

# Insulin-like Growth Factor Binding Protein-3 Induces Apoptosis in MCF7 Breast Cancer Cells

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**Insulin-like growth factors (IGFs) are known to have potent antiapoptotic activity. The antiestrogen ICI 182,780 (ICI) is a potent inhibitor of MCF7 human breast cancer cell growth and has recently been reported to act as an antiproliferative agent in part via upregulation of expression of insulin-like growth factor binding proteins (IGFBPs) -3 and -5, which attenuate the bioactivity of IGFs in many experimental systems. We show here that ICI and IGFBP-3 induce apoptosis in MCF7 cells. Treatment of MCF7 cells with 10 nM ICI or 36 nM recombinant human IGFBP-3 for 72 hours increased apoptosis ~3.5-fold relative to control as quantitated by a cell death ELISA which measures DNA fragmentation. Long R<sup>3</sup> IGF-I, an IGF-I analogue with greatly reduced affinity for IGFBPs yet similar affinity for IGF-I receptors, was a more potent inhibitor of IGFBP-3-induced and ICI-induced apoptosis than IGF-I. These results suggest that IGFBP-3 enhances apoptosis by reducing bioavailability of ligands for the IGF-I receptor and suggest that modulation of IGFBP-3 expression by ICI contributes to apoptosis induced by this compound. More generally, the data suggest that IGFBPs are regulators of apoptosis.**

Apoptosis is a physiological phenomenon involved in morphogenesis and tissue renewal (1). Transformed cells have been shown to have defects in activation of the apoptotic pathway, suggesting that derangements of apoptosis are involved in the pathophysiology of cancer. Many antineoplastic compounds induce tumor regression through their ability to activate apoptotic pathways (2,3). Recent studies ((4,5) reviewed in (6)) have demonstrated that activation of the insulin-like growth factor I (IGF-I) receptor by IGF-I or IGF-II pro-

tects cells from apoptosis, and have implicated the serine-threonine kinase Akt as a key downstream regulator of the antiapoptotic effect of IGF-IR agonists (7). However, little attention has been given to date to the role of insulin-like growth factor binding proteins (IGFBPs) (8,9) in regulating apoptosis.

The pure antiestrogen ICI 182,780 (ICI) is a potent inhibitor of breast cancer cell growth (10). We have previously reported that the antiproliferative effects of ICI on MCF7 cells are associated with increased expression and secretion of IGFBP-3 (11).

## MATERIALS AND METHODS

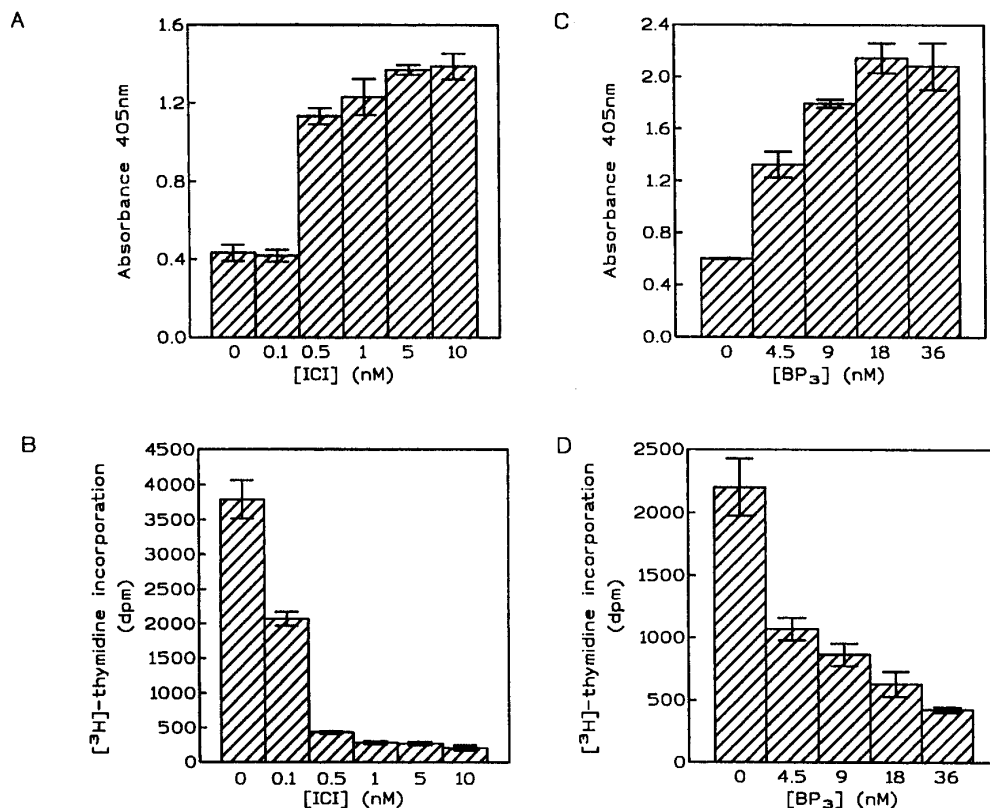
*Cell culture and growth experiments.* MCF7 cells are routinely maintained in 10% fetal calf serum (FCS) alpha modified minimal essential media ( $\alpha$ MEM) supplemented with 5  $\mu$ g/ml insulin. They were plated in 5% FCS phenol red free (PF) $\alpha$ MEM at a density of 25,000 cells/well in 24-well plates. Following attachment for 24 hours, cells were washed twice in 0.5% FCS PF- $\alpha$ MEM and incubated in this media for 3 hours. Exposure to compounds under study was for 72 hours in 0.5% FCS PF- $\alpha$ MEM. Proliferation of cells was quantitated by [<sup>3</sup>H]-thymidine incorporation as previously described (12).

