Estrogen Receptors in Human Myeloma Cells

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ABSTRACT

It has recently been reported that the human myeloma cell line U266 proceeds to undergo apoptosis after cultivation with the antiestrogen tamoxifen, thus raising the possibility that antiestrogens may be candidates for use in myeloma therapy. To obtain basic information on the effects of antiestrogens on myeloma cells, we investigated the mRNA expression levels of estrogen receptor (ER-α, ER-β, and coactivators and corepressors in nine human myeloma cell lines and compared them with those of seven human breast cancer cell lines including four ER-positive and three ER-negative lines. The alterations in cell growth and mRNA expression of the target genes of ER or those of cytokines in the myeloma lines by estradiol or antiestrogens (tamoxifen and toremifene) were also investigated. In addition, effects on membrane Fas expression, appearance of apoptosis, and cell cycle perturbation were analyzed. It was revealed that ER-β and corepressors were dominantly expressed in myeloma cells, and antiestrogens induced growth inhibition through apoptosis mediated by a Fas-related pathway and G1 arrest of the cell cycle in myeloma cell lines.

INTRODUCTION

Multiple myeloma remains an incurable hematological malignancy characterized by various clinical manifestations such as hyperproteinemia, renal dysfunction, bone lesions, and immunodeficiency (1, 2). Since the introduction of melphalan and prednisone therapy to improve the clinical response to multiple myeloma, numerous multidrug chemotherapies including Vinca alkaloid, anthracycline, and nitrosourea-based treatment have been tried (3–6). However, there has been little improvement in outcome over the past three decades. It has recently been reported that the human myeloma cell line U266 proceeds to undergo apoptosis after cultivation with the antiestrogen TAM (7). Thus, antiestrogens may provide a new strategy in myeloma therapy.

Recent advances in the study of ERs in breast cancer have demonstrated the existence of ER-β in addition to ER-α (8–11) and that ER-β may have an inhibitory effect against ER-α in breast cancer cells in terms of cellular growth, differentiation, and transcriptional activation of target genes (12–15). In addition, numerous cofactors have been identified, some of which [SRC-1 (16)/ERAP160 (17), TIF2 (18)/GRIP-1 (19)/SRC-2, AIB1 (20)/pCIP (21)/RAC3 (22)/ACTR (23)/SRC-3 (24), CBP/p300 (25), and P/CAF (26)] act as coactivators to activate the ER-induced transcription of target genes, whereas others; i.e., N-CoR (27) and SMRT (28), function as corepressors to inhibit transcription. However, the expression levels and the roles of these cofactors in the progression of breast cancer have not been clarified to date. Estrogen appears to be a negative regulator of normal hematopoiesis and lymphopoiesis (29, 30). In addition, it has been reported that estrogen loss up-regulates hematopoiesis in the mouse (31). There has been no report investigating the expression levels of ERs (ER-α and ER-β), and cofactors in myeloma cells, with the exception of two studies on ER-α expression (32, 33). If antiestrogens do cause the growth inhibition of myeloma cells, it may be important to elucidate the expression levels of these molecules in myeloma cells.

Apoptosis is a most important cell death induced by anticancer drugs and irradiation (34–40). It has recently been reported that these agents and irradiation up-regulate Fas expression through p53 activation in p53wt-type malignant cells (41). Thus, it may be important to analyze the appearance of apoptosis and alteration of Fas expression in myeloma cells cultured with antiestrogens.

In this study, we analyzed the mRNA expression levels of ER-α, ER-β, and cofactors in nine human myeloma cell lines and compared them with seven human breast cancer cell lines including four ER-positive and three ER-negative cell lines. The alteration of cell growth and mRNA expression levels of target genes of ER or those of cytokines in myeloma lines cultured with E2 or antiestrogens (TAM and TOR) were also observed. In addition, their effects on mFas expression, the appearance of apoptotic cells, and cell cycle perturbation were analyzed. These analyses indicated that ER-β is dominantly expressed in myeloma cells and that antiestrogens induced growth inhibition through apoptosis mediated by a Fas-related pathway and G1 arrest of the cell cycle in myeloma cell lines. Such analyses may provide for the future use of antiestrogens in myeloma treatment.

