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Oestrogen-mediated suppression of tumour necrosis factor alpha-induced apoptosis in MCF-7 cells: subversion of Bcl-2 by anti-oestrogens☆

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Abstract

In oestrogen receptor (ER)-positive breast carcinoma cells, 17β-oestradiol suppresses a dose-dependent induction of cell death by tumour necrosis factor alpha (TNF). The ability of oestrogens to promote cell survival in ER-positive breast carcinoma cells is linked to a coordinate increase in Bcl-2 expression, an effect that is blocked with the pure anti-oestrogen ICI 182,780. The role of Bcl-2 in MCF-7 cell survival was confirmed by stable overexpression of Bcl-2 which resulted in suppression of apoptosis induced by doxorubicin (DOX), paclitaxel (TAX) and TNF as compared to vector-control cells. The pure anti-oestrogen ICI 182,780 in combination with TNF, DOX or TAX potentiated apoptosis in vector-transfected cells. Interestingly, pre-treatment with ICI 182,780 markedly enhanced chemotherapeutic drug- or TNF-induced apoptosis in Bcl-2 expressing cells, an effect that was correlated with ICI 182,780 induced activation of c-Jun N-terminal kinase. Our results suggest that the effects of oestrogens/anti-oestrogens on the regulation of apoptosis may involve coordinate activation of signalling events and Bcl-2 expression. © 2001 Published by Elsevier Science Ltd.

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1. Introduction

The process of apoptosis is controlled by expression or activation of numerous apoptotic regulatory proteins including caspases, mitogen activated protein kinases (MAPKs), nuclear factor-kappa B (NF-κB), and members of the Bcl-2 family [1,2]. Accumulating evidence suggests that steroid hormones regulate apoptosis in hormone-responsive tissues [3,4]. Both prostate and mammary epithelial cells undergo apoptosis upon removal of testosterone and oestrogen, respectively [4–8]. This cellular dependence upon hormones for survival and proliferation extends to neoplasms arising from these tissues, as well. The MCF-7 breast cancer cell line has been shown to form tumours in nude, ovariectomized mice only in the presence of oestrogen [7,9]. Upon removal of oestrogen or with antagonism by anti-oestrogens, these malignant cells begin to undergo

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apoptosis leading to tumour regression [7,10–12]. Additionally, we along with others, have shown that pre-treatment of MCF-7 cells with oestrogen grown in vitro suppresses apoptosis induced by tumour necrosis factor alpha (TNF) and chemotherapeutic drugs, including tamoxifen [13–16]. These studies provide evidence that oestrogens play a role in both tumourigenesis and drug resistance through the suppression of apoptosis. The addition of anti-oestrogens, such as tamoxifen or ICI 182,780, has been shown to induce apoptosis in these cells presumably through the inhibition of oestrogen receptor (ER) survival signalling [13,17–19]. Reports also demonstrate that one mechanism by which oestrogens may affect apoptosis is through the increased expression of Bcl-2, a member of a family of apoptosis-regulating proteins whose expression has been shown to suppress apoptosis of MCF-7 cells [13–16]. The addition of ICI 182,780 or tamoxifen has been shown to block the Bcl-2-inducing effect of 17β-oestradiol [14–17,19], while overexpression of Bcl-2 or Bcl-xL has been shown to suppress apoptosis induced by chemotherapeutic drugs, Fas and TNF in MCF-7 cells [14,20,21]. Additionally, Pratt et al. have shown that Bcl-2-overexpressing MCF-7 cells form tumours in nude mice that do not undergo apoptosis upon oestrogen withdrawal [9]. The survival effects of 17β-oestradiol are thought to be mediated predominantly through enhanced expression of Bcl-2; however, the possibility that other anti-apoptotic signalling pathways may be involved has not been excluded. Regulation of Bcl-2 was demonstrated to occur independently of oestrogen response elements (EREs) within the promoter region suggesting an indirect effect of oestrogen on Bcl-2 expression [22], while Perillo et al. demonstrate this effect occurred through EREs within the coding region of Bcl-2 [23].

Recently, 17β-oestradiol's anti-apoptotic effect has been shown to involve rapid stimulation of cytoplasmic signalling cascades such as Erk, a member of the MAPK family, and the well-established anti-apoptotic AKT protein [24–30]. Additionally, regulation of the c-Jun N-terminal kinase (JNK) and p38 components of the MAPK pathway have been demonstrated to be critical to the anti-apoptotic effects of 17β-oestradiol [26–36]. Recent evidence has suggested that the activation of these early signalling events particularly Erk, mediate the survival signalling effects of 17β-oestradiol [26,27,32]. Therefore, the ability of the ER to regulate biological effects involves both non-genomic cytoplasmic signalling such as the MAPK cascade in addition to target genomic increases in expression of growth factors, early immediate genes (c-Fos) and survival factors (Bcl-2) that function to promote cell proliferation and cell survival [37,38].