*Quantitation of apoptosis.* Apoptotic cell death was measured using a cell death ELISA (Boehringer Mannheim) which measures cytoplasmic histone-bound DNA generated during apoptotic DNA fragmentation and not free histone or DNA that could be released during nonapoptotic cell death. This method has been shown to be reliable for quantitation of apoptotic cell death in MCF7 cells (13,14). MCF7 cells plated at a density of 25,000 cells/well and treated for 72 hours exactly as described for growth experiments were harvested by scraping in cold phosphate buffered saline. Parallel plates treated identically were used for thymidine incorporation and for determination of cell number. Cytoplasmic cell extracts were prepared according to the manufacturer's protocol and were equalized on the basis of cell number. Samples from triplicate wells were run in duplicate on the ELISA.

## RESULTS AND DISCUSSION

It has previously been shown that the antiestrogen ICI 182,780 induces apoptosis in MCF7 cells as demonstrated by cleavage of DNA to 300 and 50 kbp fragments (15), although there is controversy surrounding

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**FIG. 1.** Induction of apoptosis in MCF7 cells in response to the antiestrogen ICI 182,780 and IGFBP-3. MCF7 cells plated at a density of 25,000 cells/well were treated for 72 hours in 0.5% FCS. Apoptosis induced by various doses of ICI (A) and rhIGFBP-3 (C) was quantitated by cell death ELISA where absorbance at 405 nm is a direct measure of DNA fragmentation. Plates treated in parallel were used to measure [<sup>3</sup>H]-thymidine incorporation (B and D). All experiments were repeated at least three times. The results from one representative experiment are shown expressed as mean ± SE of triplicate wells.

the use of DNA laddering to detect apoptosis in MCF7 cells in response to antiestrogens (16,17). The cell death ELISA used to quantitate apoptosis in our experiments has been shown to detect apoptosis in MCF7 cells in response to the antiestrogens tamoxifen and toremifene (13) as well as other agents (14). Our results support the view that antiestrogens induce apoptosis in MCF7 cells. We observed a dose-dependent increase in apoptosis following exposure of MCF7 cells to ICI for 72 hours (Figure 1A). A 3.2-fold increase in apoptosis was observed in cells treated with 10nM ICI compared to vehicle-treated controls. This is correlated with the well known potent inhibitory effects of ICI on MCF7 DNA synthesis (Figure 1B). Under the same experimental conditions, treatment of MCF7 cells with recombinant human IGFBP-3 (rhIGFBP-3) for 72 hours increased apoptotic cell death in a dose-dependent manner (Figure 1C) and inhibited [<sup>3</sup>H]-thymidine incorporation (Figure 1D). Apoptosis increased 3.5-fold in the presence of 36 nM rhIGFBP-3 relative to control.

