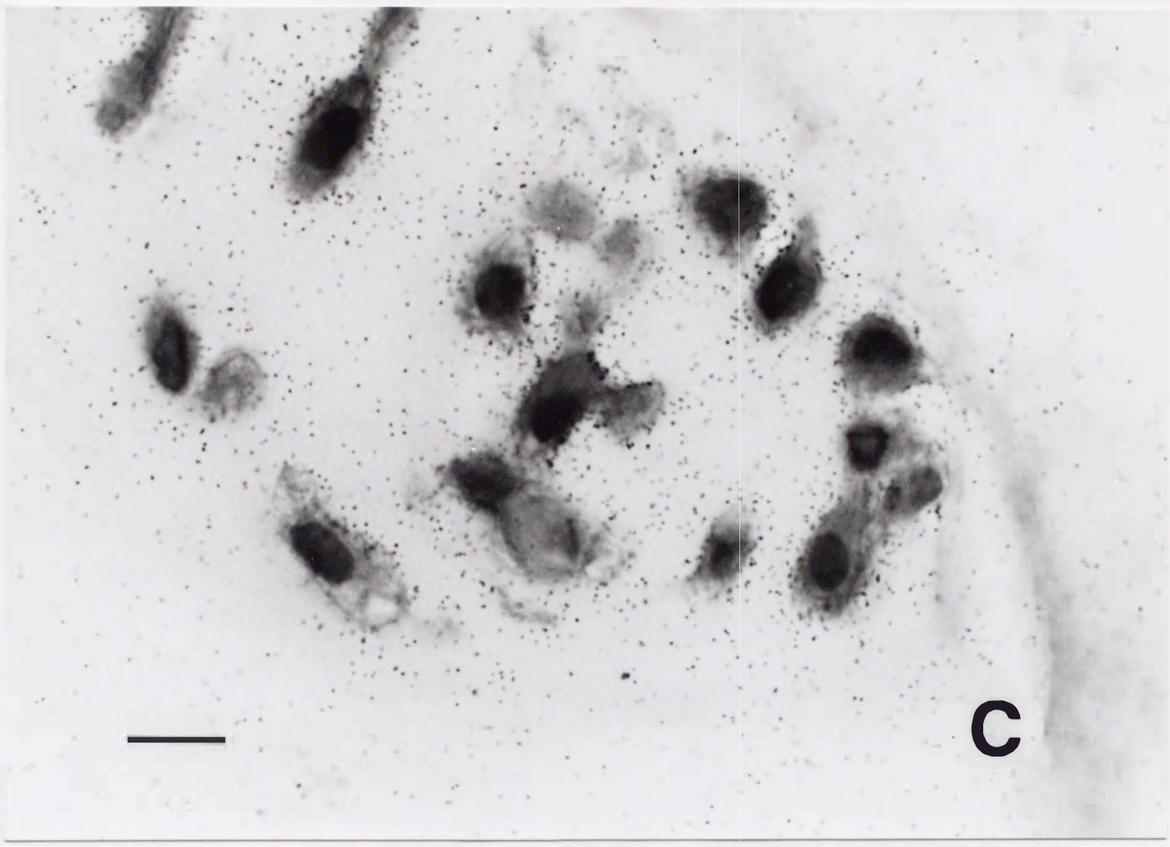


Fig.3



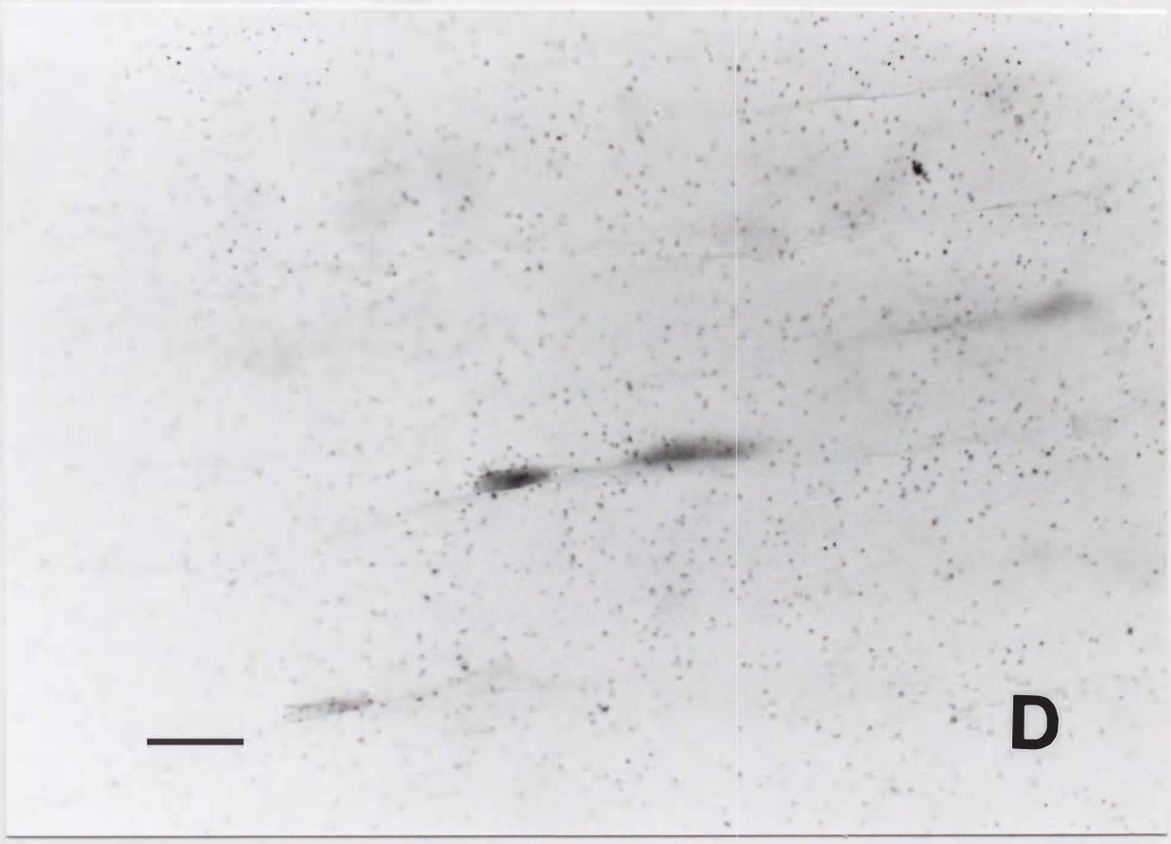
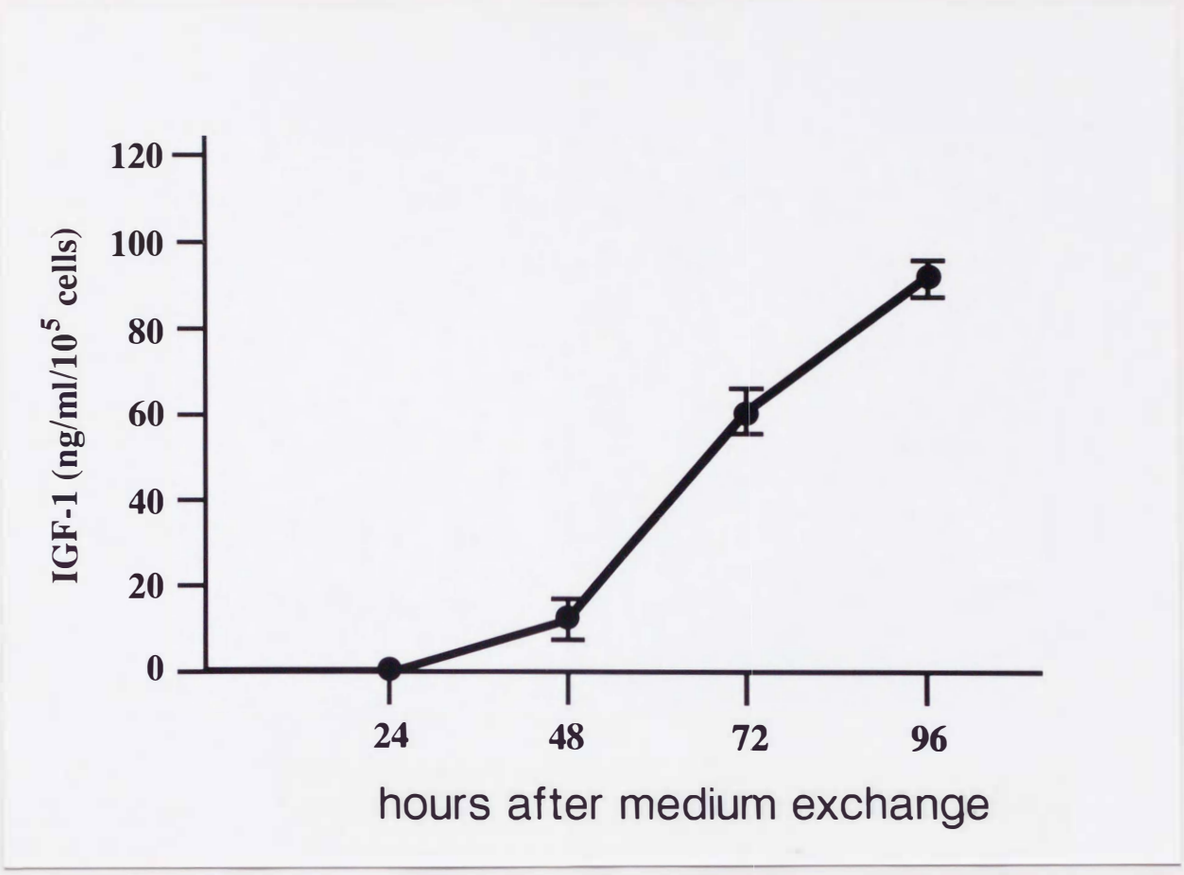