In juxtaposition to oestrogen signalling, treatment of cells with anti-oestrogens such as tamoxifen or ICI 182,780 can induce apoptosis in MCF-7 cells [10,11,17–19,39–42]. The ability of tamoxifen and ICI to promote apoptosis is partially mediated through the suppression of 17β-oestradiol-induced Bcl-2 expression and suppression of other 17β-oestradiol-dependent survival pathways. Additionally, anti-oestrogen suppression of 17β-oestradiol-mediated cell survival has also been demonstrated to occur through inhibition of early MAPK signalling, suggesting the combination of both gene expression and early MAPK signalling are critical in the regulation of cell survival by the ER [26,27]. However, reports demonstrating that tamoxifen and ICI 182,780 can function to alter other signalling pathways, such as suppression of protein kinase C (PKC), calmodulin kinase II (CamKII) or glucosyl ceramide synthase activity suggests ER-independent effects may also be involved [42–47].

In this report, we investigate the role of 17β-oestradiol and ICI 182,780 in the regulation of apoptosis by TNF and chemotherapeutic drugs in MCF-7 cells. We demonstrate the ability of 17β-oestradiol to suppress TNF-induced apoptosis, but ICI 182,780 abrogates this effect. Pre-treatment with ICI 182,780 enhanced TNF-induced apoptosis, suggesting a requirement for ER in maintaining cell viability. This enhanced sensitivity may be occurring through both the suppression of ER signalling with subsequent Bcl-2 expression, as well as through an undefined increase in a high molecular weight form of Bcl-2 suggestive of phosphorylation. Additionally, we demonstrate that ICI 182,780 can function to enhance TNF-induced cell death in both normal and Bcl-2 overexpressing cells. The ability of ICI to subvert the anti-apoptotic effects of Bcl-2 was correlated with an increased activation of JNK. Consistent with the involvement of JNK in suppression of Bcl-2, we also demonstrate that overexpression of a mutant of MEKK1 which constitutively activates the JNK pathway, sensitizes Bcl-2 expressing MCF-7 cells to TNF and chemotherapeutic drug induced cell death. The ability of ICI 182,780 to sensitize MCF-7 cells overexpressing Bcl-2 to apoptosis suggests that multiple signals generated by the ER function to regulate apoptosis including regulation of MAPK signalling in addition to expression of Bcl-2.

2. Materials and methods

2.1. Cell culture and reagents

MCF-7 cells (N variant) were maintained and grown in Dulbecco's modified eagle media (DMEM) supplemented with 10% fetal bovine serum (FBS), BME amino acids, MEM amino acids, L-glutamine, penicillin/streptomycin, sodium pyruvate (GibcoBRL, Gaitherburg, MD) and porcine insulin 1×10^{-10} M (Sigma Chemical Co., St. Louis, MO) under my-

coplasma-free conditions as previously described [48]. For oestrogen studies, MCF-7 cells were grown 3 days in DMEM (phenol red free) with 5% dextran-coated charcoal stripped FBS containing media (CS–FBS–DMEM) as above, but without insulin as previously described [49]. 17β-Oestradiol was obtained from Sigma. ICI 182,780 (7-α-[9-(4,4,5,5,5-pentafluoropentasulfiniyl)nonyl]estra-1,3,5(10) triene-3,17-β-diol was generously provided by Dr Alan E. Wakeling (Zeneca Pharmaceuticals, Macclesfield, UK). Ethanol (100%) was used as vehicle control in experiments as well as to dissolve both ICI 182,780 and 17β-oestradiol stock solutions. The expression vectors for the constitutively active MEKK1 (ΔMEKK; pEE-CMV-Δ362MEKK1 and pEE-CMV-empty) were generously provided by Dr Dennis Templeton (Case Western Reserve University) [50].

2.2. Stable transfection of Bcl-2

MCF-7 cells (N variant) were transfected with SFFV-Bcl-2-neo vector or with SFFV-neo (5 μg DNA per 1×10^6 cells) using lipofectamine (1 μg DNA/3 μl of lipofectamine, GibcoBRL) in 0% serum-containing OptiMEM (GibcoBRL) without supplements in T-75 flasks. After 6 h of transfection, the lipofectamine/DNA containing media was removed and replaced with 10% FBS-fortified DMEM (10%-DMEM) as described above. Cells were allowed to recover for 48 h after which the media was removed and replaced with 10%-DMEM containing 400 μg/ml G418 (Sigma Chemical Co.). Cells were grown for 15 days with media being replaced with fresh 10%-DMEM (400 μg/ml G418). Cells were split from T-75 flasks into multiple 100 cm^2 dishes at differing dilutions and allowed to adhere for 24 h in 10%-DMEM without G418. Following this, media was removed and replaced with fresh 10%-DMEM with 400 μg G418/ml every 3 days until visible colonies appeared. Individual colonies were isolated using a sterile cloning ring coated with petroleum jelly and removed with PBS–EDTA (100 μl). Individually removed colonies were transferred to 24-well plates and allowed to grow as separate clones. Clones were grown and maintained in 10%-DMEM (400 μg/ml/G418) until 2×10^6 cell could be used for Western blot analysis for Bcl-2 expression.