We have previously shown that the potent inhibitory effects of ICI on MCF7 cell growth are associated with

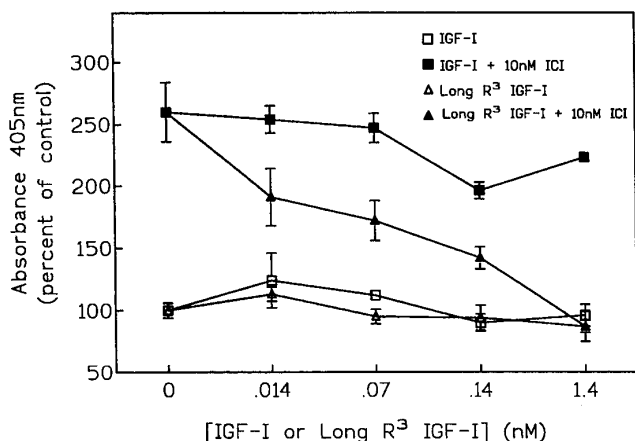
increased secretion of IGFBP-3 by the cells (11). It is therefore possible that induction of apoptosis by ICI may be related to secretion of IGFBP-3. In order to determine if ICI-induced IGFBP accumulation is functionally related to its apoptosis-inducing actions, we carried out experiments with long R<sup>3</sup> IGF-I (Gropep, Adelaide, Australia), an IGF-I analogue with greatly reduced affinity for IGF binding proteins, yet similar affinity for IGF-I receptors (18). Experiments were carried out in the presence of 0.5% FCS, as preliminary studies showed that this condition was associated with a level of apoptosis in the detection range of our assay. As shown in figure 2, baseline levels of apoptosis in the absence of either IGF-I or long R<sup>3</sup> IGF-I are more than doubled in the presence of 10nM ICI. The relatively low levels of apoptosis seen in the absence of ICI are minimally effected by addition of either IGF-I or long R<sup>3</sup> IGF-I to the media. Autocrine expression of IGF-I and IGF-II by MCF7 cells could not be detected in this system (data not shown). Furthermore, the similar results obtained with IGF-I or long R<sup>3</sup> IGF-I under these conditions indicate that the IGFBPs present in the media (from serum or autocrine production) have a negli-

gible effect on apoptosis in our experimental system in the absence of the antiestrogen.

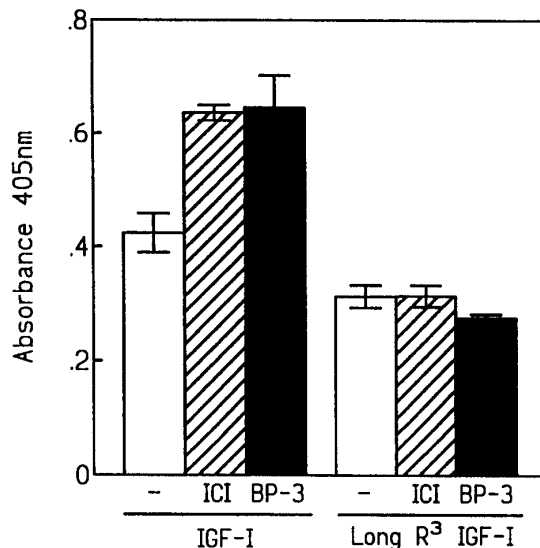
On the other hand, long R<sup>3</sup> IGF-I was a more potent inhibitor of ICI-induced apoptosis than IGF-I. 1.4 nM IGF-I reduced the apoptosis induced by 10nM ICI by 40% (Mann Whitney U-test,  $p < 0.05$ ), while the same concentration of long R<sup>3</sup> IGF-I completely abolished ICI-induced apoptosis (Figure 2). The antiapoptotic activity of IGF-I in our system extends prior work (4,19) showing attenuation of apoptosis induced by agents such as etoposide and tumor necrosis factor to antiestrogen-induced apoptosis. Furthermore, the differences between the antiapoptotic effect of IGF-I and long R<sup>3</sup> IGF-I in our experimental system suggests that the recently described induction of IGFBP-3 expression by ICI (11) contributes to the apoptotic action of the antiestrogen. The data are consistent with the view that the apoptotic action of ICI is related to competition between ICI-induced IGFBP-3 and the IGF-IR for IGF-I.

