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Up-Regulation by IGF-I of Proliferating Cell Nuclear Antigen and Bcl-2 Protein Expression in Human Uterine Leiomyoma Cells

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IGF-I has been reported to play a role in regulating proliferation of human leiomyoma cells. There is, however, little evidence to suggest that IGF-I inhibits apoptosis in the leiomyoma cells. The present study was conducted to elucidate whether IGF-I affects apoptosis and Bcl-2 protein expression, an apoptosis-inhibiting gene product, in cultured leiomyoma cells. In addition, we examined the effect of IGF-I on proliferating cell nuclear antigen (PCNA) expression in cultured leiomyoma cells. Isolated human leiomyoma cells were subcultured in phenol red-free DMEM supplemented with 10% FBS for 120 h and then stepped down to serum-free conditions for an additional 72 h in the absence or presence of graded concentrations of IGF-I (1.0, 10, and 100 ng/ml). The effects of IGF-I on Bcl-2 protein and PCNA expression in cultured leiomyoma cells were assessed by Western immunoblot analysis and immunocytochemical staining, whereas the effects of IGF-I on the cell viability and apoptosis of the cultured cells were determined by 3-(4,5-dimethylatriazol-2-yl)-2,5-diphenyltetrasodium bromide (MTT) assay and terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine 5′-triphosphate nick end labeling assay, respectively. Immunocytochemical staining demonstrated that IGF-I treatment resulted in the increase in PCNA labeling index in cultured leiomyoma cells in a dose-dependent manner. Immunoblot analysis of proteins extracted from the cultured leiomyoma cells revealed that the addition of IGF-I (10 and 100 ng/ml) significantly increased the expression of 35-kDa immunoreactive PCNA and 26-kDa Bcl-2 protein, compared with those in control cultures. Cell survival and proliferation of cultured leiomyoma cells, assessed by MTT assay, was significantly augmented by IGF-I treatment, compared with those of control cultures. Terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine 5′-triphosphate nick end labeling assay showed that the apoptosis-positive rate of leiomyoma cells treated with IGF-I was significantly decreased, compared with that in control cultures. The present results suggest that IGF-I plays crucial roles in leiomyoma cell growth, not only in promoting the proliferative potential by up-regulation of PCNA expression but also in down-regulating apoptosis by up-regulation of Bcl-2 protein expression in leiomyoma cells. (*J Clin Endocrinol Metab* 86: 5593–5599, 2001)

UTERINE LEIOMYOMA IS the most common benign tumor originated from uterine smooth muscle cells, occurring in as many as 30% of women over 35 yr of age (1). It is a frequent cause of menorrhagia, dysmenorrhea, pelvic discomfort, infertility, and recurrent pregnancy loss. The growth of leiomyoma has been known to be dependent on the biological activity of ovarian steroid hormones (2, 3) mediated, in part, by locally derived growth factors, such as epidermal growth factor (EGF) and IGF-I (4–6). In this context, we have recently demonstrated that progesterone (P_4) up-regulates the expression of immunoreactive EGF and proliferating cell nuclear antigen (PCNA), whereas 17β-estradiol up-regulates the expression of EGF receptor and PCNA in leiomyoma cells (7).

IGF-I is a major anabolic agent responsible for growth, differentiation, and mediating the biological effects of GH in many cell types. Significantly higher IGF-I concentrations and IGF-I receptor mRNA levels in leiomyoma were detected, compared with those in myometrium (8). Several reports indicated that IGF-I is responsible for leiomyoma cell proliferation, on the basis of cell number count (9–12). Moreover, IGF-I is regarded as an estromedin and progestomedin and regulates the growth-promoting effects of sex steroids in rhesus monkey uterus (13). There is, however, little evidence regarding the molecular mechanism underlying the regulation of leiomyoma cell growth by IGF-I. On the other hand, a growing body of evidence suggests that IGF-I acts as a survival factor to inhibit apoptosis in a variety of cell types, such as PC12 cells and H9C2 cardiac muscle cells (14–17). Accordingly, overexpression of IGF-I receptor in those cells increased tumorigenic potential of the cells and protected the cells from apoptosis (18, 19). To date, little information is available on the effect of IGF-I on apoptosis in leiomyoma cells. Our resent study has demonstrated the increased expression of Bcl-2 protein, an apoptosis-inhibiting gene product, in leiomyoma cells, relative to that in the adjacent normal myometrial cells, and its up-regulation by P_4 in leiomyoma cells (20). Because homeostatic control of the net growth of tumors is the result of dynamic balance between cell proliferation and cell death (21), we conducted the present study, first, to investigate whether IGF-I affects apoptosis and Bcl-2 protein expression in leiomyoma cells cultured under serum-free, phenol red-free conditions, on the basis of terminal deoxynucleotidyl transferase-mediated deoxyuridine 5′-triphosphate nick end labeling (TUNEL) assay and Western immunoblot analysis. Furthermore, we examined the effects of IGF-I on cell viability and proliferation and PCNA expression in the cultured leiomyoma cells, by MTT assay,