Fig.4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100
80
60
40
20
0
IGF-1 (ng/ml/10^5 cells)
24
48
72
96
hours after medium exchange

Fig.5

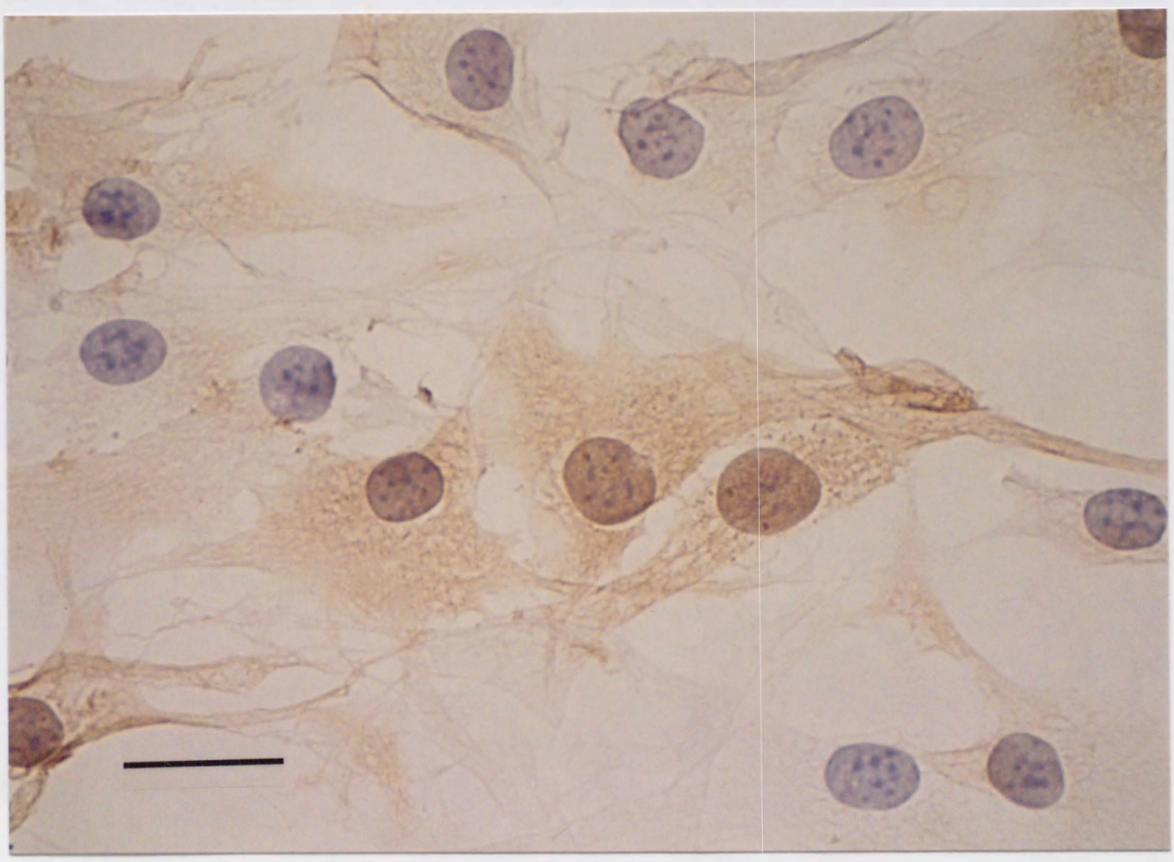
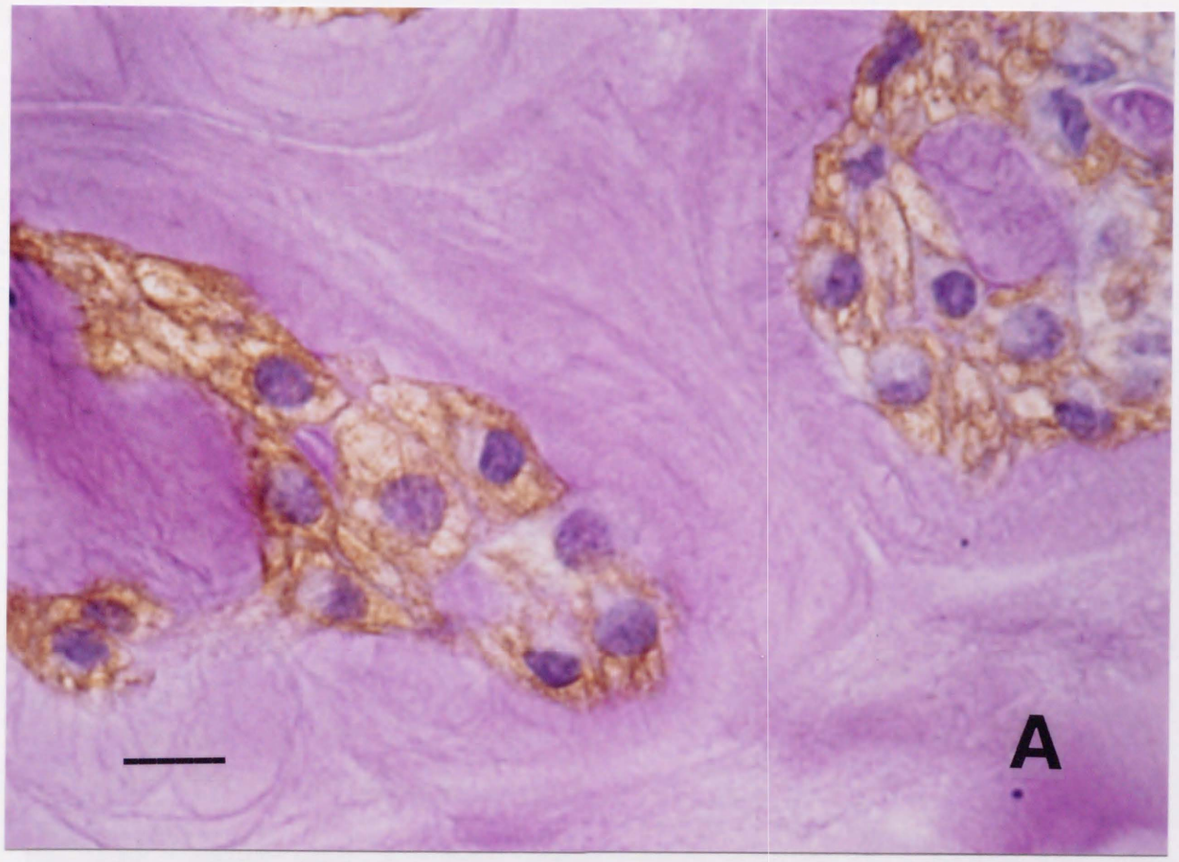


Fig.6



