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Insulin-Like Growth Factors Induce Apoptosis as Well as Proliferation in LIM 1215 Colon Cancer Cells

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Abstract The insulin-like growth factor (IGF) system plays an important role in cell proliferation and survival. However, more recently, a small number of studies have shown that IGFs induce apoptosis in some cells. Our initial studies showed this occurred in LIM 1215 colon cancer cells but not RD rhabdomyosarcoma cells. IGFs induced both proliferation and apoptosis in LIM 1215 cells, and the induction of apoptosis was dose-dependent. [R^{54}, R^{55}]IGF-II, which binds to the IGF-I receptor with normal affinity but does not bind to the IGF-II receptor, induced apoptosis to the same extent as IGF-II, whereas [L^{27}]IGF-II, which binds to the IGF-I receptor with 1000-fold reduced affinity, had no effect on apoptosis. These results suggest that the IGF-I receptor is involved in induction of apoptosis. Western blot analyses demonstrated that Akt and Erk1/2 were constitutively activated in RD cells. In contrast, phosphorylation of Akt and Erk1/2 were transient and basal expression of Akt protein was lower in LIM 1215 cells. Analysis of apoptosis-related proteins showed that IGFs decreased pro-caspase-3 levels and increased expression of pro-apoptotic Bad in LIM 1215 cells. IGFs co-activate proliferative and apoptotic pathways in LIM 1215 cells, which may contribute to increased cell turnover. Since high turnover correlates with poor prognosis in colorectal cancer, this study provides further evidence for the role of the IGF system in its progression. J. Cell. Biochem. 100: 58–68, 2007.

Key words: IGFs; colon cancer cells; proliferation; apoptosis

The insulin-like growth factor (IGF) system plays an import role in cell proliferation, mitogenesis, differentiation, and survival [LeRoith and Roberts, 2003]. It comprises IGF-I and -II, the IGF-I receptor (IGF-IR) and IGF-II/mannose-6-phosphate receptor (IGF-II/man6P R), and IGF binding proteins 1–6. The IGF-I receptor (IGF-IR), which is a tyrosine kinase receptor that is structurally homologous to the insulin receptor (IR), mediates most IGF actions whereas the IGF-IIR/man6P R regulates IGF-II levels by mediating its clearance [LeRoith and Roberts, 2003]. IGFs also bind to the IR, and the IGF-IR and IR can heterodimerize to form functional IR/IGF-IR hybrid receptors (hybrid-R) [Soos et al., 1993].

Abnormal expression of IGFs, their receptors and binding proteins has been linked to several cancers [Bach, 1999], including colorectal cancer, which is one of the most common cancers, killing 200,000 people in Europe and the USA each year. Colon cancers often overexpress IGFs [Reinmuth et al., 2002] and IGF-IR [Ouban et al., 2003]. Overexpression of IGFs and their receptors in certain cancers, in conjunction with their survival and proliferative actions, suggests that they may play an important role in tumor development and progression [LeRoith and Roberts, 2003; Durai et al., 2005]. Colon cancer cells may also express the IGF-II/man6P R [Freier et al., 1999] and IR [Watkins et al., 1990]. While IGFs are reported to stimulate colon cancer cell proliferation

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[Leng et al., 2001b], some studies showed only 50% of colon cancer cells are responsive to the mitogenic effects of exogenous IGFs [Singh et al., 1994]. Thus, there appears to be considerable heterogeneity between colon carcinoma cell lines in response to exogenous IGF stimulation.

Many studies have shown that IGF-mediated signaling plays an important role in cell survival. IGFs can rescue cells from apoptosis induced by withdrawal of growth factors or serum, or by apoptosis-inducing stimuli such as tumor necrosis factor-α, hyperosmotic stress, irradiation, and overexpression of oncogenes [O'Connor et al., 2000]. We have previously shown that IGF-II inhibits butyrate-induced apoptosis in colon cancer cells [Leng et al., 2001a]. However, a few studies have suggested that IGFs induce apoptosis in some circumstances [Granerus et al., 2001; Raile et al., 2003; Gronowicz et al., 2004; Saile et al., 2004]. The precise mechanisms underlying these apparently paradoxical actions of IGFs are yet to be elucidated. However, increased cell turnover due to high levels of proliferation and apoptosis is associated with a poor outcome in colorectal cancer [Rupa et al., 2003], so that this phenomenon may have clinical significance for cancer progression.

In the present study, we aimed to better understand these IGF-mediated proliferative and pro-apoptotic processes by comparing two human cancer cell lines, LIM 1215 colon cancer cells and RD rhabdomyosarcoma cells. We have previously shown that LIM 1215 respond to exogenous IGFs [Leng et al., 2001b], whereas RD cells have an autocrine IGF system and do not proliferate in response to exogenous IGFs [El-Badry et al., 1990; Gallicchio et al., 2001]. To further elucidate the receptors involved in IGF-induced apoptosis, two IGF analogs with differential binding affinities for IGF receptors were used. We report here that IGFs have a dual effect on LIM 1215 colon cancer cells, stimulating both proliferation and apoptosis. IGF-induced apoptosis was mediated by the IGF-IR.