Abbreviations: EGF, Epidermal growth factor; MTT, 3-(4,5-dimethylatriazol-2-yl)-2,5-diphenyltetrasodium bromide; P_4, progesterone; PCNA, proliferating cell nuclear antigen; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine 5′-triphosphate nick end labeling.

immunocytochemical staining, and Western immunoblot analysis.

Materials and Methods

Tissue collection

Sixteen uterine leiomyoma tissues were obtained from patients with regular menstrual cycles who underwent abdominal hysterectomy, for medically indicated reasons, at Kobe University Hospital. Informed consent was obtained from each patient, before surgery, for the use of uterine tissues for the present study. The institutional review board approved the use of uterine tissues for culture experiments. The patients ranged in age from 29–45 yr, and no patients received any hormonal therapy for at least six menstrual cycles before surgery. Histological diagnosis of each uterine specimen was examined. Samples were excluded from the study if accurate menstrual cycle dates could not be assigned or if unexpected pathology was found (*e.g.* adenomyosis or leiomyosarcoma).

Cell culture

Uterine leiomyoma tissues, dissected from endometrial cell layers, were cut into small pieces and digested in 0.2% collagenase (wt/vol), at 37 C for 3–5 h, as previously described (20). The leiomyoma cells were collected by centrifugation at $460 \times g$ for 5 min and washed 3 times with DMEM containing 1% antibiotic solution. The cell viability was determined by trypan blue exclusion test. The isolated leiomyoma cells were plated at densities of approximately 10^6 cells/dish in 10-cm^2 culture dishes, 4×10^4 cells/well in 2-well chamber slides, and 1×10^4/well in 96-well tissue-culture plates. The leiomyoma cells in culture dishes and 2-well chamber slides were subcultured for 120 h at 37 C in a humidified atmosphere of 5% CO_2-95% air in phenol red-free DMEM supplemented with 10% FBS (vol/vol; Life Technologies, Inc., Grand Island, NY). The isolated leiomyoma cells in 96-well tissue culture plates were subcultured for 72 h with the same conditions as mentioned above. The monolayer cultures at approximately 60% confluence were treated with graded concentrations (1.0, 10, and 100 ng/ml) of IGF-I (Chemicon International Inc., Temecula, CA) in serum-free, phenol red-free DMEM for an additional 72 h.

Immunocytochemical staining for PCNA

Leiomyoma cells cultured in 2-well chamber slides were washed for three times with PBS, fixed in methanol at 4 C for 20 min, and washed with PBS for three times once again. The fixed cells were subjected to immunostaining, by the avidin/biotin immunoperoxidase method, using a polyvalent immunoperoxidase kit (Omnitags, Lipshow, MI) according to the manual of instruction. A mouse monoclonal antibody to human PCNA protein (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used, at a dilution of 1:80, as the primary antibody in this study. To assure the specificity of the immunological reaction, control sections were subjected to the same immunoperoxidase method, except that the primary antibody was replaced by nonimmune murine IgG (Miles, Erkhardt, IN) at the same dilution as the specific antibody. The replacement of the specific primary antibody with nonimmune murine IgG resulted in a lack of positive immunostaining.

Immunostained sections were analyzed, in a blinded fashion, without knowledge of the experimental group. The PCNA labeling index was determined by observing more than 1000 nuclei for each experimented sample and was used for evaluating the proliferating activity of the cells.

Protein extraction and Western immunoblotting

Proteins were extracted from cultured cells as described previously (7). At the termination of culture, cultured cells were lysed, at 4 C for 20 min, in the presence of a lysis buffer (150 mM NaCl, 2 mM phenylmethylsulfonylfluoride, 1% Nonidet P-40, 0.5% deoxycholate, 1 mg/liter aprotinin, 0.1% SDS, and 50 mM Tris-HCl, PH 7.5). The lysates were subsequently centrifuged at $13{,}000 \times g$ for 30 min at 4 C, and the supernatants were collected. Protein content in the supernatants was determined by the Bradford assay (22), with BSA as a standard.

