Hormone-induced Apoptosis by Fas-Nuclear Receptor Fusion Proteins: Novel Biological Tools for Controlling Apoptosis in Vivo

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ABSTRACT

We have created fusion proteins between Fas and the ligand-binding domain of the estrogen or retinoic acid receptor. Murine fibrosarcoma L929 cells and human cervical carcinoma HeLa cells expressing the fusion proteins demonstrated apoptotic phenotypes in a tightly estrogen- or retinoic acid-dependent manner in vitro. Moreover, the fusion protein-expressing L929 cells transplanted into nude mice were also killed through apoptosis after injection of an estrogen agonist. This represents a novel system, "cell-targeting," that can eliminate cells not only in vitro but also in vivo through the activation of a natural suicide machinery, i.e., apoptosis, by currently used hormones. This system implies wide applications not only in developmental biology and neurobiology but also in medicine, especially for cancer gene therapy.

INTRODUCTION

Recently, a novel approach to willingly regulate the protein functions in cultured cells has been demonstrated, where the LBDs of the nuclear receptors are fused to some proteins, leading to conversion of their bona fide functions into hormone-dependent functions (1). The examples of the target proteins include oncoproteins (2–4), kinases (5, 6), and transcription factors (7–15); this approach has revealed the molecular aspects of highly complicated biological phenomena such as control of the cell cycle (13), determination of epithelial polarity (14), and differentiation of muscle (11).

We have previously identified a fused retinoic acid receptor with a novel protein, PML, in human APL and proposed a model in which chromosomal translocation t(15;17) places the PML protein under the control of the RAR LBD (15). Recently, we have demonstrated experimental evidence that supports our model (16). Because the PML-RAR fusion protein definitely contributes to the remission of APL by treatment with a high dose of retinoic acid (15–17), we wondered whether some other fusion protein, if designed adequately, could contribute to curing cancers other than APL. The most straightforward approach to curing cancers would be to kill the cancerous cells in the bodies, ideally through apoptosis, the natural suicide mechanism. To this end, we decided to create Fas-nuclear receptor fusion proteins and to examine the possibility that they might induce hormone-dependent apoptosis in cancerous cells.

Fas (18, 19), which is also referred to as APO-1 (20), is a type 1 membrane protein and is a member of the nerve growth factor receptor/TNFR family. Fas and TNFR p55 have been shown to mediate apoptotic signals depending on their ligands or stimulatory antibodies (18, 20). The intracellular domains of Fas and TNFR p55 that mediate the apoptotic signals have been identified and referred to as death domains (21, 22). Consistently, these domains are highly conserved between Fas and TNFR p55 (22, 23). In this report, we show the construction of several Fas-nuclear receptor fusion cDNAs and their expression in cultured cells and demonstrate that these Fas-nuclear receptor fusion proteins can induce hormone-dependent apoptosis not only in vitro but also in vivo.

MATERIALS AND METHODS

Plasmids. All plasmids were made following general procedures (24). The mouse Fas cDNAs (25), which encode amino acids 135 to 305 for MfasER and 184 to 305 for fasER, were amplified by PCR (15, 26) from a mouse T-cell cDNA library (26). The cDNAs for the LBD of ER was amplified from a rat ER cDNA (Ref. 27; a kind gift from Dr. M. Muramatsu). The cDNAs for the human RAR LBD (amino acids 176 to 462; Refs. 28 and 29) and for the Drosophila EcR LBD (amino acids 404 to 878; Ref. 30) were also amplified by PCR. The amplified cDNAs were cut with appropriate restriction enzymes and ligated into the expression plasmids pEF-BOS-bsr with an optimal translational initiation site (31) followed by the influenza virus hemagglutinin epitope, YPYDVPDYA, which is recognized by a monoclonal antibody, 12CA5 (Boehringer Mannheim). pEF-BOS-bsr has a basicidin S selection marker (32) in pEF-BOS (33).