MATERIALS AND METHODS

Materials

LIM 1215 colon cancer cells were kindly provided by Dr. Robert Whitehead at Ludwig Institute for Cancer Research, Melbourne, Australia. RD cells derived from a pelvic embryonal rhabdomyosarcoma were obtained from ATCC. Antibodies to pro-caspase-3, Bcl-2, Bad, phospho-Akt (Ser473), total Akt, phospho-Erk1/2, and Erk1/2 were purchased from Cell Signalling Technology. Mouse anti-actin monoclonal antibody was purchased from Chemicon International.

Cell Culture

LIM 1215 cells were cultured in RPMI 1615 medium supplemented with 10% fetal calf serum (FCS), 5 mM L-glutamine, penicillin-streptomycin (10,000 U/ml and 10 mg/ml, respectively), 0.025 U/ml insulin, 10 μM thioglycerol, and 1 μg/ml hydrocortisone in a 37°C humidified atmosphere containing 5% CO_2. IGF stimulation was carried out in the above medium without insulin but containing 1% FCS and 0.05% BSA. RD cells were cultured in DMEM supplemented with 10% FCS, 5 mM L-glutamine, and 1% penicillin-streptomycin (10,000 U/ml and 10 mg/ml, respectively). IGF stimulation was performed in serum-free DMEM medium containing 0.05% BSA.

MTT Assay

MTT assays were performed as previously described [Leng et al., 2001b]. Briefly, LIM 1215 cells were seeded in a 96-well plate (1×10^4 cells/well in 100 μl medium), cultured with or without IGF-I or IGF-II (100 ng/ml) for 72 h. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (10 μl) was then added for 3 h and this was followed by the addition of acidic isopropanol solution (200 μl). The mixtures were then agitated for 30 min. Abs_{540} was measured with a Bio-Rad Microplate reader. Validation experiments showed a linear correlation ($r = 0.996$) between Abs_{540} in the MTT assay and cell number in LIM 1215 cells (results not shown).

Cell Viability Assay

LIM 1215 cells were seeded in 24-well plates at a density of 1×10^5 cells/well. After culture with various concentrations of IGF-I for 72 h, floating and adherent cells were harvested and counted. Dead cells, as assessed by inability to exclude trypan blue, were counted and expressed as a percentage of total cells.

Cell Death Detection ELISA

Several methods were used to detect apoptosis. The first one is based on quantification of

mono- and oligo-nucleosomes in the cytoplasm. Briefly, LIM 1215 cells (~60%–70% confluence) were cultured in RPMI/1% FCS for 24 h, after which the media was aspirated and replaced with the same medium with or without IGF-I or IGF-II (100 ng/ml) for 72 h. Both floating and adherent cells were harvested and lysed. Cytoplasmic histone-associated DNA fragments from 5×10^3 cells were measured using the Cell Death Detection kit according to the manufacturer's instructions (Boehringer Mannheim, Germany).

Cytoplasmic DNA Fragmentation Assay

Cells were grown in 10 cm culture dishes until 60%–70% confluence was reached. They were then treated with 100 ng/ml IGF-I or IGF-II for 72 h. Floating and adherent cells were lysed in 10 mM Tris pH 8.0, 1 mM EDTA, 0.1 M NaCl, and 1% Triton X-100. Supernatants were collected after centrifuging at 8,000 rpm for 5 min, extracted once with phenol/chloroform and precipitated with an equal volume of isopropanol. DNA/RNA pellets were resuspended in 20 μl of TE buffer containing 50 μg/ml RNase A and incubated at 37°C for 1 h. DNA fragments were separated by 2.5% agarose gel electrophoresis and visualized with ethidium bromide staining.

Annexin V-FITC/PI (Propidium Iodide) FACS

Apoptotic cells were differentiated from viable or necrotic cells with Annexin-V-FITC and PI staining. After 72 h of treatment with IGFs (100 ng/ml), suspension cells were collected, and adherent cells were detached with 0.5 mM EDTA. Both floating and adherent cells were combined and resuspended in 200 μl Annexin V-binding buffer (10 mM HEPES pH 7.4, 140 mM NaCl and 2.5 mM CaCl, 2.5×10^6 cells/ml), then incubated with annexin V-FITC conjugate (1 μl, Sigma) for 15 min at room temperature in the dark. After the reaction, the cells were washed once with Annexin V-binding buffer and resuspended in Annexin V-binding buffer (300 μl) containing 1 μl of PI solution (50 μg/ml, Sigma). Cells were analyzed by flow cytometry using a FACScan (Becton Dickinson). Data were analyzed by CELLQuest software. Cells were categorized into four populations; vital cells (annexin V^-/PI^-, bottom left quadrant), early apoptotic cells (annexin V^+/PI^-, bottom right quadrant), late apoptotic cells and/or necrotic cells (annexin V^+/PI^+, top right quadrant), and necrotic cells (PI^+, top left quadrant).