MATERIALS AND METHODS

Cell Lines Used in This Study.

Nine human myeloma cell lines (KMM-1, KMS-11, KMS-12-PE, KMS-12-BM, KMS-18, KMS-20, KMS-21-PE, U266, and RPMI8226) and seven human breast cancer cell lines (KPL-1, KPL-4, MDA-MB-231, MCF-7, KPL-3C, T47D, and SkBr3) were used in this study. The cultures of seven former myeloma lines were initiated independently on supplemental IL-6 and established at Kawasaki Medical School between 1982 and the present (42–44). Three of the breast cancer cell lines (KPL-1, KPL-4, and KPL-3C) were also established at Kawasaki Medical School (45–47). Among the breast cancer cell lines, KPL-1, KPL-3C, MCF-7, and T47D have been demonstrated to express ER-α strongly by ELISA. These ER-positive cell lines showed growth enhancement when they were cultured with E2 in vitro and growth inhibition by antiestrogens as reported previously (45–49). All of the cell lines were cultured in RPMI 1640 supplemented with 10% (for myeloma cell lines) or 5% (for breast cancer cell lines) FBS at 37°C in a humidified atmosphere with 5% CO2, without any particular supplementation of IL-6. Because phenol red that included in RPMI 1640 and FBS have been known to possess weak estrogenic activity, we used PRF RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) with 2% FBS to analyze the effects of E2 on cell growth to minimize the estrogenic activity in the medium and maintain myeloma cell proliferation activity.

RNA Extraction, cDNA Synthesis, and MP-RT-PCR. RNA extraction and cDNA synthesis were performed as reported previously (50, 51). MP-RT-PCR

Received 8/23/99; accepted 1/5/00.
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1 Supported by Kawasaki Medical School Project Grants 9-310 and 10-305.
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3 The abbreviations used are: TAM, tamoxifen; ER, estrogen receptor; SRC, steroid receptor co-activator; N-CoR, nuclear receptor co-repressor; SMRT, silencing mediator of retinoid and thyroid receptor; E2, estradiol; FBS, fetal bovine serum; PRF, phenol red free; RT-PCR, reverse transcription-PCR; MP-RT-PCR, multiplex RT-PCR; PGFR, progesterone receptor; IGF, insulin-like growth factor; TGF, transforming growth factor; TOR, tumornf; mFas, membrane Fas; IL, interleukan; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.
PCRs was applied to examine the mRNA expression level for ERs (ER-α and ER-β) and coactivators in myeloma and breast cancer cell lines. The primer sequences used in this study, the expected size of each RT-PCR amplicon, and the annealing temperature for each primer set are listed in Table 1. To amplify β-actin, the housekeeping control gene, and the target gene in a single reaction, MP-RT-PCR was performed. The ratios of primer sets between the target gene and β-actin are also shown in Table 1. These ratios and PCR cycles were determined so as to amplify both products logarithmically and in relatively similar amounts. The detailed MP-RT-PCR procedure was as reported previously (50).

After visualization of MP-RT-PCR product electrophoresis on a 1.2% agarose gel stained with ethidium bromide, gel images were obtained using the FAS-II UV image analyzer (TOYOBO Co. Ltd., Tokyo, Japan), and the densities of the products were quantitated using Quantity One version 2.5 (PDI Inc., Huntington Station, NY). The relative expression level of each target gene in individual cell lines was calculated as the density of the product of that gene divided by the density of the product of the β-actin gene derived from the same MP-RT-PCR.

**Growth Curves of Myeloma Cell Lines Cultured with E2 or Antiestrogens**

KMM-1, KMS-11, KMS-12-PE, and RPMI8226 myeloma cell lines were cultured with 0, 10⁻⁷, 10⁻⁶, and 10⁻⁵ M E2 (Sigma Chemical Co., St. Louis, MO) in PRM medium with 2% FBS for 2 days, and cell numbers were determined so as to amplify both products logarithmically and in relatively similar amounts. The detailed MP-RT-PCR procedure was as reported previously (56).