Fractionation of Cellular Proteins. COS cells transfected with expression vectors for MfasER or fasER were collected, and cellular proteins were divided into membrane and cytoplasmic fractions as described (34). Each sample was separated on 10% SDS-PAGE and transferred to a nitrocellulose membrane (Schleicher & Schuell). The membrane was reacted with anti-HA polyclonal antibody (BAbCO) or anti-HSC70 polyclonal antibody (Ref. 35; a kind gift from Drs. M. Satoh and K. Nagata, Chest Disease Institute, Kyoto University), and the signals were detected by the biotin/avidin method (VECTASTAIN ABC kit; Vector Laboratories).

Cell Cultures. L929 cells (a kind gift from Dr. S. Nagata) and HeLa cells (American Type Culture Collection) were maintained in DMEM supplemented with 10% calf serum (HyClone). The fusion protein-expressing cells were established by the following procedures. L929 or HeLa cells were transfected with the linearized expression plasmids by the calcium-phosphate method (36, 37). After selection with blasticidin S (Funakoshi; 2 μg/ml for L929 and HeLa cells), the cells were cloned. MER3c and MER6 were cloned cells expressing MfasER, and MRAR5f were cloned cells expressing MfasER. The expression of the fusion proteins was confirmed by Western blot using the 12CA5 antibody.

Animals. BALB/c nude mice were purchased from Japan SLC Inc. (Shizuoka) and were kept at specific pathogen-free conditions. All animals were handled in accordance with guidelines established by Kyoto University. Two million cells were s.c. implanted in each 4-week-old nude mouse (38). After 16 days, the longest diameters of the tumors were measured. No clear difference of the tumor growth was observed between male and female mice. Sixteen days after implantation of the cells, 10 mg estradiol benzoate were s.c. injected into the nude mice, and 6 days later another 10 mg were injected in areas far from the tumor (39). The tumor volumes were measured every 3 days after the first injection of estradiol benzoate.

RNA Preparation and Northern Blot Analysis. RNAs were purified by the guanidinium acid phenol method (40) from tumors originated from parental L929 cells and tumors from MfasER-expressing L929 cells before and 1 month after injection of E$_2$-benzoate. Northern blot was probed with a P$^{32}$-labeled rat ER cDNA as described (41).
Assays for Cell Death. The low molecular weight DNA was purified as described (42). The nuclear morphology was examined by DAPI staining (16). Cells were photographed through a Zeiss fluorescence microscope at ×80. Transmission electron micrographs were taken using Nihon-Denshi JEM-1210. For the quantification of the dead cells, the cells were stained with DAPI, and the condensed nuclei were counted before and 2.5, 5, and 7.5 h after the addition of $10^{-7}$ m $E_2$. For the ligand dose-dependency assay, MER3c, MER6, or parental L929 cells were incubated with various concentrations of estradiol and its analogues ($10^{-13}$ to $10^{-6}$ m) at 37°C for 16 h and stained with crystal violet. The percentage of viable cells was calculated as described (18, 23). To detect the dying cells in the tumor sections, ApopTag (Oncor) was used following the manufacturer’s protocol. ApopTag is based on terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling methods (43), and the dying cells with DNA fragmentations are stained in brown.

RESULTS

Construction and Expression of a Fas-Estrogen Receptor Fusion Protein. Among the lipophilic hormones whose functions are mediated by nuclear receptors, estrogen is one of the ideal drugs for in vivo usage: it is currently used for the treatment of human diseases such as hypogonadism, postmenopausal hormone deficiency, and osteoporosis, and its pharmacokinetics as well as side effects, e.g., nausea, breast tenderness, hyperpigmentation, headaches, cholestasis, hypertension, and others, have been well characterized (44). Therefore, we made several fusion constructs between Fas and the ER and examined whether these fusion proteins could induce estrogen-dependent apoptosis in cultured cells. Fig. 1A shows the two representative constructs between Fas and ER; one construct is a fusion between the transmembrane and cytoplasmic domains of the mouse Fas and the LBD of the rat ER (referred to as MfasER) and the other between the Fas cytoplasmic portion with the death domain and LBD of the rat ER (referred to as fasER). Both MfasER and fasER are designed to have an optimal translational initiation site (31) followed by an influenza virus HA epitope (45) recognized by a monoclonal antibody, 12CA5 (16). Both constructs were verified by sequence analysis and gave rise to proteins with expected sizes, $M_r$ 57,000 for MfasER and $M_r$ 52,000 for fasER, when expressed in cultured COS cells (Fig. 1B). As expected from the structural features, MfasER and fasER were predominantly localized in the membrane and cytoplasmic fractions, respectively (Fig. 1B).