Western Blot Analysis

Expression of apoptosis-related proteins of the Bcl-2 family, pro-caspase-3, and signaling molecules were examined by Western blot analysis. After treatment with IGFs (100 ng/ml) for 3 days, both floating and adherent cells were washed with PBS and lysed in 10 mM Tris pH 8.0, 0.1 M NaCl, 1 mM EDTA, and 1% Triton X-100. The cell mixtures were vortexed for 10 s and centrifuged at 13,000 rpm for 5 min. Supernatants were collected and protein concentration was determined by Bio-Rad protein assay. About 50–60 μg of protein per lane was separated by SDS–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane (Osmonics). Blots were blocked with 5% skim milk powder in Tris-buffered saline-0.1% Tween-20 (TBS/T) for 1 h, then incubated with primary antibodies Bcl-2 (1:1,000), Bad (1:1,000), pro-caspase-3 (1:1,000), phospho-Akt (1:800), total Akt (1:1000), phospho-Erk1/2 (1:2000), or total Erk 1/2 (1:2000) in TBS/T buffer containing 5% BSA at 4°C overnight. After washing with TBS/T buffer three times, membranes were incubated with anti-rabbit IgG conjugated with horseradish peroxidase (1:2,000) in TBS/T buffer containing 5% BSA for 1 h at room temperature. They were then washed three times with TBS/T. Target proteins were detected using Super-Signal West Pico Chemiluminesent reagent (Pierce). To confirm equal sample loading, a parallel Western blot was performed using an anti-pan-actin antibody. Band intensities were quantitated using ImageJ software (Rasband, WS. ImageJ, National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij/) and corrected for loading using actin content.

Statistical Analysis

Results are shown as mean ± SEM of three to six independent experiments. Cell Death ELISA data were logarithmically transformed to stabilize variance. Data were analyzed by ANOVA, and correction for multiple post hoc comparisons was performed using Fisher's PLSD test.

RESULTS

IGFs Induce LIM 1215 Cell Proliferation

We previously demonstrated that IGFs induce LIM 1215 cell proliferation [Leng et al., 2001b]. To confirm these results, we treated LIM 1215 cells with 100 ng/ml IGF-I or IGF-II, and live cell numbers were assessed using the MTT assay. As shown in Figure 1, treatment with IGF-I or IGF-II (100 ng/ml, 72 h) resulted in a significant increase in cell number to $184 \pm 10\%$ (IGF-I) and $151 \pm 9\%$ (IGF-II) of control, respectively ($P < 0.05$).

IGFs Induce Dose-Dependent Cell Death in LIM 1215 Cells

IGF treatment of LIM 1215 cells not only induced proliferation, but also resulted in increased floating cell numbers in the culture medium. We therefore investigated cell viability by Trypan blue exclusion using a hemocytometer. As shown in Figure 2, dose-dependent cell death was observed with IGF-I after 72 h treatment ($P = 0.0004$). Compared to untreated cells ($5.8 \pm 1.2\%$), a maximal $28.4 \pm 3.7\%$ cell death was observed after 100 ng/ml IGF-I ($P < 0.001$). IGF-II similarly induced cell death (results not shown).

IGFs Induce Apoptosis in LIM 1215 Cells but not in RD Cells

IGF-dependent apoptosis of LIM 1215 cells was also determined using a Cell Death Detection ELISA. As shown in Figure 3, a dose-dependent increase in apoptosis was observed ($P = 0.042$). IGF-I (100 ng/ml, 72 h) increased apoptosis 3.7-fold compared to control ($P < 0.05$). As we reported previously [Gallicchio et al., 2001], IGFs had no effect on RD cell apoptosis (results not shown).

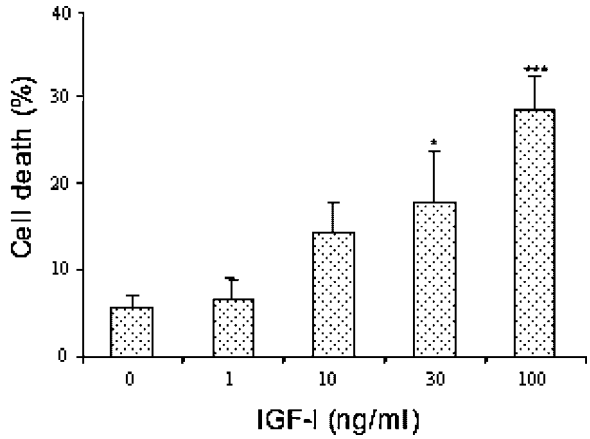

Fig. 2. IGF-1 increases LIM 1215 cell death. Adherent and non-adherent cells were pooled after 72 h treatment with IGF-I. Cell death was determined by Trypan blue exclusion assay. Results are shown mean ± SE of at least three independent experiments. $^{*}P < 0.05$ versus control, $^{***}P < 0.001$ versus control.

IGFs Induce DNA Fragmentation in LIM 1215 Cells but not in RD Cells

One of the biochemical features of apoptosis is fragmentation of genomic DNA, an irreversible event that inextricably leads to cell death. To further investigate whether the observed effects of IGFs on LIM 1215 cells were due to apoptosis or necrosis, we prepared cytoplasmic

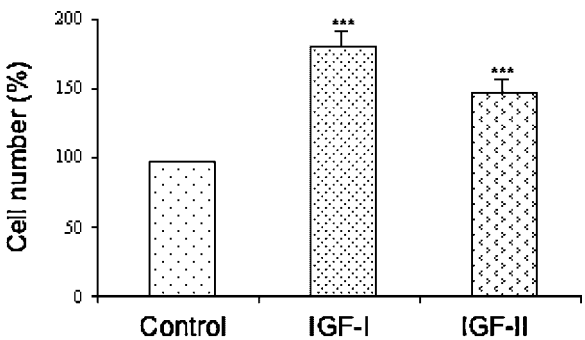

Fig. 1. IGFs increase LIM 1215 colon cancer cell number. Cells were treated with 100 ng/ml of IGF-I or IGF-II for 72 h, and viable cell number determined by the MTT assay as detailed in Materials and Methods. Control was defined as 100%. Results are expressed as mean ± SE of six independent experiments. $^{***}P < 0.001$ versus control.