In co-incubation experiments, both 36 nM rhIGFBP-3 and 10nM ICI significantly increased the levels of apoptosis seen in the presence of 1.4 nM IGF-I (Mann Whitney U-test,  $p < 0.01$ ) (Figure 3). However, in the presence of 1.4 nM long R<sup>3</sup> IGF-I, ICI and rhIGFBP-3 had no influence on apoptosis. These results suggest a functional role for IGFBPs in the induction of apoptosis by ICI.

The IGF-IR plays a key role in regulating programmed cell death and has been suggested to promote cell survival by directly inhibiting apoptosis (4,5). We previously reported that ICI downregulates IGF-IR



**FIG. 2.** Effects of IGF-I and Long R<sup>3</sup> IGF-I on apoptosis induced by ICI. MCF7 cells were plated as described in the legend to figure 1 and treated for 72 hours in 0.5% FCS with various doses of IGF-I (open squares) or long R<sup>3</sup> IGF-I (open triangles) alone or in the presence of 10 nM ICI (filled squares and filled triangles, respectively). Cytoplasmic extracts equalized on the basis of cell number were run on the ELISA as described under Materials and Methods. The experiment was repeated three times and the results from one experiment are shown expressed as mean  $\pm$  SE.



**FIG. 3.** Effects of IGF-I and Long R<sup>3</sup> IGF-I on protecting MCF7 cells from apoptosis induced by ICI and IGFBP-3. Cells plated at 25,000 cells/well were cultured for 72 hours in 0.5% FCS with or without 10 nM ICI or 36 nM rhIGFBP-3 in the presence of either 1.4 nM IGF-I or long R<sup>3</sup> IGF-I. Cytoplasmic extracts from cells were run on the cell death ELISA and A<sub>405</sub> was measured. Data are expressed as mean  $\pm$  SE from triplicate wells.

number in MCF7 cells (20). Although this raises the possibility that induction of apoptosis in MCF7 cells by ICI may be related in part to reduction of IGF-IR levels, the magnitude of changes in IGFBP-3 and IGFBP-5 expression in response to ICI are much greater than ICI-induced changes in IGF-IR expression (11,21).

Recent evidence has emerged that IGFBP-3 has direct growth inhibitory effects that are independent of its capacity to bind IGF-I, which are mediated by a putative IGFBP-3 receptor (22). In view of these observations, it is possible that IGFBP-3 can influence apoptotic pathways through mechanisms other than modulation of IGF bioactivity. However, the fact that rhIGFBP-3 attenuates the antiapoptotic effects of IGF-I but not of long R<sup>3</sup> IGF-I strongly suggests that in our experimental system IGFBP-3 enhances apoptosis indirectly by reducing binding of IGFs to the IGF-IR.

The observation that induction of apoptosis in the rat mammary gland during post-lactational involution is correlated with greatly increased IGFBP production (23) suggests that IGFBPs may be important physiological regulators of apoptosis in the breast. The finding that IGFBP-3 is upregulated by p53 in EB1 colon carcinoma cells (24) has led to speculation that IGFBP-3 regulates apoptosis by reduction of IGF bioactivity (25). Our data provide the first direct demonstration that IGFBP-3 induces apoptosis in MCF7 cells, and suggest that the apoptotic action of ICI is mediated, at least in part, by ICI-induced IGFBP expression. Our results are consistent with the hypothesis that in general, IGFBPs

regulate apoptosis by modulating IGF bioactivity. IGFBP-3 has recently been reported to mediate TGF $\beta$ -induced apoptosis in PC-3 prostate carcinoma cells (26). Our observations that IGFBPs contribute to anti-estrogen-induced apoptosis in breast cancer cells provide further evidence for a role of IGFBPs as regulators of apoptosis.

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