Each 100-μg aliquot of protein extracted from cultured cells was separated by 12% SDS-PAGE, under a reducing condition, using 20–25 mA for the stacking gel and 30–35 mA for the separating gel for 2–3 h. The proteins were then electrophoretically transferred from gels to nitrocellulose membranes (Bio-Rad Laboratories, Inc., Hercules, CA). Blots were exposed overnight to the monoclonal antibodies to Bcl-2 protein (Santa Cruz Biotechnology, Inc.) or to PCNA protein at a dilution of 1:200 in blocking buffer. The membranes were incubated for 1 h with horseradish peroxidase-conjugated goat antimouse secondary antibody (Amersham Pharmacia Biotech, Arlington Heights, IL) that was diluted at 1:2000 with blocking buffer. The antigen-antibody complexes were detected with the ECL chemiluminescence detection kit (Amersham Pharmacia Biotech). Membranes were visualized by exposure to X-Omat film (Eastman Kodak Co., Rochester, New York). The radioautograms were then scanned and quantified with ChemiImager 4400 (Astec Co., Ltd., Osaka, Japan).

MTT assay for cell growth and viability

The term "growth" is used to denote a net increase in cell numbers, reflecting both proliferation and apoptosis. In the present study, leiomyoma cell numbers and viability were evaluated with MTT [3-(4,5-dimethylahiazol-2-yl)-2,5-diphenyltetrasodium bromide] assay using the Mosmann method (23). This assay is based on the cleavage of the tetrazolium salt MTT to a blue formazan product by mitochondrial dehydrogenase in viable cells. Briefly, after being treated in the absence or presence of graded concentrations of IGF-I in serum-free DMEM for 72 h in a 96-well tissue culture plate, 10 μL MTT (Chemicon International Inc.) solution was added to each well, and the cultured cells were incubated at 37 C for another 4 h. Then, 100 μL isopropanol/HCl solution was added in each well and mixed thoroughly with EM-36N microtube mixer (Taitec, Tokyo, Japan). The absorbance was measured in an MTP-120 ELISA plate reader (Corona Electric Co., Osaka, Japan) with a test wavelength of 570 nm and a reference wavelength of 630 nm.

In situ TUNEL assay

In situ labeling of fragmented DNA in cultured leiomyoma cells was performed with the TUNEL assay, using the *in situ* apoptosis detection kit (Oncor, Gaithersburg, MD) according to the manufacture's protocol for monolayer cell cultures. Leiomyoma cells were subcultured in 2-well plastic chamber slides for 120 h and then cultured, under serum-deprivation conditions, for an additional 72 h in the absence or presence of graded concentrations of IGF-I. At the termination of cultures, nucleotide-sized DNA fragments were tailed with digoxigenin-deoxyuridine 5′-triphosphate and then bound with peroxidase-conjugated antidigoxigenin antibodies. The nuclei were counterstained with hematoxylin (Zymed Laboratories, Inc., San Francisco, CA), for determining the apoptosis-positive rate of cultured leiomyoma cells.

Data analysis

The data were expressed as the mean ± SD. Statistical analysis was performed using one-way ANOVA with the StatView 4.1 software (SAS Institute, Inc., Cary, NC) for Macintosh, followed by *post hoc* testing using Fisher's protected least-significant-difference test. Difference with a $P < 0.05$ was considered statistically significant.

Results

Effect of IGF-I on PCNA expression

Immunocytochemical staining demonstrated that PCNA-positive nuclei of leiomyoma cells treated with IGF-I (10 ng/ml, Fig. 1C; 100 ng/ml; Fig. 1D) were more abundant than those in control cultures (Fig. 1A). Treatment with 1.0 ng/ml IGF-I showed no apparent effect on the abundance of PCNA-positive nuclei in cultured leiomyoma cells (Fig. 1B). Replacement of the primary antibody with nonimmune murine IgG showed a lack of positive immunostaining in the cultured leiomyoma cell nuclei (Fig. 1E).