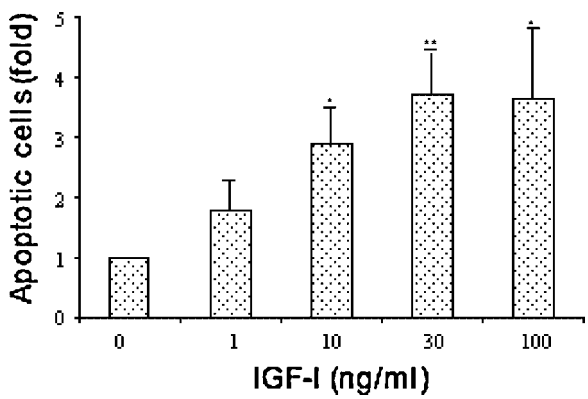

Fig. 3. IGF-I induces apoptosis in LIM 1215 colon cancer cells. Cytoplasmic DNA–histone complexes were measured by ELISA. The cells were treated with IGF-I for 72 h. Experiments were performed in triplicate and results are shown mean ± SE of at least three independent experiments. $^{*}P < 0.05$ versus control, $^{**}P < 0.01$ versus control.

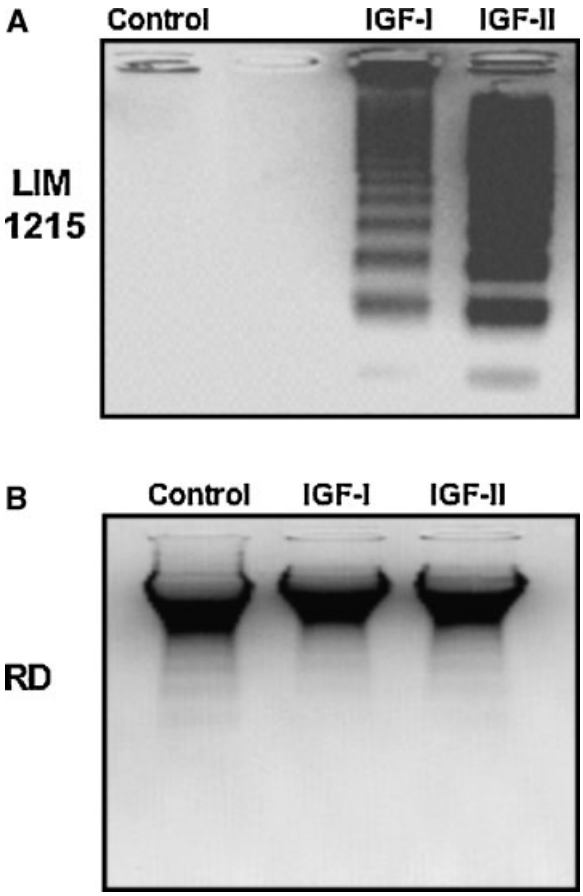

Fig. 4. IGFs induce of DNA fragmentation in (**A**) LIM 1215 colon cancer but not (**B**) RD rhabdomyosarcoma cells. Cells were treated with 100 ng/ml of IGF-I or IGF-II for 72 h, DNA was extracted from the adherent and non-adherent cell population as described in Materials and Methods and separated by 2.5% agarose gel electrophoresis. Results of a typical experiment are shown. Experiments were repeated three times.

DNA after treating cells with IGF-I or IGF-II (100 ng/ml, 72 h). Adherent and floating cells were then pooled, and cytoplasmic DNA was isolated and subjected to 2.5% agarose gel electrophoresis. LIM 1215 cells treated with IGFs but not control cells displayed DNA laddering (Fig. 4A). In contrast, no laddering was observed after IGF treatment of RD cells (Fig. 4B). The effect of IGF-induced apoptosis in LIM 1215 cells was not due to overconfluence, since DNA fragmentation was still observed with cells at 20% confluence (results not shown). These results confirm that the induction of apoptosis by IGFs is cell-type specific.

IGFs Induce LIM Apoptosis Through the IGF-I Receptor

We next investigated whether the IGF-IR or IGF-II/man6P R mediates IGF-dependent apoptosis in LIM 1215 cells. Two IGF-II analogs, [L^{27}]IGF-II and [R^{54}, R^{55}]IGF-II, were utilized. [L^{27}]IGF-II binds to the IGF-IR with 1,000-fold reduced affinity, but binds to the IGF-IIR/man6P R with normal affinity [Sakano et al., 1991]. [$R^{54}R^{55}$]IGF-II binds to the IGF-IR with normal affinity, but does not bind the IGF-IIR/man6P R [Sakano et al., 1991]. Figure 5 shows a clear apoptotic response to IGF-I, IGF-II, and [R^{54}, R^{55}]IGF-II in LIM 1215 cells (cells are rounded and floating). In contrast, [L^{27}]IGF-II had little effect (similar to control cells that are adherent and healthy). These results indicate that the IGF-IR is the primary receptor involved in IGF-dependent apoptosis in LIM 1215 cells (Fig. 5).