The antiestrogens, TAM and TOR, were added to myeloma cell cultures at a concentration of 1 × 10⁻⁶, 3 × 10⁻⁶, and 1 × 10⁻⁵ M, respectively, with standard RPMI 1640 plus 10% FBS for 2 days to avoid artificial cell death by estrogen-minimized medium, and cell numbers were compared as a percentage of control (0 M TAM or TOR). TAM and TOR were kindly provided by Zeneca Pharmaceuticals (Macclesfield, United Kingdom) and Nippon Kayaku Co. (Tokyo, Japan), respectively.

**Alteration of mFas Expression and Detection of Apoptosis in Myeloma Cells Cultured with Antiestrogens**

Three of nine myeloma cell lines, KMS-12-2M, KMS-18, and KMS-21-PE, were cultured with or without 10⁻⁵ M TAM or TOR for 2 days. mFas expression was examined as reported previously (56).

The apoptotic fraction of KMS-11 and KMS-18 cells and DNA indices of KMS-21-PE cells cultured with or without 10⁻⁵ M antiestrogen (TAM or TOR) for 2 days were examined as reported previously (56).

**Statistical Analysis**

The relative expression level of the cofactor gene from myeloma or breast cancer cell lines was analyzed statistically using the Mann-Whitney test. The growth-inhibitory effects of antiestrogens on myeloma cell lines were analyzed using Student’s t test.

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**RESULTS**

**ER-α and ER-β mRNA Expression in Myeloma Cell Lines.** As shown in Fig. 1a, MP-RT-PCR clearly demonstrated that all of the myeloma cell lines except RPMI8226 expressed ER-α message, and all nine myeloma cell lines expressed ER-β to some degree. However, ER-α expression levels were lower in the myeloma cell lines than in the four ER-positive breast cancer cell lines (KPL-1, KPL-3C, MCF-7, and T47D). KPL-4 cells also expressed a relatively weak ER-α message on MP-RT-PCR as shown in Fig. 1a. Fig. 1b shows the relative expression levels of ER-α and ER-β in each cell line used in this study. All four ER-positive cell lines had high ER-α and low ER-β expression levels, whereas all of the myeloma cell lines with the exception of KMS-11, KMS-18, and KMS-20 had high ER-β and low ER-α expression levels. Fig. 1c shows a comparison of ER-α and ER-β expression levels and the balance of these levels (represented as the expression level of ER-α divided by that of ER-β in individual lines) among ER-positive and ER-negative breast cancer lines and myeloma cell lines. The ER-α expression levels were significantly higher in ER-positive breast cancer cell lines than in ER-negative breast cancer lines and myeloma lines. In contrast, ER-β expression trended to be stronger in myeloma cell lines than in breast cancer lines. Thus, the expression balance of both ERs was significantly higher in ER-positive breast cancer cell lines than in myeloma cell lines. It is noteworthy that the level of ER-α expression was highest in KMS-11 of all the myeloma cell lines, and the expression balance was close to that of ER-positive breast cancer cell line T47D. These results indicated that both ER-α and ER-β were expressed in myeloma cell lines, and the expression balance in the KMS-11 cell line was similar to that seen in the ER-positive breast cancer cell lines.

Therefore, we tried to detect ER by ELISA (Otsuka Assay Laboratories, Tokyo, Japan) using cellular extracts from myeloma cell lines. However, values were below the detectable level (0.5 fmol/mg protein). These results may reflect relatively low ER-α expression levels in myeloma cell lines as compared to ER-positive breast cancer cell lines.

**Expression of Cofactors in Myeloma Cell Lines and ER-positive and ER-negative Breast Cancer Cell Lines.** Fig. 2 shows MP-RT-PCR gel images for coactivators (SRC-1, TIF2, AIB1, CBP, and P/CAF) and corepressors (N-CoR and SMRT) in four ER-positive and three ER-negative breast cancer cell lines and nine myeloma cell lines.