Figure 2 shows the PCNA-positive rate of leiomyoma cells

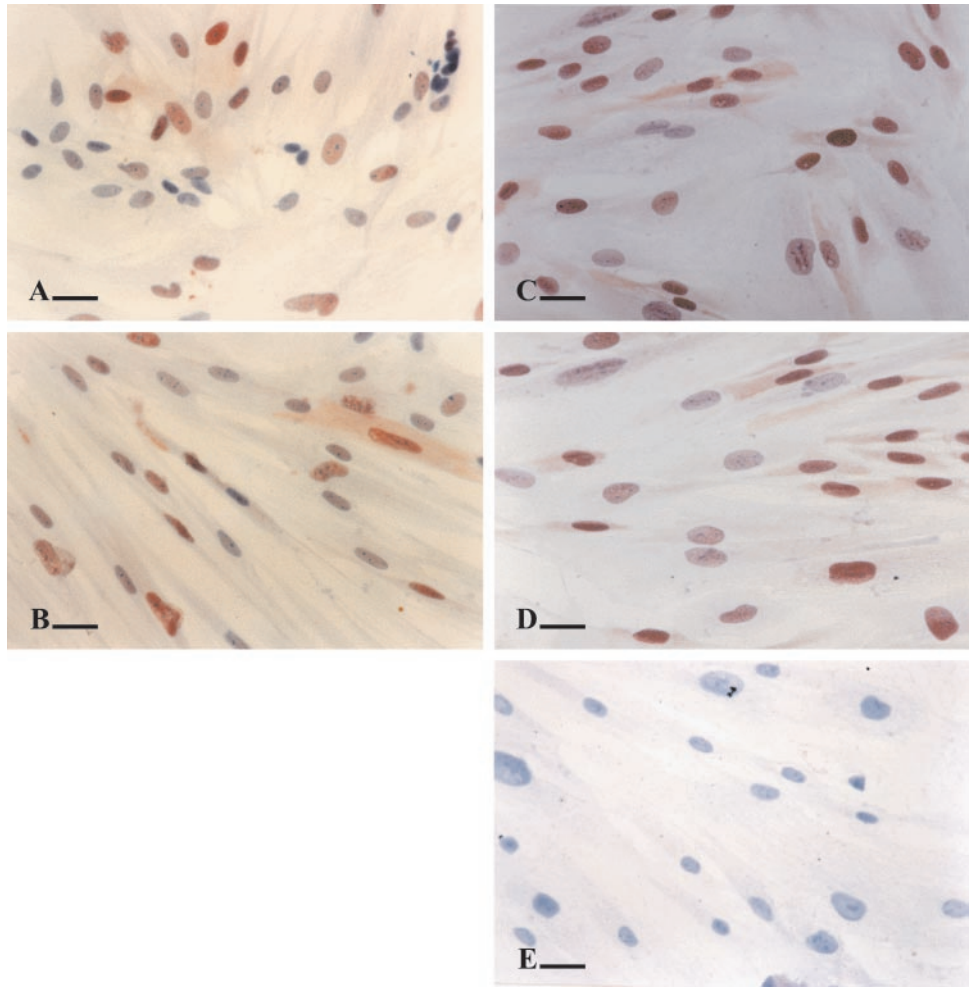

FIG. 1. Immunocytochemical staining of PCNA in leiomyoma cells cultured for 72 h under serum-free, phenol red-free conditions, in the presence or absence of IGF-I. It was apparent that PCNA-positive nuclei were more abundant in the cultured leiomyoma cells treated with either 10 ng/ml IGF-I (C) or 100 ng/ml IGF-I (D), relative to those in control cultures (A). Treatment with 1.0 ng/ml IGF-I (B) did not show an apparent difference in the abundance of PCNA-positive nuclei, compared with that in control cultures. Replacement of the primary antibody with nonimmune murine IgG showed a lack of positive immunostaining in the cultured leiomyoma cell nuclei (E). *Bars*, 5 μm; original magnification, $\times$400.

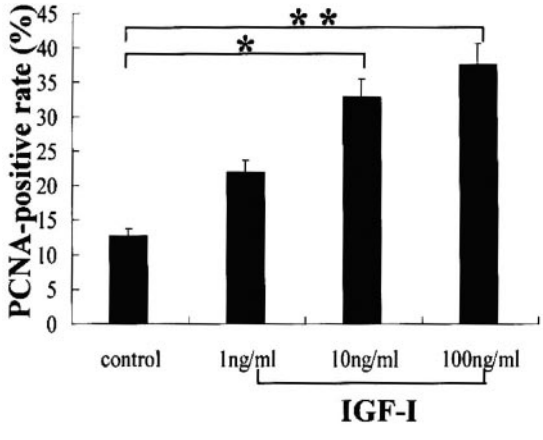

FIG. 2. The mean percentage of PCNA-positive nuclei in cultured leiomyoma cells, as assessed by immunocytochemical analysis. Although treatment with 1.0 ng/ml IGF-I showed no significant effect on the PCNA-positive rate of cultured leiomyoma cells, treatment with IGF-I (10 ng/ml or 100 ng/ml) significantly increased the PCNA-positive rate, compared with that in control cultures. Values were presented as the mean $\pm$ SD. **, $P < 0.01$; *, $P < 0.05$.

cultured in the absence or presence of graded concentrations of IGF-I for 72 h. The addition of IGF-I augmented the PCNA-positive rate of the cultured cells in a dose-dependent manner, compared with that in control cultures. Significant increase in the PCNA-positive rate of cultured leiomyoma cells was obtained by IGF-I treatment with higher concentrations than 10 ng/ml (10 ng/ml, $P < 0.05$; 100 ng/ml, $P < 0.01$).