IGFs Increase Annexin V-FITC Staining on LIM 1215 Cells

Translocation of phosphatidylserine (PS) from the inner leaflet of the plasma membrane to the outer leaflet of the plasma membrane is another feature of apoptosis that can be detected by annexin V-FITC binding. To further confirm whether the IGF-induced LIM cell death was due to apoptosis, annexin V-FITC/PI flow cytometry was used to determine apoptotic and viable cells. Figure 6 shows representive results. Incubation of LIM 1215 cells with IGF-I or IGF-II (100 ng/ml, 72 h) increased the proportion of early apoptotic cells (Annexin V^+/PI^-) from 14% to ~25%. The results are consistent with those obtained in the Cell Death Detection ELISA and DNA fragmentation assays. Treatment of LIM 1215 cells with [R^{54}, R^{55}]IGF-II (100 ng/ml, 72 h) but not [L^{27}]IGF-II also increased apoptosis (Fig. 6), further confirming that IGF-induced apoptosis is mediated by the IGF-I receptor.

IGF-I Activates the Akt/PKB Signaling Pathways in LIM 1215 cells

IGFs induce cell survival signals by activating PI3K/Akt and Erk1/2 signaling pathways [O'Connor, 2003]. Constitutive activation of these pathways provides signals to protect cancer cells from apoptosis. Initial time-course experiments showed that Akt phosphorylation was maximal after 5 min of IGF stimulation in LIM 1215 cells (results not shown), which is similar to that observed in U9 colon carcinoma cells [Ewton et al., 2002]. We next compared phosphorylation of Akt and Erk1/2 after IGF-I

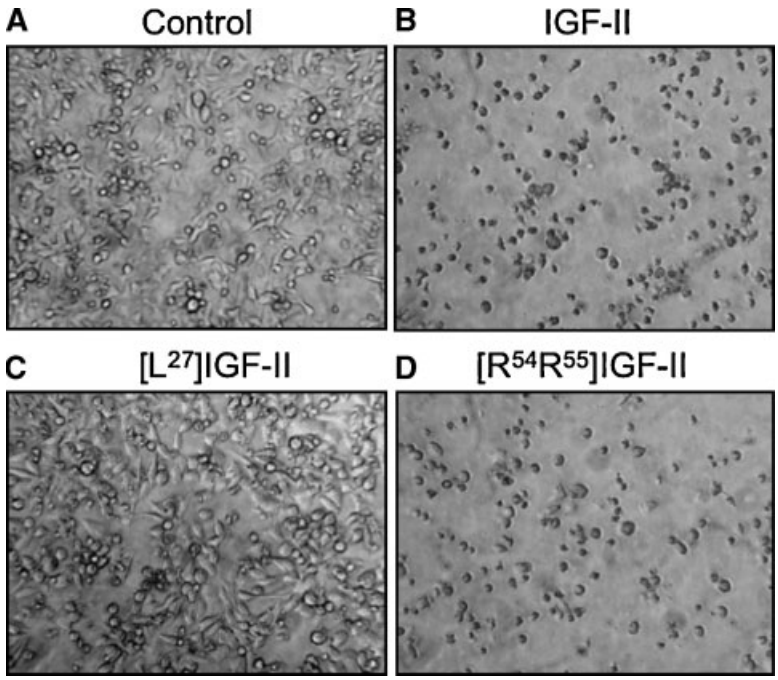

Fig. 5. Effect of IGF-II and IGF-II analogs on the LIM 1215 colon cancer cells. Cells were treated with vehicle (**A**) or 100 ng/ml IGF-II (**B**), [L^{27}]IGF-II (**C**), or [R^{54},R^{55}]IGF-II (**D**) for 6 days. After incubation, cells were photographed. Dead cells are rounded and suspended.