We examined the relative expression levels of each cofactor gene in individual cell lines and compared them between breast cancer cell lines and myeloma cell lines. As shown in Fig. 3a, the SRC-1

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**Table 1**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences</th>
<th>Temperature (°C)</th>
<th>Product size (bp)</th>
<th>Ratio (β-actin: target gene) for MP-RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER-α</td>
<td>F, 5'-AGACATGAGACGTCGCAACC-3' and R, 3'-GCCACGTCTTCGAGGACGTAAGG-5'</td>
<td>59</td>
<td>299</td>
<td>0.3:2.0</td>
</tr>
<tr>
<td>ER-β</td>
<td>F, 5'-TCACATCTCTATGCAGGACC-3' and R, 3'-CGTAACTTCTGAGGACGTAAGG-5'</td>
<td>58</td>
<td>346</td>
<td>0.3:2.0</td>
</tr>
<tr>
<td>PgR</td>
<td>F, 5'-AGTGGAGACGACTGGATG-3' and R, 3'-GACGCTGGATGGATG-5'</td>
<td>56</td>
<td>475</td>
<td>NA*</td>
</tr>
<tr>
<td>pS2</td>
<td>F, 5'-TGGGAACACAGGTGATG-3' and R, 3'-ATCCGTGATGGATG-5'</td>
<td>60</td>
<td>392</td>
<td>NA</td>
</tr>
<tr>
<td>SRC-1</td>
<td>F, 5'-GCTCTTGCTATGAGTGG-3' and R, 3'-ACGTTGTGGATGGATG-5'</td>
<td>57</td>
<td>372</td>
<td>0.4:2.0</td>
</tr>
<tr>
<td>SMRT</td>
<td>F, 5'-TGTTGTTCTACGATG-3' and R, 3'-CCGGAACTTCTGATG-5'</td>
<td>58</td>
<td>179</td>
<td>0.4:2.0</td>
</tr>
<tr>
<td>N-CoR</td>
<td>F, 5'-GTGGAGACGACTGGATG-3' and R, 3'-GCTGGATGGATG-5'</td>
<td>58</td>
<td>349</td>
<td>0.6:2.0</td>
</tr>
<tr>
<td>TIF2</td>
<td>F, 5'-TAACTGACAGATGGATGCGG-3' and R, 3'-TGTGGATGGATG-5'</td>
<td>58</td>
<td>314</td>
<td>0.4:2.0</td>
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<tr>
<td>AIB1</td>
<td>F, 5'-GCTCCTGATCCATGAGGACG-3' and R, 3'-TGCTGATCCATGAGGACG-5'</td>
<td>57</td>
<td>256</td>
<td>0.4:2.0</td>
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<tr>
<td>P/CAF</td>
<td>F, 5'-AGACATGAGACGTCGCAACC-3' and R, 5'-TGCTGATCCATGAGGACG-5'</td>
<td>57</td>
<td>345</td>
<td>0.3:2.0</td>
</tr>
<tr>
<td>CBP</td>
<td>F, 5'-TGGGAACACAGGTGATG-3' and R, 3'-GACGCTGGATGGATG-5'</td>
<td>58</td>
<td>272</td>
<td>0.3:2.0</td>
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<tr>
<td>IGF-II</td>
<td>F, 5'-AATCTCTGCTGATG-3' and R, 3'-TGCTGATCCATGAGGACG-5'</td>
<td>60</td>
<td>122</td>
<td>NA</td>
</tr>
<tr>
<td>IL-6</td>
<td>F, 5'-TCTCTGCTGATG-3' and R, 3'-TGCTGATCCATGAGGACG-5'</td>
<td>58</td>
<td>316</td>
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</tr>
<tr>
<td>IL-10</td>
<td>F, 5'-AATCTCTGCTGATG-3' and R, 3'-TGCTGATCCATGAGGACG-5'</td>
<td>57</td>
<td>202</td>
<td>NA</td>
</tr>
<tr>
<td>TGF-βF</td>
<td>F, 5'-TCCACCATGAGGATGCGG-3' and R, 3'-TGCTGATCCATGAGGACG-5'</td>
<td>58</td>
<td>190</td>
<td>NA</td>
</tr>
<tr>
<td>TGF-βb</td>
<td>F, 5'-AGTCTGCTGATG-3' and R, 3'-TGCTGATCCATGAGGACG-5'</td>
<td>59</td>
<td>192</td>
<td>NA</td>
</tr>
</tbody>
</table>

*P, forward primer; R, reverse primer.