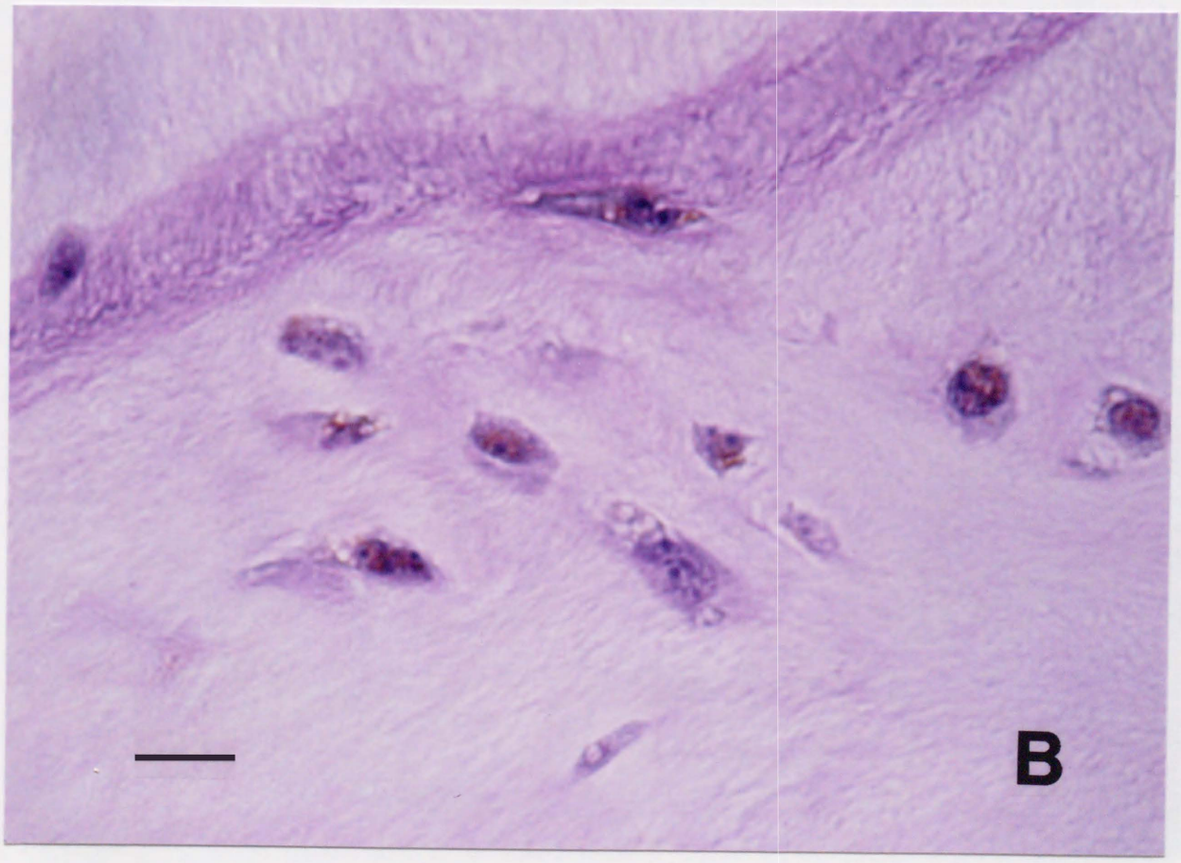
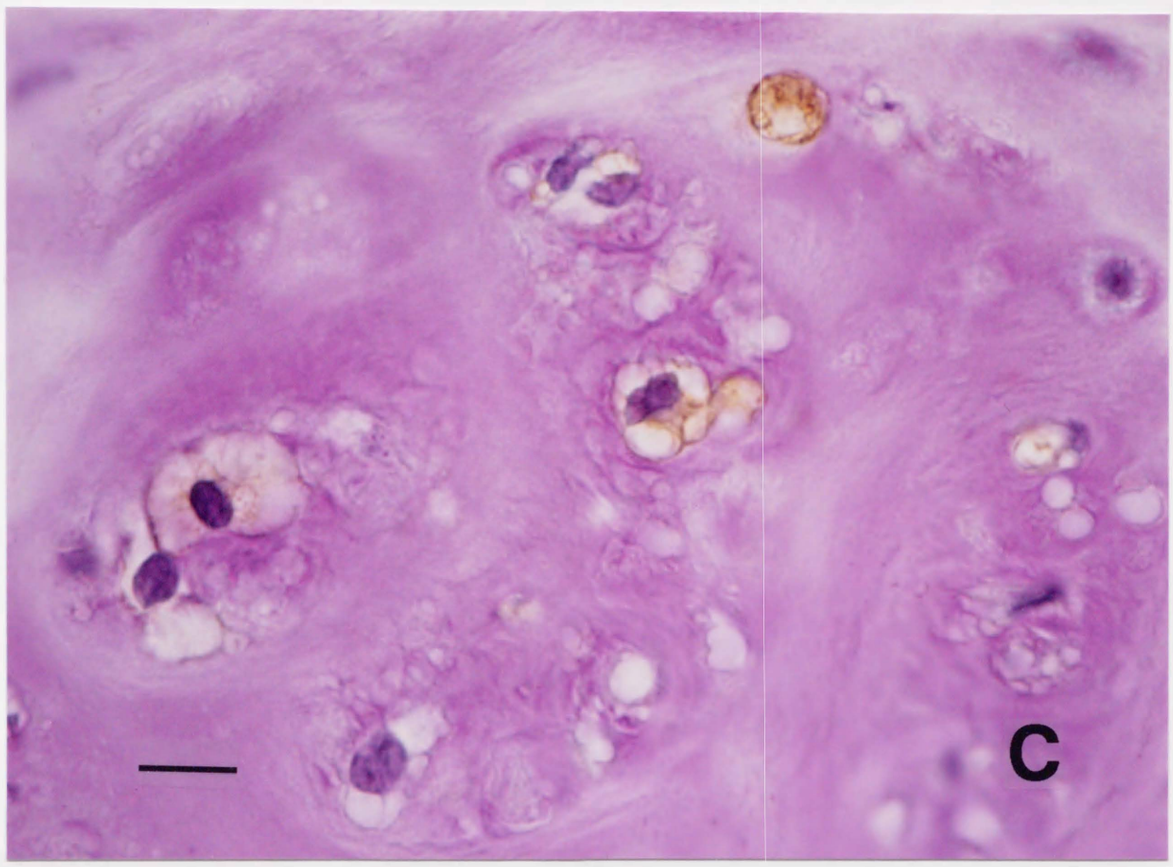


Fig.6



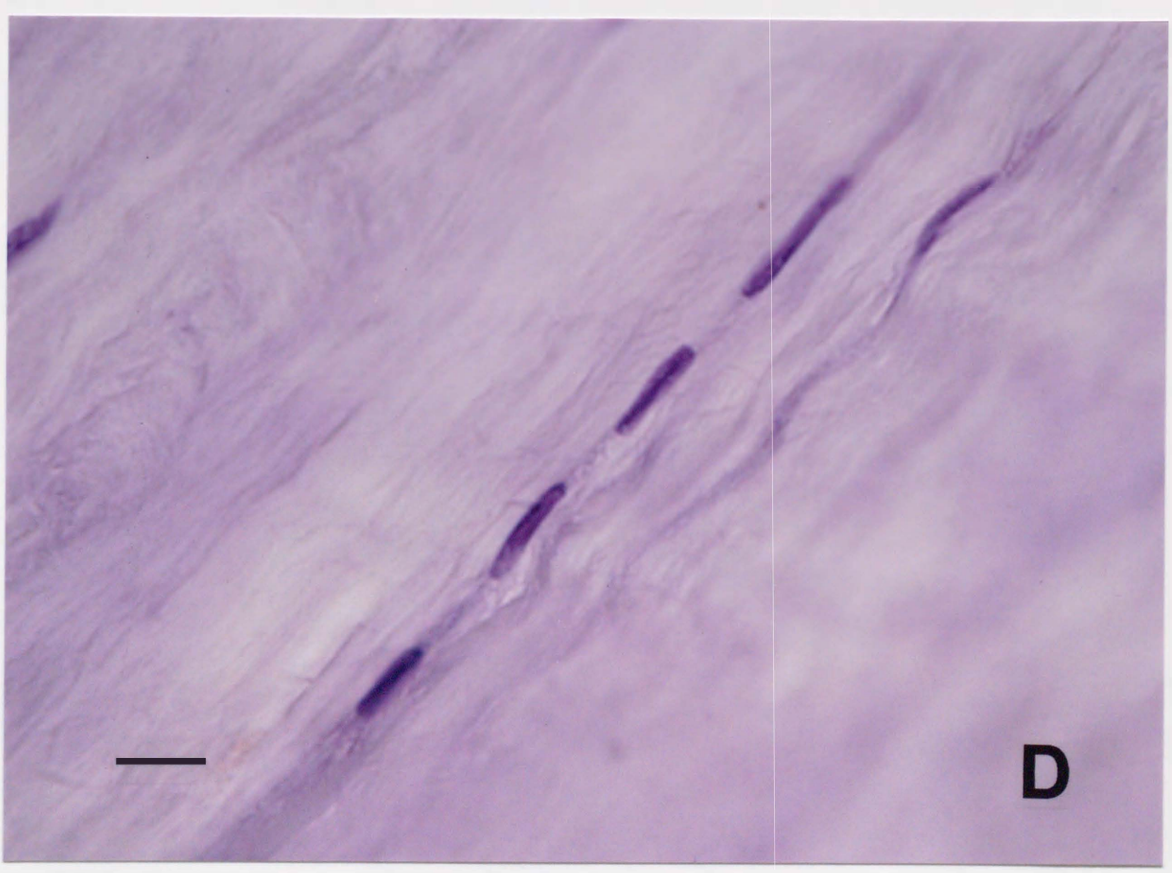