Western immunoblot analysis of proteins extracted from leiomyoma cells cultured for 72 h revealed that cultured leiomyoma cells contained immunoreactive PCNA with a molecular mass of approximately 36 kDa. The 36-kDa PCNA expression was significantly ($P < 0.01$) higher in leiomyoma cells treated with IGF-I with higher concentrations than 10 ng/ml, compared with that in control cultures (Fig. 3).

Effect of IGF-I on cell growth and viability

Figure 4 shows IGF-I-induced changes in cell numbers and viability of cultured leiomyoma cells, as determined by MTT assay. The addition of IGF-I with higher concentrations than 10 ng/ml significantly ($P < 0.05$) increased the cell numbers and viability of cultured leiomyoma cells, compared with that in control cultures; whereas the addition of 1.0 ng/ml IGF-I showed no stimulatory effect on leiomyoma cell growth and viability.

To address whether the increase in cell number with IGF-I, as measured by MTT assay, was a result of proliferation or an inhibition of apoptosis, leiomyoma cells treated with IGF-I were examined for PCNA expression and apoptosis-positive

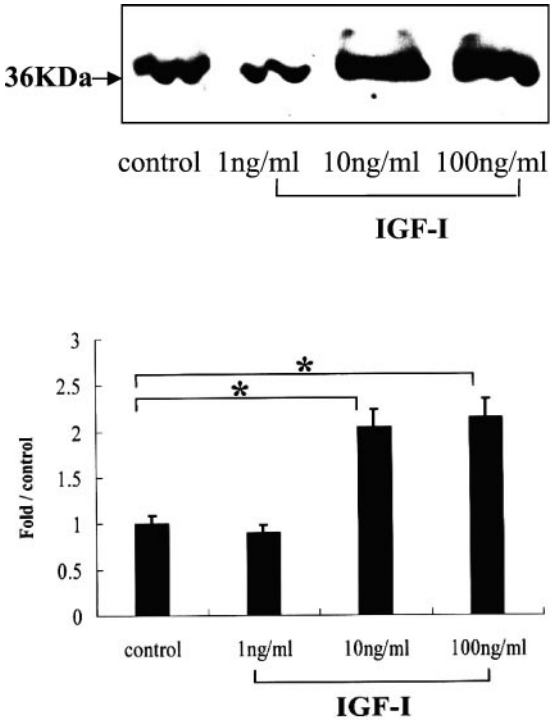

FIG. 3. Effect of IGF-I on PCNA protein expression in cultured leiomyoma cells, as assessed by Western immunoblot analysis. Isolated leiomyoma cells were cultured for 72 h in serum-free DMEM, in the absence or presence of graded concentrations of IGF-I. Each 100-μg aliquot of proteins extracted from cultured leiomyoma cells was subjected to Western immunoblotting with a monoclonal antibody to PCNA. The 36-kDa PCNA expression observed in untreated leiomyoma cells was augmented by treatment with either 10 ng/ml IGF-I or 100 ng/ml IGF-I, whereas treatment with 1.0 ng/ml IGF-I did not affect the 36-kDa PCNA protein expression in the cells. Experiments were repeated three times, with similar results for each. Densitometric analysis of PCNA protein level in cultured leiomyoma cells was performed as described in *Materials and Methods*. Data were presented as the fold increase over the control value and as the mean ± SD. *, $P < 0.01$.

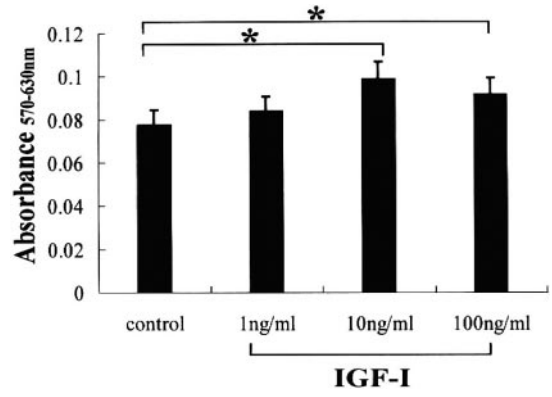

FIG. 4. Effect of IGF-I on cell growth and viability of cultured leiomyoma cells, as assessed by MTT assay. Isolated leiomyoma cells were cultured for 72 h in serum-free DMEM, in the absence or presence of graded concentrations of IGF-I. After addition of MTT solution (10 μL/100 μL medium) in each well, the cells were incubated at 37 C for 4 h, and then 100 μL isopropanol/HCl solution was added to dissolve the dark blue crystals. The absorbance was measured in an ELISA plate reader with a test wavelength of 570 nm and a reference wavelength of 630 nm. The increases in cell number and viability in leiomyoma cells treated with either 10 ng/ml or 100 ng/ml IGF-I were significantly higher than that in control cultures. Values were presented as the mean ± SD. *, $P < 0.05$.

rate in the present study. The data show that IGF-I stimulated proliferation and decreased the apoptosis-positive rate in cultured leiomyoma cells.