Hormone-Induced Apoptosis in Cultured Cells. We have established several L929 cells constitutively expressing the chimeric receptors. L929 cells have been reported to be sensitive to Fas-mediated apoptosis, indicating that L929 cells have the intracellular signaling machinery to mediate the apoptotic signal from the death domain. The expression of MfasER and fasER were confirmed by the Western blot using the anti-HA antibody 12CA5. Both parental L929 and MfasER- or fasER-expressing L929 cells grew equally well in the standard cell culture conditions, where the cells were maintained in DMEM supplemented with 10% calf serum. The parental and fasER-expressing L929 cells did not demonstrate any phenotypic changes in the presence or absence of $E_2$ (Fig. 2B), whereas in contrast, the MfasER-expressing cells underwent clear phenotypic changes in the presence of $E_2$. The cells became shrunken, demonstrated cytoplasmic fragmentation, and were detaching from the dishes. All of these morphological changes were indistinguishable from those seen in apoptosis mediated by the wild-type Fas (Ref. 18; Fig. 2A). Consistently, the MfasER-expressing cells but not the parental or fasER-expressing L929 cells treated with $E_2$ demonstrated DNA fragmentation, a well-known marker for apoptotic cell death, as early as 1 h after the addition of $E_2$ (Fig. 2, C and D).

To confirm that the observed morphological changes are due to

![Figure 1](#)
addition of 10^{-7} \text{ m} \text{E}_2, and almost all nuclei were condensed 7.5 h after the addition of \text{E}_2 (Fig. 3F).

Ligand Dose Dependency Effect on Apoptotic Cell Death. We next examined the effect of ligand dose dependency on cell death. As shown in Fig. 4A, two independent clones expressing MfasER showed similar responses to different concentrations of \text{E}_2, and the \text{E}_2 concentrations above 10^{-10} \text{ m} resulted in significant cell death, with the \text{IC}_{50} of \sim 3 \times 10^{-10} \text{ m}. The sensitivity did not change in the presence of actinomycin D (0.5 \mu g/ml; Fig. 4A). This result was unexpected since the L929 cells expressing human Fas have been reported to show enhanced cell death with actinomycin D (18). Although the reason for this discrepancy remains to be elucidated, MfasER seemed to be more effective in mediating the apoptotic signal than the wild-type Fas. One possibility would be that the LBD of ER fused at the COOH-terminal portion following the death domain and might sterically interfere with the access of a negative regulator of Fas-mediated signaling, which has been reported to be actinomycin D sensitive (23, 46). We also observed that HeLa cells expressing MfasER died in an \text{E}_2 dose-dependent manner with a similar \text{IC}_{50} (data not shown). Next, we examined the potency of different \text{E}_2 analogues to induce apoptosis (Fig. 4B). The rank order of inducing apoptosis by the \text{E}_2 analogues well paralleled their agonistic activities on the wild-type ER (47, 48). Interestingly, a competitive partial agonist inhibitor of \text{E}_2, tamoxifen (44, 49), showed an agonistic activity at a high concentration (10^{-7} \text{ m}; Fig. 4B).