2.3. Viability assay

Viability/cell death is determined using trypan blue exclusion as previously described [48]. MCF-7 cells were plated at 5.0×10^4 cells/ml in 10 cm^2 wells in 5% CS–FBS–DMEM. The cells were allowed to adhere for 18 h before treatment with or without 17β-oestradiol (Sigma Chemical Co.) for 24 h followed by recombinant human TNF-α (10 ng/ml; R&D systems, Minneapolis, MN). Cells were then counted at 48 h post-TNF treatment for viability assay. For Bcl-2 viability studies, cells were plated in 10%-DMEM for 18 h, and the following day media was changed to 0%-DMEM followed by treatment with or without ICI 182,780 for 60 min, followed by treatment with either TNF, paclitaxel (TAX; Biomol, Plymouth Meeting, PA), or doxorubicin (DOX; ICN, Aurora, OH). Cells were harvested 24, 48 and 72 h later, and viability was measured by trypan blue exclusion. The results are represented as the number of viable cells/ml. Apoptosis is expressed as the percentage of trypan blue-stained cells in treated samples compared to control viability (100%).

2.4. DNA fragmentation analysis

Following treatment, cells were harvested for DNA as described previously [48]. Briefly, $1–2 \times 10^6$ cells were pelleted and resuspended in lysis buffer [10 mM Tris–HCl, 10 mM EDTA, 0.5% SDS (w/v) pH 7.4] to which RNAse A (100 μg/ml) was added. After incubation for 2 h at 37 °C, proteinase K (0.5 mg/ml) was added and the lysates were heated to 56 °C for 1 h. Sodium chloride (NaCl) was then added (final concentration, 1 M) and lysates were incubated overnight at 4 °C. Lysates were centrifuged at $15{,}000 \times g$ for 30 min, and nucleic acids in the supernatant were precipitated in two volumes of ethanol with 50 mM Na acetate. Isolated DNA was then separated by electrophoresis on 1.5% agarose gels for 2 h and visualized by staining with ethidium bromide.

2.5. Western blot analysis

MCF-7 cells were grown for 2 days as described above, and then 5×10^6 cells were harvested in sonicating buffer (62.5 mM Tris–HCl, pH 6.8, 4% (w/v) SDS, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, 2.5 μg/ml aprotinin, and 1 mM Na Orthovanadate) and sonicated for 30 s. Following centrifugation at $1000 \times g$ for 20 min, 50 μg of protein was resuspended in sample loading buffer (62.5 M Tris–HCl, pH 6.8, 2% (w/v) SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue), boiled for 3 min and electrophoresed on a 15% polyacrylamide gel. The proteins were transferred electrophoretically to a nitrocellulose membrane. The membrane was blocked with PBS–Tween (0.05%)-5% low fat dry milk solution at 4 °C overnight. The membrane was subsequently incubated with rabbit Bcl-2 antisera (1:4000) [48,51] (generously provided by John Reed), with monoclonal antibodies to Bcl-2 (1:1000) [48] (Pharmingen, San Diego, CA), or phospho-specific monoclonal antibodies to P-Erk1/2 (1:1000), P-JNK1/2 (1:1000), P-p38α (1:1000; New England Biolabs, Beverly, MA). Follow-

ing incubation for 2 h at room temperature, blots were washed in PBS-Tween solution and incubated with goat anti-rabbit antibodies conjugated to horseradish peroxidase (1:30,000 dilution; Oxford, Oxford, MI) or with goat anti-mouse antibodies conjugated to horseradish peroxidase (1:5000 dilution; Oxford, Oxford, MI) for 60 min at room temperature. Following four washes with PBS-Tween solution, immunoreactive proteins were detected using the ECL chemiluminescence system (Amersham, Arlington Heights, IL) and recorded by fluorography on Hyperfilm (Amersham), according to the manufacturer's instructions. Fluorograms were quantitated by image densitometry using the Molecular Analyst program for data acquisition and analysis (Bio-Rad, Hercules, CA).

3. Results

Several reports have demonstrated that oestrogens promote cell survival in oestrogen-responsive cells [13–16]. We examined the ability of 17β-oestradiol to promote cell survival in MCF-7 cells treated with TNF (Fig. 1(a)). TNF is a potent inducer of apoptosis in MCF-7 cells resulting in a decrease in viability to 59 ± 5.9, 41.9 ± 4.6 and $39.4 \pm 5.4\%$ with 0.1, 1.0 and 10 ng/ml, respectively, at 24 h after treatment. Consistent with previous results [49], pre-treatment of MCF-7 cells with 1 nM 17β-oestradiol for 24 h circumvents a dose-dependent induction of cell death by TNF (0.1–10 ng/ml) to 72.6 ± 3.1, 61.2 ± 4.3 and $60.0 \pm 2.4\%$ viability. Interestingly, 1 nM 17β-oestradiol pre-treatment for only 1 h was sufficient to partially suppress TNF-induced cell death (data not shown). No difference in viability was observed between vehicle alone and oestradiol alone. The ability of 17β-oestradiol to suppress TNF-induced apoptosis was blocked by the pre-treatment with the pure anti-oestrogen ICI 182,780 (1 μM), restoring TNF-induced cell death (Fig. 2(a)). The ability of 17β-oestradiol to suppress and ICI 182,780 to restore TNF-induced apoptosis was demonstrated using DNA fragmentation analysis (Fig. 2(b)). These studies also demonstrate that ICI 182,780 alone is capable of slightly increasing DNA-fragmentation as compared to vehicle treated control cells. The ability of 17β-oestradiol to suppress cell death has been correlated with