Effect of IGF-I on apoptosis

Figure 5 represents the distribution of apoptotic cells in leiomyoma cells cultured in the presence or absence of IGF-I, as detected by TUNEL assay. Most of the TUNEL-positive cells were labeled in black-brown confined to the nuclei, whereas all nuclei were counterstained with hematoxylin. TUNEL-positive cells were less in leiomyoma cells treated with IGF-I with higher concentrations than 10 ng/ml (Fig. 5, C and D), relative to those in control cultures (Fig. 5A). There was no significant difference in the appearance of TUNEL-positive cells between control cultures and cultures treated with 1.0 ng/ml IGF-I (Fig. 5B).

Figure 6 shows the quantitative analysis of apoptosis in cultured leiomyoma cells on the basis of apoptosis-positive rate. Determination of the mean percentage of apoptotic cells stained positively by the TUNEL assay in cultured leiomyoma cells revealed that the apoptosis-positive rate in leiomyoma cells treated with IGF-I with higher concentrations than 10 ng/ml was significantly ($P < 0.01$) less than that in control cultures. There was, however, no difference in the apoptosis-positive rate between control cultures and cultures treated with 1.0 ng/ml IGF-I.

Western immunoblot analysis of proteins extracted from leiomyoma cells cultured for 72 h indicated that cultured leiomyoma cells contained immunoreactive Bcl-2 protein with a molecular mass of 26 kDa and that the 26-kDa Bcl-2 protein expression was significantly ($P < 0.05$) more abundant in leiomyoma cells treated with IGF-I with higher concentrations than 10ng/ml, relative to that in control cultures (Fig. 7). The treatment with 1.0 ng/ml IGF-I showed no significant effect on Bcl-2 protein expression in cultured leiomyoma cells.

Discussion

Apoptosis is an important biological mechanism through which tissues shape normal developmental patterns and adapt to new environmental changes. Bcl-2 protein has been proposed to serve as a signaling receptor for intracellular organelles in which serine phosphorylation of both apoptotic and antiapoptotic family members are hypothesized to affect cell survival. The bcl-2 protooncogene was discovered in lymphoma tumors composed of B cells and encodes a 26-kDa protein, localized to mitochondrial and perinuclear membranes (24). Numerous studies have reported on the ability of enhanced Bcl-2 expression to promote the survival of various cell types, as well as the ability to block apoptotic cell death (25–28). This study is believed to be the first to demonstrate that IGF-I inhibits the apoptotic way in human leiomyomas through up-regulating Bcl-2 protein expression in leiomyoma cells. The antiapoptotic effects of IGF-I seem to be mediated by the function of its receptor tyrosine kinase (29, 30). In fact, a decrease in IGF-I receptor number induces