To examine the possibility that the LBDs of different nuclear receptors could substitute the LBD of ER, we next fused the portion of Fas to the LBD of either the RAR (28, 29) or the EcR (30), an insect

Fig. 2. Estrogen-dependent cell death by the Fas-estrogen receptor fusion protein in cultured L929 cells. The morphologies of the MfasER-expressing cells (A) and the parental L929 cells (B) after treatment of 10^{-7} \text{ m} \text{estradiol for 8 h}. Low molecular weight DNAs recovered from the MfasER-expressing cells (C) and the parental cells (D) are shown in 2% agarose gel after 0 h (Lane 2), 1 h (Lane 3), 2 h (Lane 4), and 4 h (Lane 5) treatment with 10^{-7} \text{ m} \text{estradiol}. The DNA cut by HindIII is included as size markers (Lane 1). Note that estradiol treatment did not induce any morphological changes or DNA fragmentations in the fasER-expressing L929 cells (data not shown).

Fig. 3. The apoptotic features of estrogen-induced cell death. A-C, morphologies of the MfasER-expressing cloned L929 cells (MER3c) before (A) and after the addition of 10^{-7} \text{ m} \text{estradiol for 2.5 h} (B) and 5 h (C). The nuclei were stained with DAPI (A, B, and C, left panels). Parental L929 cells did not show any such morphological changes in the presence of \text{E}_2 (data not shown). The apoptotic morphologies were further confirmed by transmission electron microscopy. In the presence of \text{E}_2, the MfasER-expressing cells (Fig. 3D), but not parental L929 cells (Fig. 3E), demonstrated chromatin condensation, chromatin margination along the inner nuclear membrane, cytoplasmic fragmentation, and membrane blebbing, all of which are also typical morphological features of apoptotic cell death. We then quantified the dead cells through the treatment of \text{E}_2 by counting the condensed nuclei. About 25% of the nuclei were condensed 2.5 h after

Fig. 4. Ligand dose dependency effect on apoptosis. A, two independent clones expressing MfasER showed similar responses to different concentrations of \text{E}_2, and the \text{E}_2 concentrations above 10^{-10} \text{ m} resulted in significant cell death, with the \text{IC}_{50} of \sim 3 \times 10^{-10} \text{ m}. The sensitivity did not change in the presence of actinomycin D (0.5 \mu g/ml; Fig. 4A). This result was unexpected since the L929 cells expressing human Fas have been reported to show enhanced cell death with actinomycin D (18). Although the reason for this discrepancy remains to be elucidated, MfasER seemed to be more effective in mediating the apoptotic signal than the wild-type Fas. One possibility would be that the LBD of ER fused at the COOH-terminal portion following the death domain and might sterically interfere with the access of a negative regulator of Fas-mediated signaling, which has been reported to be actinomycin D sensitive (23, 46). We also observed that HeLa cells expressing MfasER died in an \text{E}_2 dose-dependent manner with a similar \text{IC}_{50} (data not shown). Next, we examined the potency of different \text{E}_2 analogues to induce apoptosis (Fig. 4B). The rank order of inducing apoptosis by the \text{E}_2 analogues well paralleled their agonistic activities on the wild-type ER (47, 48). Interestingly, a competitive partial agonist inhibitor of \text{E}_2, tamoxifen (44, 49), showed an agonistic activity at a high concentration (10^{-7} \text{ m}; Fig. 4B).
Hormone-Induced Apoptosis in Vivo. Our in vitro data prompted us to examine whether this system is valid in vivo. The MfasER-expressing and parental L929 cells were s.c. transplanted into nude mice. Sixteen days after the transplantation, the tumor sizes were examined. In the physiological condition, where the animals were not treated with any drugs, both types of cells grew equally well and made visible tumors with similar sizes (Fig. 5A). Next, all animals were treated with estradiol benzoate, a metabolically stable E2 agonist. During the treatment with estradiol benzoate, all animals looked healthy, and no signs of severe side effects were observed; they ate well and moved well, although most mice seemed to have dry skin. The tumors from the parental L929 cells continuously grew, even in the presence of estradiol benzoate (Fig. 5B). In contrast, little growth of the tumors from the MfasER-expressing cells was observed (Fig. 5B). After a long period, however, E2-resistant tumors became apparent and kept growing. We observed that the long-cultured, MfasER-expressing L929 cells lose their sensitivities to E2 due to loss of MfasER expression. Similar inactivation of the promoters have been reported in the long-cultured cells (38, 53). We, therefore, speculated that the observed in vivo resistance to E2 also resulted from the loss of MfasER expression. We compared the expression levels of MfasER mRNAs between E2-resistant tumors and either control tumors or MfasER-expressing tumors before E2 treatment (Fig. 5C). Accordingly, E2-resistant tumors showed greatly decreased or little expression of MfasER mRNA. These results indicate that the loss of E2 sensitivities in the residual tumors is due to lack of MfasER proteins.