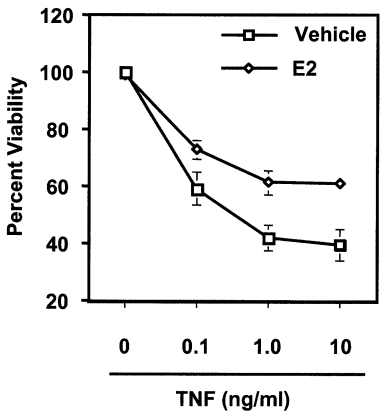

Fig. 1. Oestrogen suppression of TNF induced cell death. MCF-7 cells were plated in 5% CS-containing media for 18 h followed by treatment with or without 17β-oestradiol (1 nM) for 24 h. Cells were then treated with TNF (0.1–10 ng/ml) for 24 h and harvested for viability. Data are represented as the mean ± SEM > of six experiments.

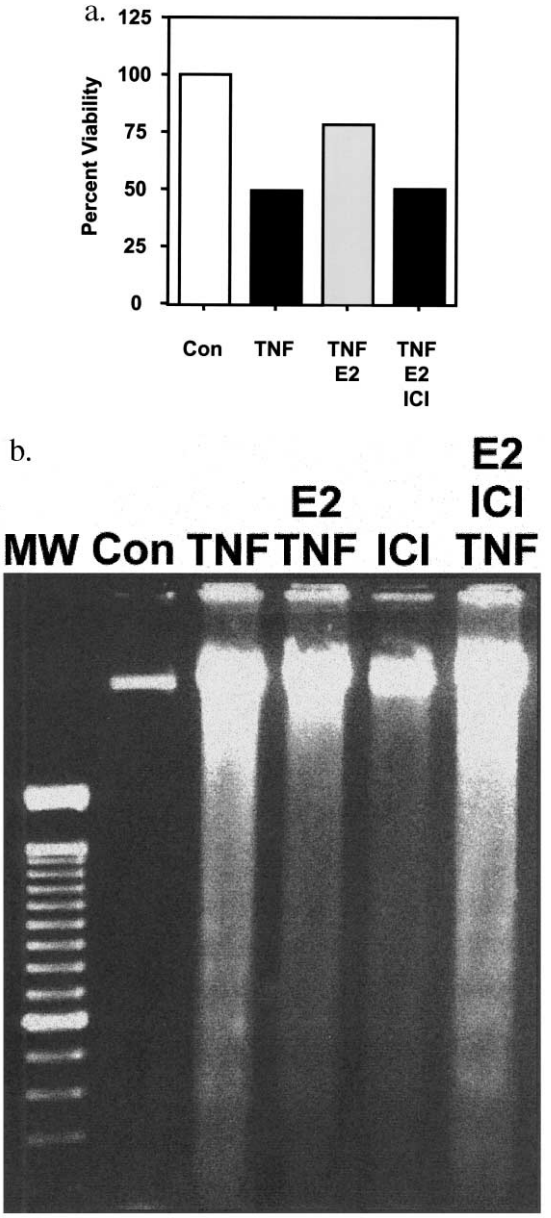

Fig. 2. Suppression of oestrogen-mediated cell survival by ICI 182,780. MCF-7 cells were plated in 5% CS media for 18 h, pre-treated with vehicle (Con) or ICI 182,780 (1 μM; ICI 182,780) for 30 min followed by vehicle or 17β-oestradiol (1 nM; E2) for 24 h. Cells were then treated with TNF (10 ng/ml) for 24 h and harvested for viability assay (a) or DNA fragmentation analysis (b). Data displayed are representative of three experiments.

increased expression of Bcl-2. Consistent with these reports, 1 nM 17β-oestradiol was shown to increase expression of Bcl-2, as compared to vehicle treated controls cells (Fig. 3). Additionally, ICI 182,780 suppressed both basal and 17β-oestradiol-stimulated induction of Bcl-2 expression. Interestingly, an increased level of the 32 kDa molecular weight Bcl-2 was observed prominently with ICI 182,780 treatment. Previous studies have demonstrated this to be a phosphorylated form of Bcl-2 [52–55], suggesting an increased phosphorylation of Bcl-2 upon ICI 182,780 treatment. Bcl-2 expression has been associated with significant resistance to apoptosis by numerous agents. However, contradictory reports [20,56] addressing the role of Bcl-2 in resistance to apoptosis exist regarding MCF-7 cells, and we wished to determine if Bcl-2 suppressed apoptosis in the MCF-7 cell variant used in our studies.