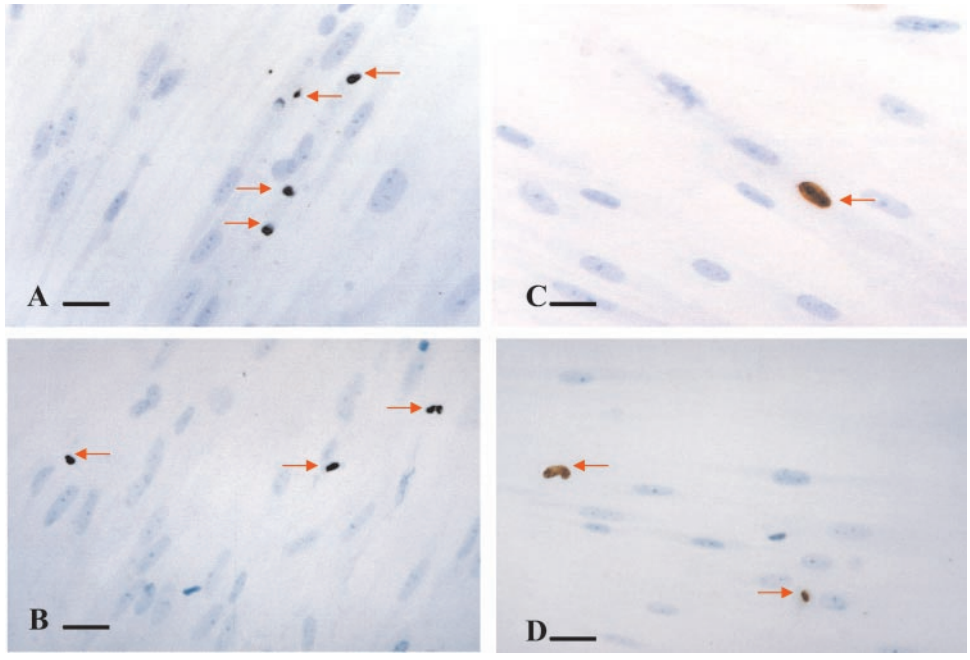

FIG. 5. Apoptotic cells in cultured leiomyoma cells, as assessed by TUNEL assay. Isolated leiomyoma cells were cultured for 72 h in serum-free DMEM, in the absence or presence of graded concentrations of IGF-I. The fragmented DNA of apoptotic cells was labeled in black-brown (*arrows*), whereas all nuclei were counterstained with hematoxylin. It was apparent that apoptotic cells were less in cultured leiomyoma cells treated with either 10 ng/ml (C) or 100 ng/ml (D) IGF-I, relative to those in control cultures (A). No apparent difference in the distribution of apoptotic cells was observed between control cultures and cultures treated with 1.0 ng/ml IGF-I (B). *Bars*, 5 μm; original magnification, ×400.

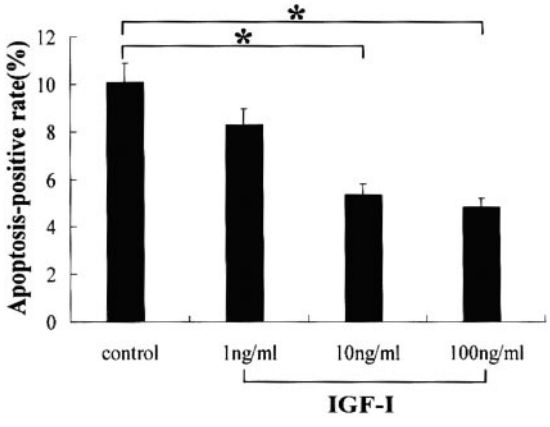

FIG. 6. Effect of IGF-I on apoptosis-positive rate in cultured leiomyoma cells, as assessed by TUNEL assay. The apoptosis-positive rate in cultured leiomyoma cells treated with either 10 ng/ml IGF-I or 100 ng/ml was significantly decreased, compared with that in control cultures, whereas no apparent difference in the apoptosis-positive rate was observed between control cultures and cultures treated with 1.0 ng/ml IGF-I. Values were presented as the mean ± SD. *, $P < 0.01$.

apoptosis in tumor cells (18). The molecular events in IGF-I modulating apoptosis in leiomyoma cells, however, is largely unknown. Our findings suggest the possibility that the greater abundance of Bcl-2 protein in leiomyoma cells, caused by IGF-I treatment, may be responsible, in part, for the growth of leiomyoma by preventing the cells from apoptosis.

Furthermore, this study has also revealed that PCNA expression in cultured leiomyoma cells is markedly increased by IGF-I treatment. PCNA is an essential protein found in proliferating eukaryotic cells; and it carries out crucial roles in DNA replication, repair, and control of cell proliferation (31). This protein is involved in synthesis of both leading and lagging DNA strands, providing an anchorage site and increasing the processivity of DNA pol δ and DNA pol ϵ, which is the basis of PCNA serving as a proliferative marker for evaluating DNA synthesis (32, 33). It is, therefore, likely that the presence of IGF-I may be mitogenic for the DNA synthesis in leiomyoma cells through up-regulating PCNA expression in the cells. The results obtained by MTT assay, in the present study, support the concept of IGF-I up-regulating proliferative activity of leiomyoma cells. Because the concentrations of IGF-I in leiomyoma tissues ranged from 50–150 ng/g tissue (8), the concentrations of IGF-I (10 ng/ml and 100 ng/ml), which were found in the present study to be effective in inhibiting apoptosis and stimulating proliferation of leiomyoma cells, seem to be within the physiological tissue concentration range.

In the present study, the addition of IGF-I (10 ng/ml and 100 ng/ml) resulted in a 20–25% increase in the PCNA-positive rate (Fig. 2) and an even-more marked increase in PCNA protein expression (Fig. 3); whereas the addition of IGF-I (10 ng/ml and 100 ng/ml) inhibited apoptosis, with a 5–6% decrease in apoptotic-positive rate (Fig. 6) and with approximately 70% increase in Bcl-2 protein expression (Fig. 7) in cultured leiomyoma cells. These results suggest that the proliferation of leiomyoma cells by IGF-I may be a central feature in the regulation of leiomyoma cell growth, whereas the inhibition of cell death by IGF-I may play a minor role in regulating leiomyoma cell growth. It seems likely that the effects of IGF-I on leiomyoma cell growth are mediated much more through effects on proliferation than on apoptosis.