After treatment of estradiol benzoate, the animals were sacrificed, and the tumors were examined for evidence of apoptosis using the terminal deoxytransferase-mediated deoxyundine-digoxigenin nick end labeling assay (43), where the nuclei with fragmented DNA were stained in brown. The cells from the non-growing tumor demonstrated apoptotic phenotypes. More than 60% of the cells in the non-growing tumor section were clearly positive, and an additional 30% of the cells were weakly positive for the apoptotic marker; some showed typical condensed nuclei (Fig. 5, C and D). These differences would be due to different levels of MfasER expression. Consistently, the DNA recovered from the non-growing tumors specifically demonstrated clear DNA fragmentations (Fig. 5E).

DISCUSSION

In this report, we presented evidence that Fas-nuclear receptor fusion proteins can induce hormone-dependent apoptosis not only in cultured cells but also in vivo. MfasER induced apoptosis in cultured cells at a very low level of E2, around $10^{-10}$ M (Fig. 3), which is, however, still higher than the physiological concentration of E2 in female mice ($1 \sim 10^{-11}$ M; Ref. 54). Consistently, both male and female mice could develop tumors from the MfasER-expressing cells. This result is further supported by the evidence that only free estrogen manifests its hormonal actions; the plasma contains more estrogen-binding proteins than the culture medium (55). Eventually, the addition of a high dose of exogenous hormones to the animals could easily induce apoptosis in vivo (Fig. 5). This observation is reminiscent of the PML-RAR in APL; the PML-RAR protein shows similar binding affinity to retinoic acid as compared to the wild-type RAR, but the PML-RAR react only with a high concentration of retinoic acid, thus inducing differentiation of the APL cells in vivo (56).

This study also demonstrated that the intracellular signals from the death domain of the membrane-bound receptor could be regulated with the combination of the nuclear receptor LBDs and their ligands. This is the first demonstration that the LBDs are exchangeable between membrane-bound and nuclear receptors to transduce the signals originally mediated from the membrane-bound receptors. The TNF

![Fig. 4. Dose response of nuclear receptor ligands on the induction of apoptosis. A, the cell-killing effects of various concentrations of estradiol were analyzed on two independently cloned cells expressing MfasER (MER3c and MER6) as well as the parental L929 cells. B, the cell-killing effects of various concentrations of estradiol analogues, estradiol benzoate, estriol, and tamoxifen on the MfasER-expressing cells (MER3c). Note that parental L929 cells were refractory to all estradiol analogues (data not shown). C, the cell-killing effects of various concentrations of retinoic acid were analyzed on cloned cells expressing MfasRAR (MRAR5f) as well as the parental L929 cells. The percentage of viable cells is presented. The mean values of triplicated experiments were calculated; bars, SD. Where bars are not shown, they are smaller than the symbols.

nuclear receptor. To reduce side effects in vivo, the insect hormones such as ecdysone might be ideal, since human cells do not have receptors for them (50). The L929 and HeLa cells expressing MfasRAR also died in an RA-dependent manner (Fig. 4C and data not shown). However, MfasEcR could not mediate ligand-dependent apoptosis in these cells, which would be explained by the observation that the EcR requires a heterodimer partner, ultraspiracle, to bind its ligand (51, 52). Co-expression of ultraspiracle or its human counterpart, retinoid X receptor, might allow the MfasEcR to induce apoptosis in an ecdysone-dependent manner (50).
reduce the death signal from Fas to interleukin 1k-converting enzyme family of cysteine protease, key molecules involved in apoptosis (65, 66).