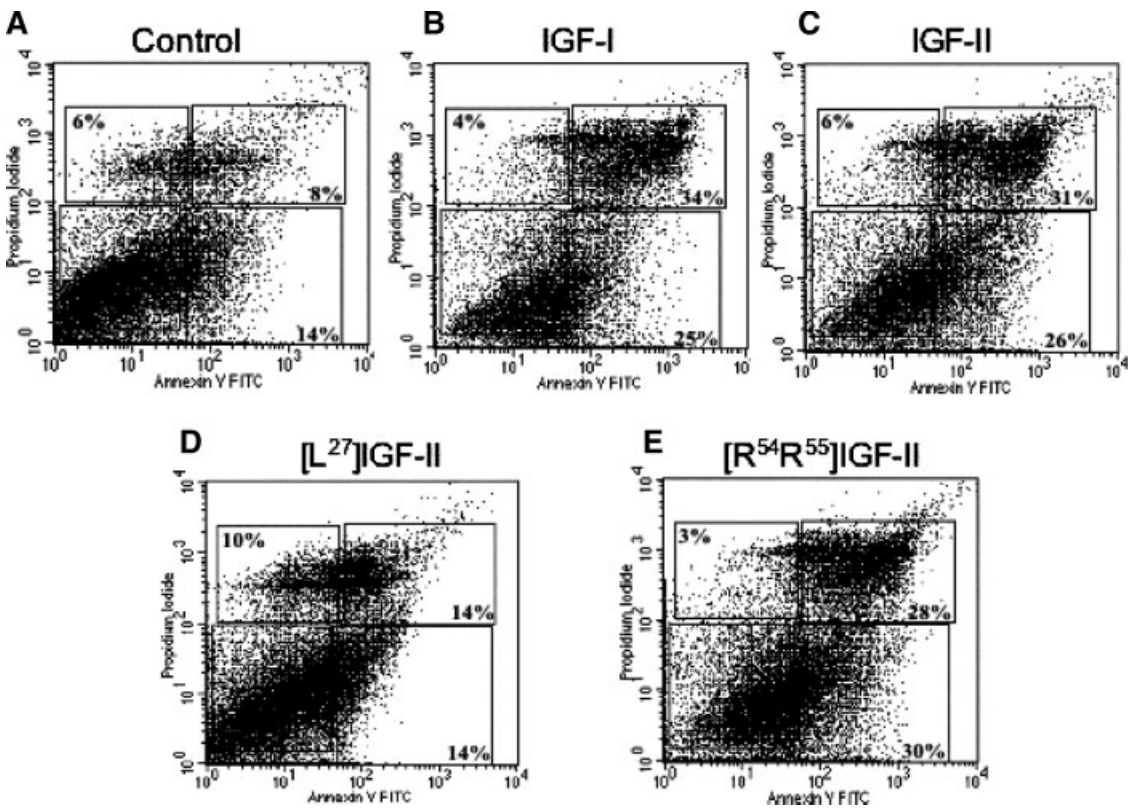

Fig. 6. Induction of apoptotic and necrotic cell death by IGFs and IGF-II analogs as revealed by the annexin V-FITC binding/PI exclusion assay. Cells were treated with vehicle (**A**), or 100 ng/ml of IGF-I (**B**), IGF-II (**C**), [L^{27}]IGF-II (**D**), or [R^{54},R^{55}]IGF-II (**E**). 10,000 cells per treatment condition were analyzed by flow cytometry. The figure represents one of three independent experiments.

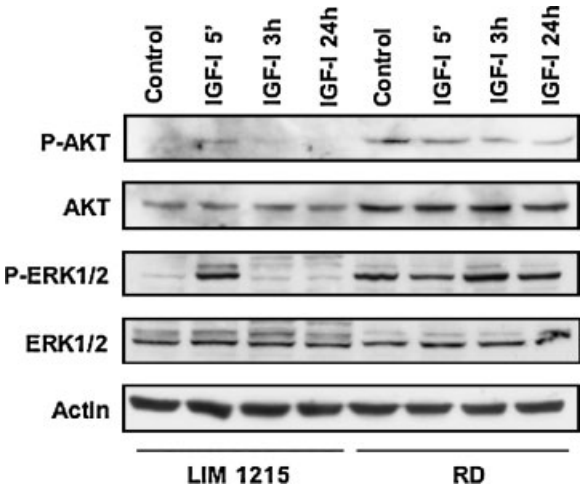

Fig. 7. Effect of IGFs on Akt and Erk1/2 signaling pathways. Western blots were carried out using antibodies against phospho-Akt, total Akt, phospho-ERK1/2, and total ERK1/2. Fifty to sixty micrograms of total cell lysates were used for each lane.

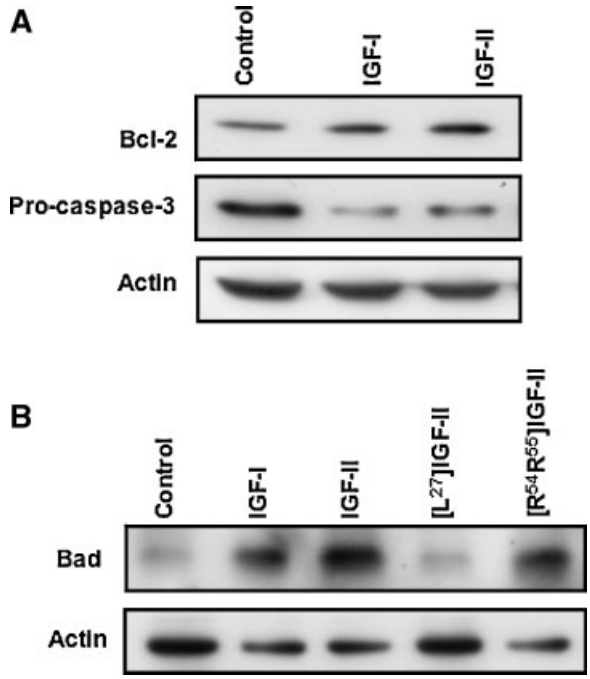

Fig. 8. IGFs (**A**) downregulate pro-caspase-3 and (**B**) upregulate Bad expression in LIM cells. After IGF-treatment, cell lysates were prepared as described in Material and Methods. Fifty to sixty micrograms of total cell lysates were used for Western blot analyses with antibodies directed against pro-caspase-3, Bcl-2, Bad, and pan-actin.