*NA, not available.
expression level was significantly higher in breast cancer cell lines than in myeloma cell lines ($P = 0.0229$), whereas the levels of corepressors N-CoR and SMRT were significantly higher in myeloma cell lines ($P = 0.0036$ and 0.0129, respectively). In addition, when we divided the breast cancer cell lines into two groups (four ER-positive and three ER-negative cell lines), SRC-1 was significantly higher in the ER-positive cell lines than in myelomas, whereas TIF2 expression was significantly lower in ER-negative breast cancer cell lines than in myeloma cell lines. AIB1 expression was significantly lower in ER-negative cell lines than in ER-positive breast cancer or myeloma cell lines (Fig. 3b). These results indicated that corepressors were expressed more in myeloma cell lines and that the coactivator SRC-1 was expressed less in myeloma cell lines than in the breast cancer cell lines. Thus, the effects of E$_2$ on transcriptional activation may be repressed in myelomas compared to breast cancer cells.

Effect of E$_2$ on Myeloma Cell Growth and Expression of Target or Cytokine Genes. KMM-1, KMS-11, KMS-12-PE, and RPMI8226 cell lines were cultured with 0, 10$^{-7}$, 10$^{-8}$, and 10$^{-9}$ M of E$_2$ in PRF medium with 2% FBS for 2 days. KMS-11 was selected for its ER-$a$ dominant expression, similar to the ER-positive breast cancer cell lines, and RPMI8226 was selected as an ER-$a$ negative myeloma line. As shown in Fig. 4, the growth of KMS-11 cells was stimulated by E$_2$ in a dose-dependent manner, whereas that of other cell lines examined was not. These results indicate that the balance between ER-$a$ and ER-$b$ was repressed in myelomas compared to breast cancer cells.

Fig. 1. The gel images of MP-RT-PCR products for ER-$a$ and ER-$b$ in seven breast cancer cell lines and nine multiple myeloma cell lines are shown in $a$. The relative expression levels of each target gene in individual cell lines were calculated as the density of the product of the target gene divided by the density of the product of the $b$-actin gene derived from the same MP-RT-PCR. $b$ gives a comparison of the relative expression levels of ER-$a$ and ER-$b$ in individual breast cancer cell lines (top) and myeloma cell lines (bottom). $c$ shows ER-$a$ and ER-$b$ relative expression levels among ER-positive and ER-negative breast cancer cell lines and myeloma cell lines (left and middle graphs, respectively). The balance between the two ERs was calculated by dividing the relative expression level of ER-$a$ by that of ER-$b$ from the same line (see right graph in $c$). BCCL, breast cancer cell lines; MMCL, multiple myeloma cell lines.

Fig. 2. The gel images of MP-RT-PCR products for cofactors, ligand-dependent coactivators (i.e., SRC-1, TIF2, AIB1, CBP, and P/CAP), and corepressors (i.e., N-CoR and SMRT) are shown. The primer set and annealing temperatures, product sizes, and ratios of primers for $b$-actin and target genes are listed in Table 1. The ratio and cycles were determined so as to amplify both genes (the $b$-actin gene and the target gene) logarithmically.

[1436]
ER-β may play an important role on the effect of E₂ on myeloma cell growth. Moreover, less expression of the coactivator and more expression of corepressors may explain the low level of growth enhancement seen in myeloma cells cultured with E₂ in vitro.