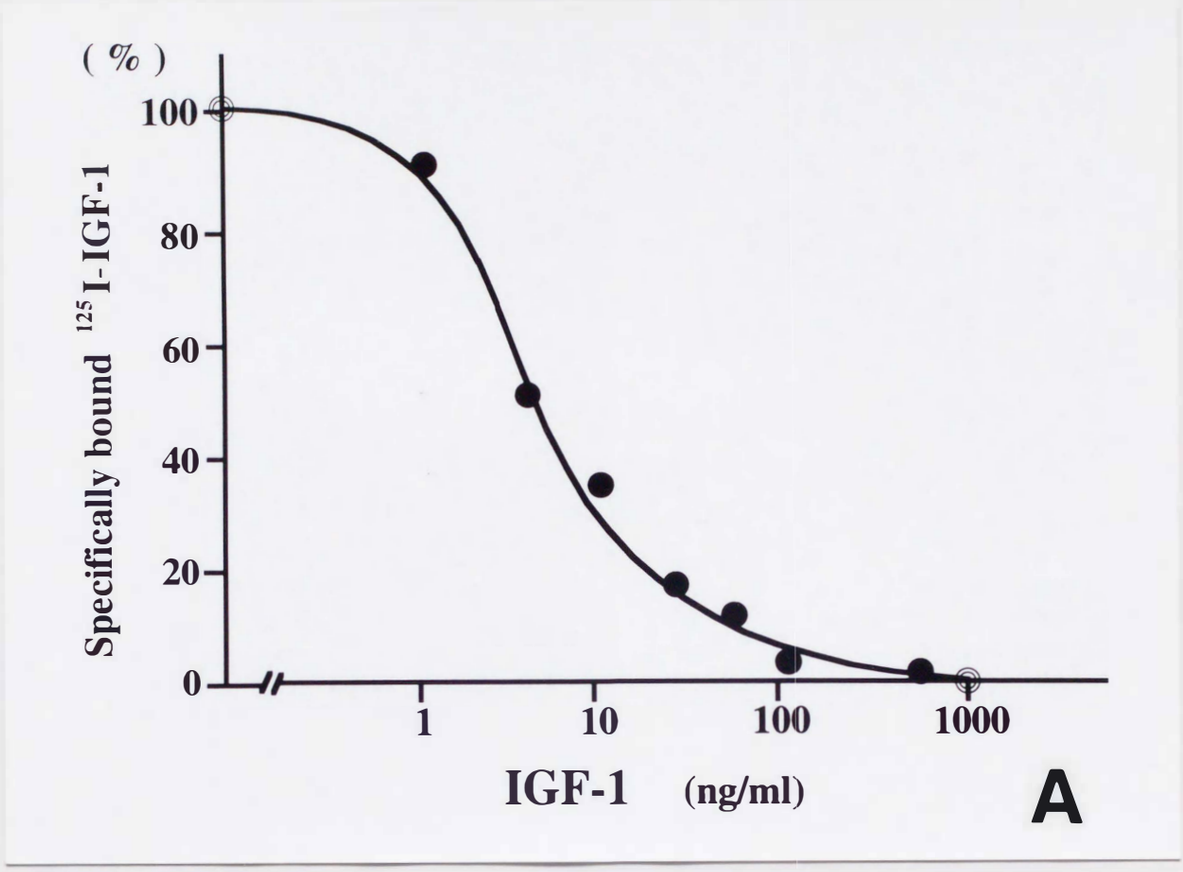
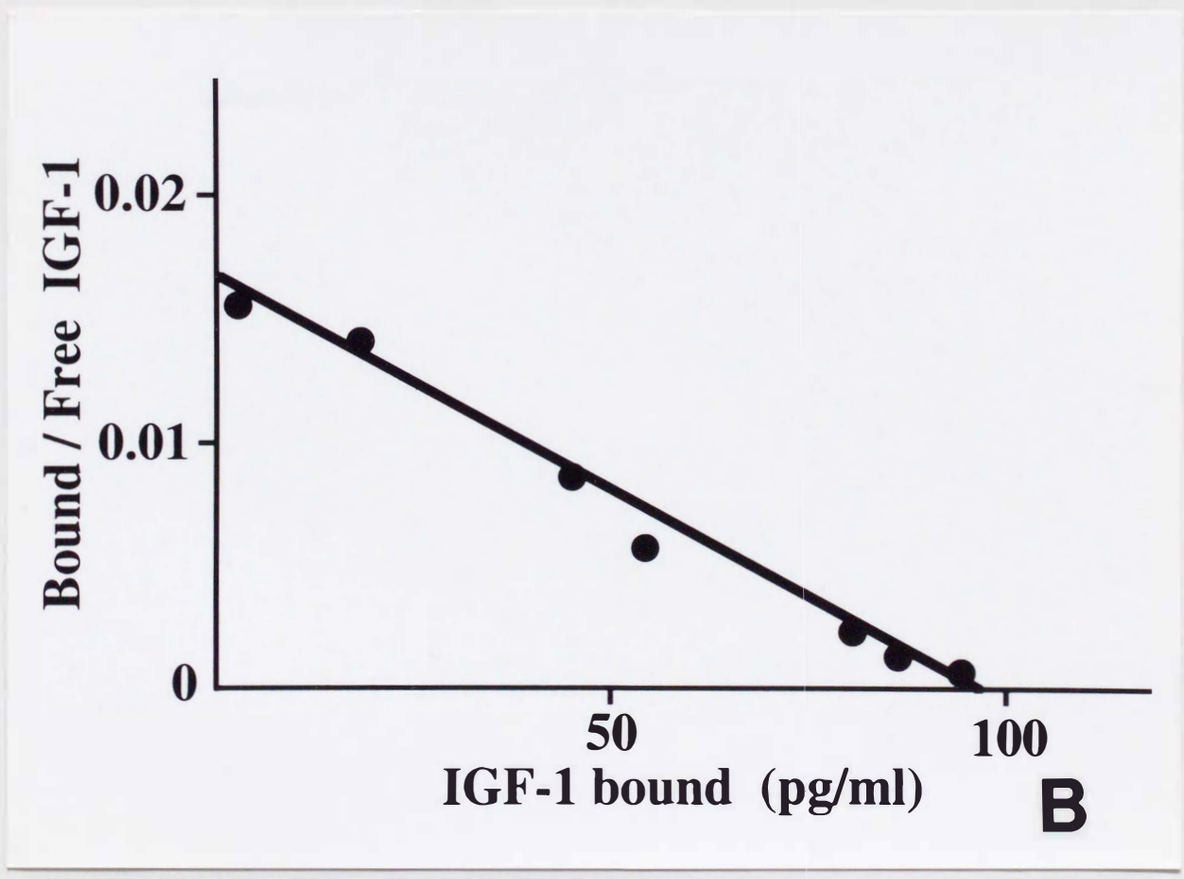
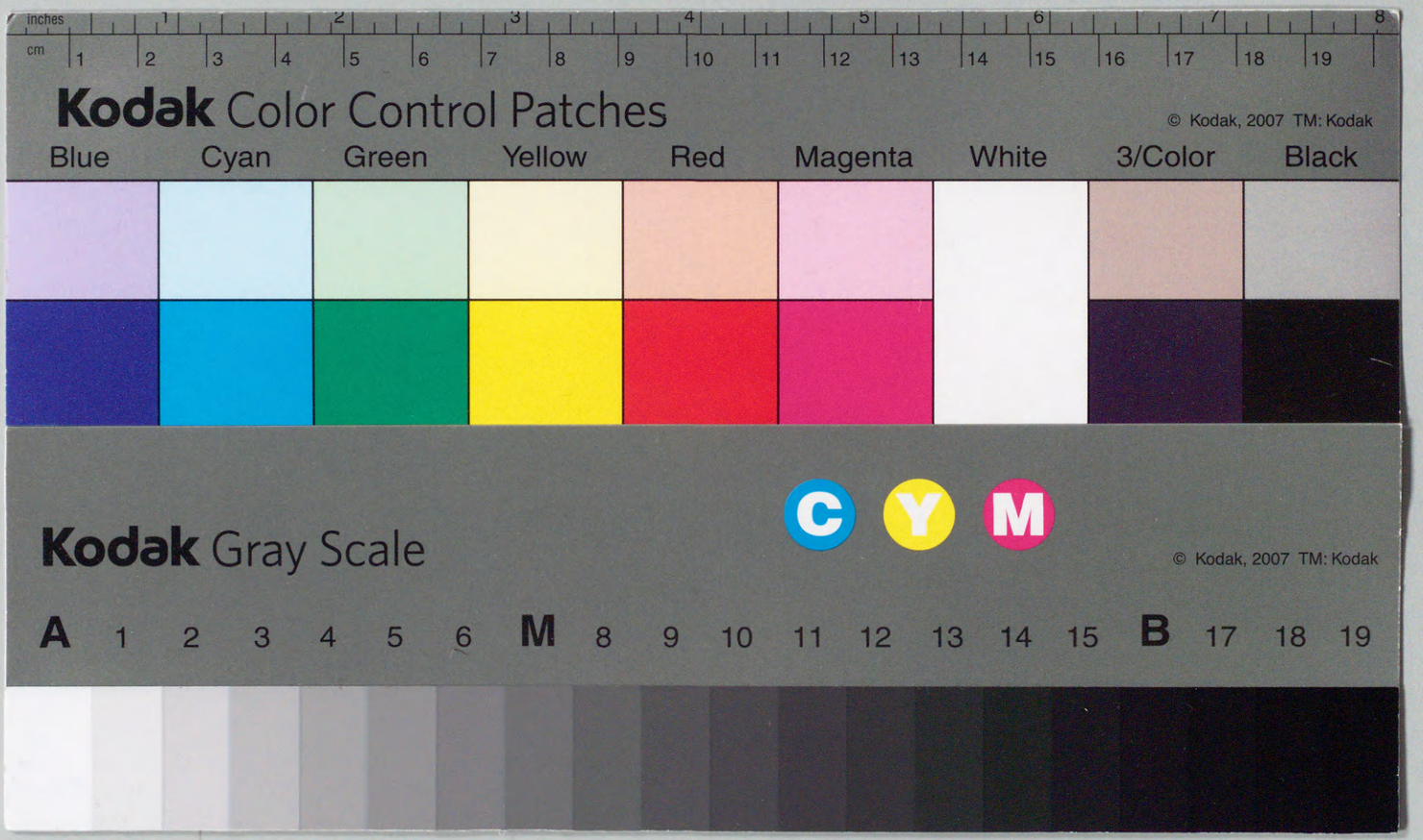


Fig.7


(%)
100
80
60
40
20
0
Specifically bound ^{125}I-IGF-1
1
10
100
1000
IGF-1 (ng/ml)
A


0.02
0.01
0
Bound / Free IGF-1
50
100
IGF-1 bound (pg/ml)
B


inches
cm
Kodak Color Control Patches
© Kodak, 2007 TM: Kodak
Blue
Cyan
Green
Yellow
Red
Magenta
White
3/Color
Black
Kodak Gray Scale
C
Y
M
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A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19