treatment in LIM 1215 and RD cells. The results in Figure 7 demonstrated no basal phosphorylation of Akt in LIM 1215 cells. Treatment with IGF-I increased phosphorylation of Akt in LIM 1215 cells, which reached a maximum at 5 min and then decreased afterwards (Fig. 7). In contrast, Akt was constitutively phosphorylated in RD cells, and IGF stimulation had no further effect. Furthermore, levels of total Akt in RD cells were 1.8-fold those in LIM 1215 cells (Fig. 7). Erk1/2 was also constitutively activated in RD cells, whereas transient phosphorylation following IGF stimulation, peaking at 5.5-fold higher than control after 5 min, was observed in LIM 1215 cells. These findings demonstrate that exogenous IGF-I transiently stimulates both the PI3K/Akt and ERK1/2 pathways in LIM 1215 colon cancer cells, while these pathways are constitutively active and non-responsive to exogenous IGFs in RD cells.

Molecular Mechanisms of IGF-Induced Apoptosis

Caspase-3 is an important mediator of mammalian cell apoptosis. Consequently, we examined the proteolytic processing of procaspase-3 by Western blot analysis. As shown in Figure 8A, treatment with IGF-I and -II for 72 h decreased procaspase-3 levels by 54% and 32%, respectively, suggesting that caspase-3 is activated by IGFs and that IGF-induced apoptosis is caspase dependent.

We next investigated whether the apoptotic response observed in LIM 1215 cells after treatment with IGFs is mediated through the Bcl family. Expression levels of pro- and anti-apoptotic Bcl-2 family members in LIM 1215 cells were determined by Western blot analysis. IGF treatment had little effect on the expression of anti-apoptotic Bcl-2 (Fig. 8A), but increased levels of pro-apoptotic Bad (Fig. 8B) four-to sixfold. [R^{54}, R^{55}]IGF-II similarly increased Bad expression sixfold, but [L^{27}]IGF-II had no effect, suggesting that this effect was mediated by the IGF-I receptor (Fig. 8B).

DISCUSSION

The present study has revealed three key findings: (a) IGFs not only induce proliferation, but also result in apoptosis in LIM 1215 colon cancer cells. The apoptotic effects of IGFs were demonstrated by increased cytoplasmic DNA–histone complexes, PS externalization, and DNA fragmentation. (b) IGF-induced apoptosis in LIM 1215 cells is mediated through the IGF-IR, as [L^{27}]IGF-II, which binds to this receptor

with 1,000-fold lower affinity, had no effect on apoptosis. (c) IGFs increased levels of the pro-apoptotic protein Bad in LIM 1215 cells. Excess Bad may translocate to the mitochondria where it forms oligomers with the anti-apoptotic proteins Bcl-X_L/Bcl-2 and initiates apoptotic signaling.

We have demonstrated that IGFs dose-dependently induce apoptosis in LIM 1215 colon cancer cells. Although a large number of studies have shown that IGFs induce cell proliferation and survival, only a few have suggested that IGFs may also induce apoptosis. The effect has been seen in normal rat liver hepatic stellate cells and osteoblasts [Gronowicz et al., 2004; Saile et al., 2004] as well as in osteosarcoma and Wilms' tumor cells [Granerus et al., 2001; Raile et al., 2003]. Therefore, the dogma that growth factors are survival factors is not unconditional. Another example of this is insulin, which can activate caspase-3 and induce apoptosis of myeloma cells [Godbout et al., 1999].

It has not previously been clear whether IGF-induced apoptosis occurs through one of the IGF receptors or through another mechanism. Studies using IGF-IR antibodies have produced contradictory results. In osteoblasts, the addition of an IGF-IR antibody had no effect on IGF-II-induced apoptosis [Gronowicz et al., 2004], whereas an IGF-IR antibody actually increased IGF-induced apoptosis in human MG63 osteosarcoma cells [Raile et al., 2003]. In contrast, an IGF-IR antibody abrogated IGF-induced cell death in Wilm's tumor cells [Granerus et al., 2001]. We used an alternative approach using IGF-II analogs with differential binding affinity for IGF receptors [Sakano et al., 1991] to show that the IGF-IR and not the IGF-II/man6P R mediates the apoptotic effects of IGFs in LIM 1215 cells. Another possibility is that the IGF effect was mediated through the IR or hybrid receptors. However, the binding affinity of IGF-I for the IR is >100 nM [Frasca et al., 1999; Denley et al., 2004], and we observed a significant effect at a concentration of 1.3 nM (10 ng/ml), making this highly unlikely. Hybrid receptors are expressed in a variety of human cancers, including colon cancer [Garrouste et al., 1997]. The binding affinities of IGFs for the IGF-IR and hybrid receptors are similar [Soos et al., 1993], so we cannot absolutely rule out the possibility that IGFs-induced apoptosis in LIM 1215 cells through these receptors. To further examine this possibility, a specific blocking antibody for hybrid receptors would be required.

The IGF-IR is also responsible for IGF-induced cell proliferation and survival [O'Connor, 2003]. In the present study, IGFs increased cell number as measured by MTT assay, so that the proliferative response must be greater than the cell death response. Since it is highly likely that proliferation was mediated by the IGF-IR, an important question to emerge from our study is how this receptor might simultaneously mediate seemingly opposite effects.