Table 2 shows the alterations in gene expression in eight ER-α-positive myeloma cell lines cultured with 10⁻⁷ M E₂ for 2 days. The cell lines are listed top to bottom according to the relative expression levels of each gene examined. Table 2 shows that PgR and pS2 genes have been considered as typical target genes of ER, and they are usually up-regulated by E₂ in breast cancer cells. However, PgR and pS2 gene expressions were restricted in three myeloma cell lines and one myeloma cell line, respectively. In addition, the up-regulation of expression was only observed in PgR of the U266 cell line, whereas PgR in KMS-18 and KMS-11 cells was markedly down-regulated. TGF-α, TGF-β, and IGF-II are also known as target genes of ER. An up-regulation of expression was observed in KMS-12-PE, KMS-12-BM, and KMS-20 for TGF-α, in KMS-20 and KMS-21-PE for TGF-β, and in KMS-18 and KMS-21-PE for IGF-II gene, whereas down-regulation was also demonstrated in KMS-12-PE and KMS-12-BM for TGF-β and in KMS-11, KMS-20, and KMS-12-BM for IGF-II. On the other hand, E₂ treatment induced an up-regulation of the IL-6 gene only in the U266 cell line, whereas IL-10 was observed in three of six IL-10-expressing lines; again, U266 was the only exception that showed down-regulation of IL-10.

Fig. 3. a, the relative expression levels of cofactors demonstrated in Fig. 2 were calculated as described in “Materials and Methods.” a shows the relative expression levels of cofactors in each cell line. Those of myeloma lines (MMCL) and breast cancer cell lines (BCCL) were compared. Significance was found in SRC-1 (breast cancers > myelomas, \( P = 0.0229 \)), N-CoR (breast cancers < myelomas, \( P = 0.0036 \)), and SMRT (breast cancers < myelomas, \( P = 0.0129 \)) expressions. b shows the results of similar analyses with the breast cancer cell lines divided into two groups, ER-α-positive and ER-α-negative cells. A significant difference was demonstrated in the expression of SRC-1 (ER-positive breast cancers > myelomas, \( P = 0.0440 \)), TIF2 (ER-negative breast cancers < myelomas, \( P = 0.0126 \)), AIB1 (ER-positive breast cancers > ER-negative breast cancers, \( P = 0.0339 \)), ER-negative breast cancers < myelomas, \( P = 0.0208 \)), N-CoR (ER-positive breast cancers < myelomas, \( P = 0.0335 \); ER-negative breast cancers < myelomas, \( P = 0.0135 \)), and SMRT (ER-negative breast cancers < myelomas, \( P = 0.0335 \)).
Therefore, there is no obvious tendency in the alteration of target gene expression induced by E2 treatment in the myeloma cell lines examined, according to the ER-α/ER-β relative expression levels (Table 2) or expression of corepressors (data not shown). However, it may be noteworthy that KMS-12-PE, KMS-12-BM, and KMS-11 had up-regulated IL-10 messages after cultivation with E2, and KMS-18 showed up-regulation of IGF-II. If these cytokines affect cellular biological characteristics including the growth-promoting activity of myeloma cells (57–59), then E2 may modulate myeloma cell biology. In addition, IL-10 is considered to be one of the most important regulatory cytokines when myeloma cells escape from IL-6-mediated growth regulation (57); it may be interest that IL-6 and IL-10 expression showed an inverse pattern in most of the myeloma cells examined.

**Effect of Antiestrogens on Myeloma Cell Growth.** Fig. 6 shows the growth inhibition in seven myeloma cell lines cultured with 10^{-6}, 3 \times 10^{-6}, and 10^{-5} M TAM (Fig. 6a) or TOR (Fig. 6b) for 2 days. Cultivation with 10^{-5} M TAM resulted in a significant growth inhibition in all of the myeloma cell lines examined. TOR (10^{-5} M) had significant inhibitory effects on all cell lines, and 3 \times 10^{-6} M TOR inhibited cell growth in five of seven cell lines examined. U266 cell growth was significantly suppressed by 10^{-6} M TOR. These results indicate that it may be possible to use antiestrogens as chemotherapeutic drugs for clinical treatment of myeloma, although most of the myeloma cell lines did not show growth enhancement when they were cultured with E2.

To elucidate whether or not the growth inhibition of myeloma cell lines induced by antiestrogens is mediated by the binding between ER and antiestrogens, four myeloma cell lines were cultured with both TAM (10^{-6} M) and E2 (10^{-6} M). However, additional E2 caused further growth inhibition in all cell lines examined as shown in Fig. 6c. Although PRF medium plus 2% FBS was used in this experiment, the effects of the antiestrogens on myeloma cell growth seem to be mediated by mechanisms other than the competitive binding of these agents to ER against E2.