MCF-7 (N variant) cells were transfected with a pSFFV-Bcl-2 construct or empty vector (Fig. 4). Cells were selected in DMEM-10% media containing G418 and clones were examined for Bcl-2 expression. All vector-transfected clones examined expressed very low to undetectable levels of Bcl-2. In contrast, all SFFV-Bcl-2 transfected clones (B1-6 represented) displayed significant levels of Bcl-2 levels. Vector and Bcl-2 containing clones were compared to another variant of MCF-7 (M variant) cells. The MCF-7M cells were previously shown to endogenously express high levels of Bcl-2 protein compared to the MCF-7N cells used here [48]. Arbitrarily, MCF-7N-Bcl-2 (B3; MCF-7/Bcl-2) and MCF-7N-Vec (MCF-7/Vec) were selected to examine the effects of Bcl-2 expression on TNF-, TAX- and DOX-induced apoptosis. Both clones were examined for sensitivity to either TNF-, DOX- or TAX-mediated cell death at 24, 48 and 72 h (Fig. 5(a), (b) and (c)). These results demonstrate that TNF treatment resulted in a potent loss of viability in MCF-7N-Vec cells with a 63 ± 5.5, 37 ± 11.7 and 23 ± 10.7% viability at 24, 48 and 72 h post-treatment, respectively. However, MCF-7/Bcl-2 cells were resistant to TNF-induced cell death with only a slight loss of viability to 90 ± 5.7, 87 ± 3.8 and 86 ± 7.6% at 24, 48 and 72 h post-treatment, respectively. Similarly, both DOX and TAX treatment resulted in significant cell death in MCF-7/Vec cells. TAX produced a loss of viability to 69 ± 1.2, 67 ± 1.7 and 60 ± 0.68% at 24, 48 and 72 h post-treatment, respectively, while DOX-induced a loss of viability to 68 ± 4.2, 49 ± 3.1, 35 ± 8.5 at 24, 48 and 72 h, respectively. In contrast, MCF-7/Bcl-2 cells displayed resistance to cell death with a viability of 97 ± 3.1, 99 ± 0.5 and 95 ± 2.5% with TAX at 24, 48 and 72 h post-treatment, respectively, and percent viability of 99 ± 1.1, 94 ± 6 and 75 ± 4.6% with DOX at 24, 48 and 72 h post-treatment, respectively. Increased expression of Bcl-2 protein levels by oestrogens has been a proposed mechanism by which this steroid hormone promotes cell survival. Interestingly, the ability of ICI 182,780 to suppress Bcl-2 expression also revealed an increase in a higher (32 kDa) molecular weight form of Bcl-2 suggestive of phosphorylation, an event associated with decreased anti-apoptotic activity of this protein. Using MCF-7/Bcl-2 and MCF-7/Vec cells, we wished to examine the possibility that ICI 182,780 may function to partially suppress the survival effect of Bcl-2 in the MCF-7/Bcl-2 cells or enhance apoptosis in the MCF-7/Vec cells. Specifically, MCF-7/Vec cells were pre-treated with ICI 182,780 (1 μM) for 1 h, followed by treatment with TNF (10 ng/ml), TAX (0.04 μg/ml) or DOX (0.1 μg/ml). Subsequent viability outcomes demonstrated that the combined TNF and ICI 182,780 (TNF/ICI) treatments resulted in a decrease in viability to 21 ± 4.6, 6.6 ± 3.4 and 8 ± 2.6% at 24, 48 and 72 h post-treatment in MCF-7/Vec cells, respectively, while the TAX/ICI-treated MCF-7/Vec cells resulted in a loss of viability to 53 ± 1.7, 47 ± 4 and 37 ± 6% at 24, 48 and 72 h post-treatment, respectively. The DOX/ICI-treated MCF-7/Vec cells resulted in a loss of viability to 43 ± 4.7, 18 ± 3.8 and 35 ± 20% at 24, 48 and 72 h post-treatment, respectively. Similar to

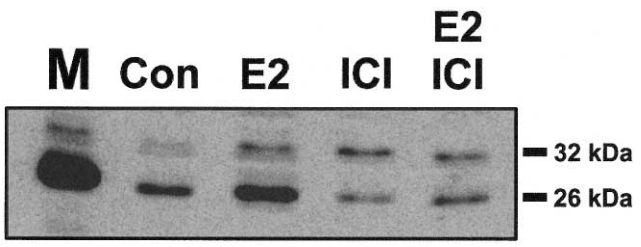

Fig. 3. Regulation of Bcl-2 expression and phosphorylation by oestrogen signalling. MCF-7 cells were plated in 5% CS media for 18 h, pre-treated with or without ICI 182,780 (1 μM; ICI) for 30 min followed by 17β-oestradiol (1 nM; E2) for 24 h. Cells were then harvested for Western blot analysis of Bcl-2 expression using Bcl-2 antisera. High Bcl-2-expressing MCF-7M cells were used as a standard (M).