In several signal transductions through membrane-bound receptors, recruitment of signal-mediating molecules into or near the plasma membrane has been shown to be important (60, 61). Consistently, the MfasER protein was found in a membrane fraction when examined by subcellular fractionation and Western blot techniques, and a transmembrane domain-lacking fusion protein, fasER, could not induce apoptosis nor was it anchored in the membrane. Therefore, the intracellular localization of the MfasER protein may also be important in its activation mechanism. The requirement of the membrane attachment for death signaling suggests the existence of target molecules in or near the plasma membrane. In fact, several proteins interacting with the death domain have been cloned by the yeast two-hybrid system (62–64). Among them, MORT1/FADD has been proposed to transduce the death signal from Fas to interleukin 1β-converting enzyme family of cysteine protease, key molecules involved in apoptosis (65, 66).

and its receptor have been shown to form a trimeric structure (57) when mediating the signal, and it has also been shown that antibodies to Fas must link multiple receptors to activate the apoptotic signals; the dimer formation of Fas is not enough for its activation (21). These observations suggest the possibility that ER and RAR could form higher order complexes in a ligand-dependent manner, either when separated from the response elements or when the receptors are chimeric as indicated in this study, although ER and RAR have been well demonstrated to make homodimers and heterodimers, respectively, on their hormone response elements (58). Alternatively, the ligand-bound MfasER may allow the death domain to multimerize, since the death domains have the intrinsic ability to multimerize by themselves (59).

In several signal transductions through membrane-bound receptors, recruitment of signal-mediating molecules into or near the plasma membrane has been shown to be important (60, 61). Consistently, the MfasER protein was found in a membrane fraction when examined by subcellular fractionation and Western blot techniques, and a transmembrane domain-lacking fusion protein, fasER, could not induce apoptosis nor was it anchored in the membrane. Therefore, the intracellular localization of the MfasER protein may also be important in its activation mechanism. The requirement of the membrane attachment for death signaling suggests the existence of target molecules in or near the plasma membrane. In fact, several proteins interacting with the death domain have recently been cloned by the yeast two-hybrid system (62–64). Among them, MORT1/FADD has been proposed to transduce the death signal from Fas to interleukin 1β-converting enzyme family of cysteine protease, key molecules involved in apoptosis (65, 66).

The functions of genes have been examined by disrupting the genes by the gene targeting technique. Our system, in combination with the tissue-specific promoter and transgenic animal technique, would make it possible to delete the specific cells at any time in vivo, which we refer to as cell targeting. For this purpose, the lipophilic hormones, e.g., estradiol and retinoic acid, have several advantages in triggering cell death in vivo. They can be easily delivered throughout the body; they can even go through the blood-brain barrier, which blocks the peptide ligand. In addition, they are less antigenic, resulting in prevention of inactivation by antibodies produced in vivo. It is easily imagined that this system implies wide applications in developmental biology and neurobiology. Furthermore, targeting specific cells by this system may allow the production of animal models with degenerative diseases such as muscular dystrophy, Parkinsonism, or amyotrophic lateral sclerosis.

Another medical application would be for cancer gene therapy. For treating cancer, two big questions remain to be answered, i.e., what gene to use and how to introduce the gene to the cancers. We have observed that the apoptosis is induced in many, if not all, cancerous cells by the liganded MfasER. Therefore, MfasER is an ideal molecule for cancer gene therapy. Although it seems very difficult to introduce exogenous genes exclusively into cancer cells, it would be possible to express the exogenous gene products in a cancer-specific manner. Recently, we have succeeded in expressing β-galactosidase in almost all mouse liver cells in vivo by a recombinant adenovirus vector, and more and more promoters have become available that allow gene expression in a cancer cell-specific manner. Therefore, the
combination of highly efficient gene introduction systems, together with the cancer-specific promoters, theoretically makes it possible to express the exogenous gene specifically in the cancer cells in vivo. As another approach, the exogenous gene could be restrictedly expressed with the cancer-specific promoters, theoretically making it possible to achieve a contribution toward treatment.

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