**Increase of Apoptotic Cells Cultured with Antiestrogens in Myeloma Cell Lines.** To examine the role of apoptosis in growth inhibition of myeloma cells induced by antiestrogens, apoptotic cells were analyzed using the TUNEL method. As shown in Fig. 7a, KMS-11 and KMS-18 cells cultured with 10^{-5} M TAM or TOR for 2 days showed an increase in the TUNEL-positive apoptotic cell fraction. Because it has been recently reported that anticancer drugs or irradiation induced mFas up-regulation in malignant cells, the alteration of mFas expression was also examined in myeloma cells. Fig. 7b shows the results of flow cytometric analyses of mFas expression in the mFas-negative and weakly mFas-positive myeloma cell lines KMS-12-BM and KMS-18, respectively, and the mFas-positive KMS-21-PE cell line. The expression of mFas was up-regulated in KMS-18 cells but was unaltered in the other two cell lines. These results suggest that the growth inhibition in myeloma cells induced by antiestrogens was mediated in part by a Fas-related apoptotic pathway.

Because antiestrogens are considered to act as cytostatic drugs, the alteration in cell cycle progression was examined using KMS-21-PE cells, which are originally Fas positive and do not show up-regulation of mFas expression by antiestrogens. As shown in Fig. 8, KMS-21-PE cells cultured with 10^{-5} M TAM or TOR for 2 days clearly exhibited not only an increase in the hypodiploid, apoptotic fraction but also an accumulation of G_0-phase cells with a decrease in the S-phase fraction. Thus, it is possible that the growth-inhibitory effects of antiestrogens on myeloma cells are mediated by a G_0-S-phase blockade and a Fas-related apoptotic pathway.

**DISCUSSION**

Estrogen appears to be a negative regulator of normal hematopoiesis and lymphopoiesis (29, 30). In addition, it has been reported that estrogen loss up-regulates hematopoiesis in the mouse (31). However,
much less is known about the role of estrogens and ERs in terminally differentiated plasma cells and transformed myeloma cells.

ER-α was expressed in most of the myeloma cell lines studied. However, the expression levels were lower than those of ER-positive breast cancer cell lines. In contrast, ER-β expression was observed in all of the myeloma cell lines examined, although only the T47D breast cancer cell line expressed a level of ER-β similar to the myeloma cell lines. Because it has been considered that ER-β may function as an inhibitory molecule against ER-α in terms of ER-α-induced transcriptional activation (8–15), myeloma cell lines that dominantly express ER-β rather than ER-α may reduce ER-α-induced gene transcription and subsequent growth stimulation. As expected, only the ER-α dominant myeloma line KMS-11 was growth-stimulated by E₂ in vitro.

The analyses of mRNA expression levels of ER-related cofactors in myeloma cell lines and in breast cancer cell lines demonstrated that coactivators N-CoR and SMRT were more abundant and that coactivator SRC-1 was less abundant in myelomas than in breast cancer cell lines. Although we have not examined the transcriptional activities and expression levels of these molecules at the protein level, our results would indicate that ER-mediated gene transcription and subsequent growth stimulation by E₂ are inhibited by high expression levels of corepressors as well as the dominant expression of ER-β. This may explain why E₂ did not enhance myeloma cell growth except in the KMS-11 line, although a detectable level of ER-α mRNA was expressed. Moreover, expression levels of PgR and p53 genes, known as typical target genes for ER-α and known to be unregulated by E₂ (47–50), were not shown any definite tendency of their regulation in myeloma cells by E₂. However, the KMS-18 cell line exhibited up-regulation of the IGF-II gene by E₂, and the IL-10 gene (which was negative in control cultures) was also up-regulated in the KMS-12-PE and KMS-12-BM cell lines after cultivation with E₂. Because IGFs and IL-10 have been reported to function as growth factors for myeloma cells (57–59), E₂ may alter the growth or cellular biological characteristics of some myeloma cells. In any case, further subcategorization of myeloma cells according to the gene alteration caused by chromosomal translocation and the use of cytokines such as IL-6 and IL-10 for growth enhancement may be required to estimate the regulational differences among the various myeloma cells shown in this study.