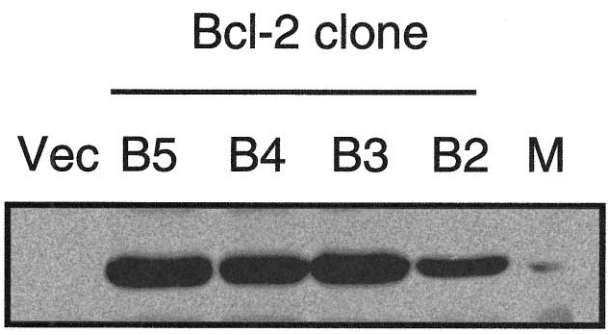

Fig. 4. Generation of stable Bcl-2 overexpression in MCF-7 cells. MCF-7 cells were transfected with either pSFFV-Bcl-2 or empty vector. Selected clones of MCF-7N-Vec and MCF-7N-Bcl-2 were examined for Bcl-2 expression by Western blot analysis. High Bcl-2-expressing MCF-7M cells were used as a standard (M).

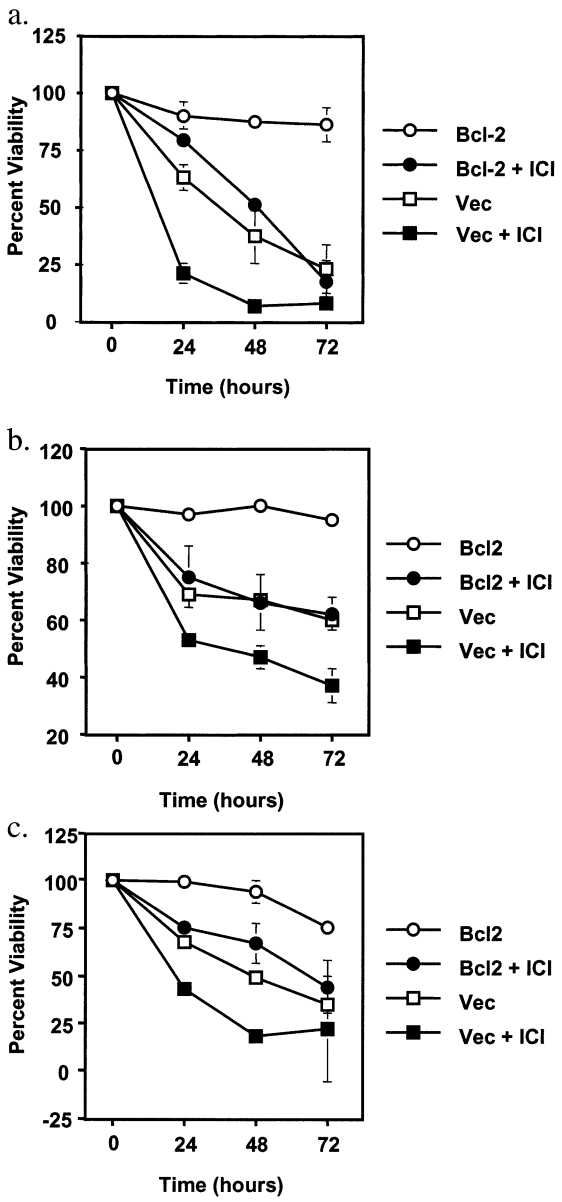

Fig. 5. Bcl-2-mediated suppression of TNF-, TAX- and DOX-induced cell death and reversal by anti-oestrogens. MCF-7N/Vec (clone 1; squares) and MCF-7N/Bcl-2 (clone 3) cells (circles) were treated with or without ICI 182,780 (10 μM; filled) for 1 h followed by treatment with either TNF (10 ng/ml) (a), TAX (0.04 μg/ml) (b) or DOX (0.1 μg/ml) (c) for 24, 48 or 72 h. Cells were then harvested for viability assay. Data are representative of three independent experiments ± SEM.

the MCF-7/Vec cells, the MCF-7/Bcl-2 cells pre-treated with ICI 182,780 also showed an increased loss of viability when compared to control MCF-7 cells. Specifically, viability outcomes from the TNF/ICI treatments resulted in a decrease in viability to 79 ± 1.7, 51 ± 2.1 and 17 ± 9.3% at 24, 48 and 72 h post-treatment in MCF-7/Bcl-2 cells, respectively, while the TAX/ICI-treated MCF-7/Bcl-2 cells resulted in a loss of viability to 75 ± 10.6, 66 ± 9.8 and 62 ± 5.7% at 24, 48 and 72 h post-treatment, respectively. The DOX/ICI-treated MCF-7/Bcl-2 cells resulted in a loss of viability to 75 ± 0.58, 67 ± 10.4 and 44 ± 14% at 24, 48 and 72 h post-treatment, respectively.