IGF-I receptor signaling involves activation of the PI3 kinase/Akt pathway. The PI3 kinase/Akt signaling pathway is a key pathway in the regulation of cell survival [Yao and Cooper, 1995], due to its ability to regulate multiple downstream apoptosis-related targets [Song et al., 2005]. Akt prevents apoptosis by directly phosphorylating and inhibiting proteins that mediate apoptosis including the forkhead transcription factors, p53 family members, pro-apoptotic Bad, and caspase-9. Akt also promotes cell survival by indirectly activating NF-κB, which upregulates some survival genes. Therefore, reducing Akt signaling might induce apoptotsis by many mechanisms. In contrast, constitutive activation or aberrant upregulation of Akt is a common feature in many human tumors [Nicholson and Anderson, 2002]. Not only was Akt activation lower in LIM 1215 cells when compared to RD cells, but also overall expression levels were lower in both basal and stimulated cells. It may be that Akt signaling is partially compromised in LIM 1215 cells, thereby sensitizing them to apoptosis. Conversely, the constitutively activated Akt observed in RD cells may provide resistance to apoptosis. Constitutive expression of active Akt has been reported to prevent apoptotic induction in prostate carcinoma [Chen et al., 2001; Panka et al., 2001] and to facilitate long-term growth factor independent cell survival [Kohn et al., 1996].

The MAP kinase Erk1/2 pathway also plays a role in cell survival. Phosphorylation of Erk1/2 leads to modulation of the activity of several downstream proteins, including protein kinases and transcription factors that regulate genes that increase proliferation and survival [Lewis et al., 1998]. Similarly to Akt activation, we found that IGFs stimulated transient Erk1/2 phosphorylation in LIM 1215 cells, but Erk1/2 was constitutively activated in RD cells.

Constitutive activation of Erk1/2 has been found in a number of cancers [Scholl et al., 2005], including RD cells [Mauro et al., 2002].

Apoptosis occurs via two activation pathways; the mitochondrial pathway and the death receptor pathway. Bcl-2 family proteins regulate cell apoptosis through the first pathway [Burlacu, 2003]. The family consists of a number of pro- and anti-apoptotic proteins that form homo- or heterodimers. The balance between pro- and anti-apoptotic dimers acts as a molecular "switch," regulating the cell's fate. The pro-apoptotic protein Bad is regulated by phosphorylation, which modulates its protein–protein interactions and subcellular localization. Akt phosphorylates Ser136 of Bad, thereby inhibiting its pro-apoptotic role [Zha et al., 1996; Ayllon et al., 2002], whereas Erk 1/2 modulates apoptosis by phosphorylating Ser112 [Scheid et al., 1999; Jin et al., 2004]. Colon cancer cells express several Bcl-2 family proteins, including Bad [Rice et al., 2003; Erler et al., 2004] and Bcl-2 [Kitamura et al., 2000; Lin et al., 2005]. In our study, the induction of apoptosis by IGFs in LIM 1215 colon cancer cells was accompanied by increased expression of Bad. However, we did not detect phosphorylated Bad in LIM 1215 cells using two different antibodies to Ser112 and Ser136 (results not shown). This may be due to inadequate sensitivity of the antibodies. Another possibility is that compromised Akt and/or Erk 1/2 signaling in LIM 1215 cells may impair Bad phosphorylation. The increase in Bad expression may then result in increased Bad/Bcl-xL/Bcl-2 oligomer formation and initiation of apoptosis. In contrast, IGF-induced apoptosis in LIM 1215 cells was not accompanied by changes in Bcl-2 levels. These results contrast with those in osteoblasts where IGF-II-induced apoptosis was associated with downregulation of Bcl-2 [Gronowicz et al., 2004], and hepatic stellate cells where IGF-I-induced apoptosis was accompanied by upregulation of Bcl-2 and downregulation of Bax [Saile et al., 2004]. IGF-induced apoptosis through Bcl-2 family members therefore appears to be cell-type specific. Further studies are necessary to define the role played by Bad and other members of Bcl-2 family in IGF-induced LIM 1215 apoptosis.

The IGF-induced increase in Bad expression suggests that IGFs induced apoptosis via the mitochondrial pathway. Recent studied have shown that IGF-I may also enhance apoptosis initiated by death receptors [Niesler et al., 2000; Foulstone et al., 2001; Remacle-Bonnet et al., 2005]. Colon cancer cells frequently co-express Fas and FasL [O'Connor, 2003] so the death receptor pathway may also be involved in IGF-induced LIM 1215 colon cancer cell apoptosis. It is also possible that cross-communication exists between the two apoptotic pathways [Zha et al., 2000; Jin and El-Deiry, 2005].

In summary, we have shown that IGFs simultaneously promote proliferation and apoptosis of LIM 1215 cells, which would lead to enhanced cell turnover. Since colorectal cancers with high turnover have a markedly poorer prognosis than those with low turnover [Rupa et al., 2003], this study provides further evidence for a role of the IGF system in progression of this cancer. In this context, it is perhaps noteworthy that LIM 1215 cells were derived from the colon cancer of a young man with aggressive metastatic disease [Whitehead et al., 1985]. An improved understanding of the simultaneous induction of proliferation and apoptosis by IGFs may allow the identification of new target molecules to improve colon cancer therapy.

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