The growth-inhibitory effects of TAM and TOR were investigated, and the lower concentration (10⁻⁶ M) of TOR exhibited a significant growth inhibition when compared with the same concentration of TAM. Although the concentrations of TAM at which the growth of myeloma cells was significantly decreased were relatively high compared to those in clinical use, those concentrations of TOR may be achievable. In addition, it may be possible to use antiestrogens in a multidrug combined chemotherapeutic regimen. In addition, it is still unclear why a further inhibition of growth occurred in the myeloma cell lines examined after treatment with antiestrogens in the presence of estrogen. Although dominant expression of ER-β and corepressors found in myeloma cells may be one of the reasons, the other mechanisms involved in growth inhibition caused by antiestrogens plus estrogen in myeloma cells should be clarified.

To explore the mechanism responsible for the growth inhibition caused by antiestrogens, we analyzed the appearance of apoptosis, up-regulation of mFas expression, and cell cycle perturbation. We found that in the KMS-18 cell line, which basically expresses a low level of mFas, the expression was up-regulated by antiestrogens, resulting in an increase in the apoptotic fraction after cultivation. In addition, KMS-21-PE cells showed G₁ accumulation and S-phase reduction with an increase in the hypodiploid, apoptotic fraction. These results suggest that the growth inhibition of myeloma cells induced by antiestrogens is caused by a combination of mechanisms, a cytostatic effect, i.e., G₁-S-phase blockade, and an induction of apoptosis that is probably mediated by a Fas-related pathway. In addition, KMS-11 cells, which dominantly express ER-α, showed a much higher appearance of the apoptotic fraction after cultivation with antiestrogens. However, future investigations may be required to elucidate the role of competition with estrogens...
and apoptotic and cytostatic mechanisms induced by antiestrogens on myeloma cells with subcategorization of those cells.

Although the expression analyses of ER-α and ER-β and cofactors, based on comparisons in ER-positive breast cancer cell lines, do not encourage the clinical use of antiestrogens in myeloma therapy, this study clearly demonstrated that antiestrogens suppress myeloma cell growth through cytostatic and apoptotic mechanisms. Antiestrogens, in combination with other chemotherapeutic agents such as anthracyclins and nitrosoureas, may have applications in myeloma chemotherapy.

Fig. 7. a, KMS-11 and KMS-18 cells cultured with or without 10^{-5} M TAM or TOR for 2 days were subjected to TUNEL analysis to detect the apoptotic fraction. As indicated in the figure, apoptotic cell numbers are increased in cultures with antiestrogens. b, the mFas-negative KMS-12-BM, weakly mFas-positive KMS-18, and mFas-positive KMS-21-PE cell lines were cultured with or without 10^{-5} M TAM or TOR for 2 days, and the alteration of mFas expression was analyzed by flow cytometry. The numbers in the panels indicate the mean intensity of mFas expression. Although the expression in KMS-12-BM and KMS-21-PE cells was not altered by either agent, expression in KMS-18 cells was up-regulated to 4.31 and 3.80 from 1.93 by cultivation with TAM and TOR, respectively.

Fig. 8. KMS-21-PE cells cultured with or without 10^{-5} M TAM or TOR for 2 days were subjected to a flow cytometric cell cycle analysis using propidium iodine staining. The X axis shows the DNA indices, and the Y axis shows the cell number. The percentage of hypodiploid (apoptotic) cells among the total cells is indicated in each panel. In addition, the percentage of cells in G1, S phase, and G2-M phase in the diploid fraction is exhibited in each panel. An increase in the hypodiploid fraction and the G1 phase percentage along with a reduction in the S-phase fraction was exhibited in cells cultured with TAM or TOR.
ACNOWLEDGEMENTS

We thank YumiKA Isozaki, Sakura Eda, and Yukani Tanaka for skillful technical assistance.

REFERENCES

Estrogen Receptors in Human Myeloma Cells
Takemi Otsuki, Osamu Yamada, Junichi Kurebayashi, et al.


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