The ability of ICI 182,780 treatment to subvert the anti-apoptotic effect of Bcl-2 overexpression suggested that suppression of an ER-dependent survival signal or activation of an apoptotic signal by ICI 182,780 was capable of subverting cell survival mediated by Bcl-2. Several studies have shown that activation of JNK is capable of phosphorylating and inactivating Bcl-2. The ability of ICI to activate a Bcl-2 phosphorylating cascade such as JNK may be in part responsible for the apoptotic enhancement seen with ICI in Bcl-2 over expressing cells. Previous reports have indicated that oestrogen treatment mediates cell survival through rapid activation of Erk signalling and recently, a coordinate suppression of JNK activation [26,27,33–36]. Additionally, tamoxifen has been shown to both suppress oestrogen-ER activation of Erk as well as to stimulate JNK activation [33–36]. The regulation of early MAPK singling by oestrogen–anti-estrogens may be critical in cell survival independently or in conjunction with oestrogen stimulation of Bcl-2 expression. We, therefore, examined the ability of ICI 182,780 treatment to activate the Erk, JNK and p38 members of the MAPK family (Fig. 6). Our results showed that in a time-dependent manner (0–60 min), ICI 182,780 (1 μM) treatment resulted in a rapid increase in phosphorylation of JNK and p38. In contrast, ICI 182,780 resulted in a rapid partial suppression of basal Erk phosphorylation.

The observation that ICI 182,780 treatment results in increased activation of JNK suggests a potential role for this pathway in mediating the survival subverting effect of ICI in MCF-7/Bcl-2 (clone 3) cells. Using a constitutive active mutant of MEKK1 (ΔMEKK) which has been previously shown to potently stimulate the JNK cascade [50], we investigated the ability of

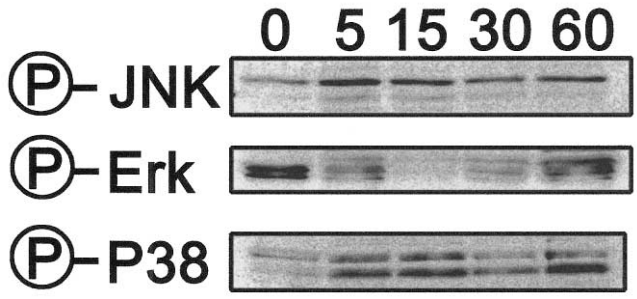

Fig. 6. Activation of JNK by the anti-oestrogen ICI 182,780. MCF-7 cells were treated with ICI 182,780 (1 μM), harvested at the times indicated and analysed for the activation of Erk, JNK and p38 MAPKs. Phosphorylated forms of Erk 1,2 (P-Erk), JNK 1,2 (P-JNK) or p38 (P-P38) were detected using immunoblot analysis with phospho-specific antibodies as indicated in Section 2. The figure is a representative blot of three experiments.

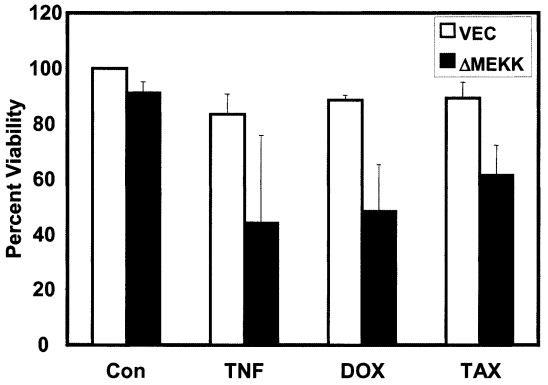

Fig. 7. MEKK activation potentiates TNF-, TAX and DOX-induced cell death in MCF-7/Bcl-2 cells. MCF-7/Bcl-2 (clone 3) cells were transfected with 3 μg of empty pEE-CMV vector (VEC) or with constitutive active MEKK, pEE-CMV-ΔMEKK1 (ΔMEKK), using the lipofectamine method followed by treatment with either 10 ng/ml TNF, 0.04 μg/ml TAX, or 0.1 μg/ml DOX and harvested 24 h later for viability assay. Data are represented as percent viability compared to untreated (CON) VEC transfected cells (100%) from two independent experiments ± SEM.

MEKK–JNK activation to subvert Bcl-2 cell survival. MCF-7/Bcl-2 (clone 3) cells were transfected with either empty vector or a ΔMEKK construct and treated with TNF, TAX or DOX (Fig. 7). Consistent with the above results, MCF-7/Bcl-2 (clone 3) cells exhibited weak responses to TNF (83.5 ± 7.5), TAX (88.5 ± 1.6) or DOX (89.1 ± 5.6) treatment. In contrast, expression of the ΔMEKK in MCF-7/Bcl-2 (clone 3) cells markedly enhanced the ability of TNF (44.2 ± 31.5), TAX (48.5 ± 16.6) or DOX (61.5 ± 10.7) to induce cell death. This suggests that molecular activation of the MEKK–JNK pathway, similar to ICI 182,780 treatment, subverts the survival effect of Bcl-2.