Recent studies have demonstrated that IGF-I induced by estrogen in the uterus (34–36) can replace estrogen, not only in mediating mitogenesis but also in inducing the PR (37, 38). Furthermore, several reports represented that sex steroids increase IGF-I concentrations and IGF-I mRNA expression but have no effect on IGF-I receptor mRNA expression in leiomyoma and myometrial cells (39, 40). These findings suggest that IGF-I may be involved in the regulation of leiomyoma growth as a local mediator of the growth-

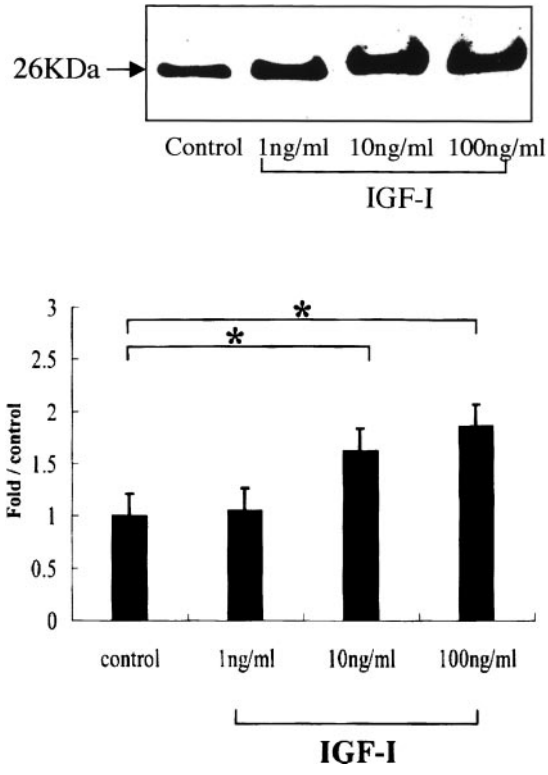

FIG. 7. Effect of IGF-I on Bcl-2 protein expression in cultured leiomyoma cells, as assessed by Western immunoblot analysis. Isolated leiomyoma cells were cultured for 72 h in serum-free DMEM, in the absence or presence of graded concentrations of IGF-I. Each 100-μg aliquot of proteins extracted from cultured leiomyoma cells was subjected to Western immunoblotting with a monoclonal antibody to Bcl-2 protein. The 26-kDa Bcl-2 protein expression observed in untreated leiomyoma cells was augmented by IGF-I treatment with higher concentrations than 10 ng/ml, whereas treatment with 1.0 ng/ml IGF-I did not affect the 26-kDa Bcl-2 protein expression in the cells. Experiments were repeated three times, with similar results for each. Densitometric analysis of Bcl-2 protein level in cultured leiomyoma cells was performed as described in *Materials and Methods*. Data were presented as the fold increase over the control value and as the mean ± SD. *, $P < 0.05$.

promoting actions of sex steroids. Our resent study demonstrated the increased expression of Bcl-2 protein in uterine leiomyoma cells, relative to normal myometrial cells of the same individual uterus, and its up-regulation by P_4 in leiomyoma cells (20), and that treatment with either P_4 or E2 increase the PCNA expression in leiomyoma cells (7). It seems that IGF-I may have similar biological effects on Bcl-2 protein and PCNA expression, as observed with P_4 in leiomyoma cells. This finding may, to some extent, support the view that IGF-I plays a role as a progestomedin in mediating the effects of P_4 on leiomyoma growth. Interestingly, several reports described that IGF-I concentrations were either very low (41) or undetectable (39) in human uterine leiomyoma tissues from patients treated with GnRH agonist. This implies that the effect of GnRH agonist on leiomyomas may be regulated, in part, by a decrease in the local concentrations of IGF-I in leiomyoma tissues. Taking these notions into account, it seems that a complex signaling network, in which sex steroid hormones are cooperated with IGF-I and other local mediators, may exist and regulate the net growth of leiomyoma.

In conclusion, we have demonstrated, for the first time, that IGF-I stimulates leiomyoma cell proliferation through augmenting PCNA protein expression in the cells but inhibits apoptosis of leiomyoma cells, in part, through increasing Bcl-2 protein expression in the cells. This suggests that IGF-I plays a vital role in regulating leiomyoma growth. Further studies will be needed to determine the detailed molecular mechanism by which IGF-I and ovarian steroid hormones interact in the regulation of human uterine leiomyoma growth.

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