4. Discussion

Oestrogen represents a potent survival factor in a number of biological systems [3,32]. In the MCF-7 breast carcinoma cell line, numerous reports have implicated oestrogen and ER-signalling in the suppression of apoptosis [7,10–16]. However, treatment of MCF-7 cells with anti-oestrogens, such as tamoxifen and ICI 182,780, is associated with the induction of apoptosis in these cells [14–17,19,57]. Similarly, removal of the oestrogen source in nude, ovariectomized mice results in apoptosis of MCF-7 tumour implants [7,19]. This phenomenon suggests that oestrogen may play a permissive role in cell proliferation and survival, similar to the role of cytokines, such as IL-3 and GM-CSF, in haematopoietic cell survival [58]. Therefore, in the presence of oestrogen, breast carcinoma cells are allowed to proliferate, but upon the removal of oestrogen or upon ER-signalling inhibition, these cells die by the loss of a necessary survival signal. The ability of oestrogen to induce cell survival is, in part, dependent upon Bcl-2 expression. In agreement with previous results, we demonstrate in this report that an increase in Bcl-2 expression is seen with oestrogen treatment, which is suppressed by ICI 182,780 treatment. ICI 182,780 not only suppresses 17β-oestradiol-induced Bcl-2 expression, but ICI 182,780 also appears to increase the presence of a 32 kDa higher molecular weight form of Bcl-2 previously described as a phosphorylated form of Bcl-2. Phosphorylation of Bcl-2 has been associated with decreased anti-apoptotic effects by the Bcl-2 molecules themselves [52–55]. The ability of ICI to suppress 17β-oestradiol-induced Bcl-2 expression is expected, however, the observed increase in a higher molecular weight Bcl-2 form suggestive of phosphorylation is an interesting and novel observation. These effects of ICI 182,780 may be in part due to additional ER-mediated, survival-signalling pathways. Recently, a report by Dong et al. demonstrated that oestrogen-mediated activation of Bcl-2 expression did not occur through direct regulation of the Bcl-2 promoter by ER, but rather through an oestrogen-dependent induction of cAMP, which then stimulates Bcl-2 expression through a CREB/ATF-I-dependent activation of cAMP-response-elements (CREs) [22]. However, a report by Perillo et al. demonstrated an ER-dependent regulation of Bcl-2 through ERE sites within the coding region [23]. The potential exists that Bcl-2 may only represent one pathway by which oestrogen and the ER function to promote cell survival. Therefore, the effects of oestrogens on survival signalling may occur through activation of other cellular signalling cascades.

Several reports have demonstrated that 17β-oestradiol can rapidly stimulate Erk activation in MCF-7 and other cell lines and is involved in mediating anti-apoptotic effects of 17β-oestradiol [26–30,32]. In contrast to Erk signalling to cell survival, the JNK and p38 members of the MAPK cascade have been demonstrated to induce or enhance apoptosis in many systems. Also of note is a recent report by Zhang et al. that demonstrates an increased activation of p38 MAPKs with 17β-oestradiol treatment [36]. Collectively, these results demonstrate the ability of Erk, JNK and p38 MAPKs to be regulated by ER signalling. Evidence now suggests that 17β-oestradiol signalling to cell survival involves both a rapid up-regulation of Erk activation coordinate to suppression of JNK activity [26,27]. Therefore, given that Erk may be an important survival signal, the ability of ICI 182,780 or tamoxifen to suppress 17β-oestradiol-induced Erk activities may inhibit ER-survival signalling. Additionally, treatment with ICI 182,780 or tamoxifen may block 17β-oestradiol's effects on JNK thus leading to both a decrease in Erk activation while increasing JNK activation.

The involvement of these rapid signalling events in the regulation of cell survival are consistent with our observation that a 1 h pre-treatment with 17β-oestradiol results in partial suppression of TNF-induced apoptosis. The ability of ICI 182,780 treatment to suppress the anti-apoptotic effects of Bcl-2 overexpression suggests both gene expression and rapid signalling are critical to the survival effects of 17β-oestradiol. Consistent with the involvement of the MAPK pathways in regulation of survival by the ER, we observed that ICI 182,780 treatment rapidly enhances JNK activation in MCF-7 cells. Activation of the JNK pathway has been demonstrated to induce Bcl-2 phosphorylation, an event that suppresses the anti-apoptotic effects of Bcl-2 [53,55]. Consistent with these findings we observed ICI treatment induced an increased molecular weight band of Bcl-2 suggestive of phosphorylation. Therefore, direct promotion of Bcl-2 phosphorylation by ICI 182,780 or tamoxifen [59] may be through a JNK-dependent pathway or through an indirect suppression of the 17β-oestradiol/ER-dependent pathway that functions to maintain Bcl-2 in an unphosphorylated state. This concept is supported by the ability of ICI 182,780 to override the Bcl-2-mediated survival signal. It has however been previously demonstrated that tamoxifen can suppress kinases, such as PKC and CamKII, as well as glucosyl ceramide synthase, and that this may be a mechanism by which tamoxifen or ICI 182,780 can influence other ER-independent effects in cells [42–47]. Activation or suppression of these other pathways by ICI 182,780 treatment may be similar to the ability of TAX to increase Bcl-2 phosphorylation, and thereby shift the survival balance towards cell death [52,54].

In conclusion, our results demonstrate a critical role for ER signalling in MCF-7 cell survival and suppression of chemotherapeutic drug- and TNF-induced apoptosis. Additionally, our observations demonstrate that ICI 182,780 functions to suppress both oestrogen- and Bcl-2-mediated cell survival in ER-positive cells, suggesting the existence of both gene expression (Bcl-2) and cytoplasmic signalling (MAPK) mechanisms of ER-mediated cell survival in MCF-7 cells.

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