Fas and Mutant Estrogen Receptor Chimeric Gene: A Novel Suicide Vector for Tamoxifen-inducible Apoptosis

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Several cancer gene therapy strategies involve suicide genes to kill the neoplasm, or to regulate effector cells such as lymphocytes. We have developed an inducible apoptosis system with a Fas-estrogen receptor fusion protein (MfasER) for rapid elimination of transduced cells. In the present study, we further improved this molecular switch for estrogen-inducible apoptosis to overcome concerns with the wild-type estrogen receptor and its natural ligand, 17β-estradiol (E2). The ligand-binding domain of MfasER was replaced with that of a mutant estrogen receptor which is unable to bind estrogen yet retains affinity for a synthetic ligand, 4-hydroxytamoxifen (Tm). The resultant fusion protein (MfasTmR) and MfasER were expressed in L929 cells for examination of their ligand specificities. Tm induced apoptosis in MfasTmR-expressing cells (L929MfasTmR) at $10^{-8}$ M or higher concentrations, but induced no apoptosis in MfasER-expressing cells (L929MfasER) at up to $10^{-6}$ M. On the other hand, E2 induced apoptosis in L929MfasER at concentrations as low as $10^{-10}$–$10^{-9}$ M, while it did so partially in L929MfasTmR at concentrations greater than $10^{-7}$ M. Thus, L929MfasTmR cells were highly susceptible to Tm, but refractory to E2, with 100–1,000 times more tolerance than L929MfasER. These results suggest that the MfasTmR/Tm system would induce apoptosis in the target cells more safely in vivo, working independently of endogenous estrogen.

Key words: Fas — Mutant estrogen receptor — Tamoxifen — Inducible apoptosis — Gene therapy

Application of suicide genes has been explored in several gene therapy strategies against cancer. Such approaches include direct killing of neoplastic cells, eradication of donor lymphocytes responsible for severe complications of allogeneic bone marrow transplantation such as graft-versus-host disease, and elimination of cytokine producer cells to terminate supplement gene therapy.1–6) So far the herpes simplex virus-1 thymidine kinase (HSV-TK) gene has been most widely used as a suicide gene, which confers sensitivity to antiviral agents such as ganciclovir (GCV) on mammalian cells.1, 7–9) Several clinical studies involving HSV-TK are currently under way, but this virus-directed enzyme/prodrug system has some limitations.10) For instance, it may not promptly kill slowly-growing cells since the cytocidal activity of HSV-TK/GCV depends on DNA synthesis; GCV may cause non-specific bone marrow suppression; immunological reactions may be elicited against HSV-TK to result in premature elimination of the transduced cells.

Recently, an alternative approach to the HSV-TK/GCV system has been developed.11, 12) This strategy is based on the fact that the ligand-binding domain (LBD) of a nuclear receptor can work as a molecular switch to control heterologous proteins. Various effector proteins have been converted to function in a ligand-dependent manner, by fusing them with the LBD of several nuclear receptors.13, 14) Among them, an apoptosis-inducing system was designed with Fas and the LBD of estrogen receptor (ER). When the transmembrane and cytoplasmic domains of Fas (Mfas) were fused with ER, expression of the chimeric molecule resulted in estrogen-induced apoptosis in L929 cells both in vitro and in vivo.11) The transmembrane domain of Fas was required for the fusion protein to transmit death signals, since the chimera without it failed to do so. The estrogen-induced apoptosis was rapid and extensive, whether the challenged cells were proliferating or resting, unlike the HSV-TK/GCV system that was ineffective against non-proliferating cells. Immunological reactions against the fusion protein are less likely to be elicited than against HSV-TK, because MfasER is composed of endogenous proteins.

However, the MfasER/estrogen system has raised several practical concerns. To minimize background apoptosis in cultured MfasER-expressing cells, one must remove serum estrogen and other agonistic substances such as phenol red from the media.15) Similar concerns will apply for in vivo treatment when LBDs of wild-type nuclear receptors are used as molecular switches. As for ER, elevated endogenous estrogen in females may evoke unwanted activation of the effector proteins. In addition,
exogenously administered estrogen may have adverse effects, including stimulating the growth of carcinoma cells from estrogen-dependent tissues.46

To overcome these concerns, we employed a mutant murine ER, G525R (TmR).17,18 This mutant selectively binds to a synthetic steroid, 4-hydroxytamoxifen (Tm), to be dimerized as the wild-type ER is activated upon binding of 17β-estradiol (E2). In this study, we constructed a cDNA encoding the fusion protein of Mfas and the LBD of TmR (MfasTmR), and examined whether this chimera selectively induces apoptosis in the target cells.

MATERIALS AND METHODS

Plasmids Retroviral expression vectors containing MfasER and MfasTmR were constructed as follows. MfasER cDNA was derived from pEF-BOS/MfasER.11 An internal ribosome entry site (IRES) sequence was amplified by polymerase chain reaction (PCR) on encephalomyocarditis virus genome (nucleotides 259–833), using primers A (5′-GACAATGACGCTGTGATGAAATGTTGAACAAGAT-3′) and D (5′-GGAGACATGAGAGCTG-3′) and F (5′-GCATTCCTAGGGGTCTTTCC-3′).21) Primers B and C were designed to create an overlap between the 3′-end of the IRES and the 5′-end of the neo PCR products, and IRESneo fragment was PCR-amplified on them with primers A and D. The prepared MfasER and IRESneo fragments were cloned into pMX retroviral vector (a gift from Dr. T. Kitamura, University of Tokyo, Tokyo).22) The resulting bicistronic retroviral vector was designated as pMX/MfasER-IRESneo. An MfasTmR expression vector (pMX/MfasTmR-IRESneo) was constructed by replacing a portion of mouse Fas (amino acids 135–305) and the carboxyl-half of murine TmR (amino acids 287–599).18,25) Primers E (5′-GATCTGGATCCGGCGACATTCA-GGAGACATGAGAGCTG-3′) and F (5′-GCATTCCTAGGGGTCTTTCC-3′), with primer E designed to modify the junctional sequence of MfasTmR to be identical to that of MfasER.

Cells L929 mouse fibroblasts (from Dr. M. Hayakawa, Setsunan University, Osaka) were maintained in Dulbecco’s modified minimum essential medium supplemented with 10% fetal calf serum (Filtron, Brooklyn, Australia).23) L929 cells were transfected with either pMX/MfasTmR-IRESneo or pMX/MfasER-IRESneo using Lipofectamine transfection reagent (Life Technologies, Grand Island, NY), following the manufacturer’s protocol. After selection with 1 mg/ml G418 (Life Technologies), G418-resistant clones were selected and MfasTmR-expressing (L929MfasTmR) and MfasER-expressing (L929MfasER) transfectants were established.

Western blot analysis L929MfasTmR cells were harvested and lysed with NP-40 buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% NP-40, 100 IU/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). Cell lysates (10 μg per lane) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to Immobilon-P polyvinylidene fluoride membrane (Millipore, Yonezawa). The membrane was incubated with an anti-ER monoclonal antibody, MC-20 (Santa Cruz Biotechnology, Santa Cruz, CA), and MfasTmR was visualized with an ECL detection kit (Amersham, Little Chalfont, UK).

Morphological examinations L929MfasTmR cells were plated (1×10⁶ cells per 100 mm dish) the day prior to assay. After designated periods of incubation with no ligand, 10⁻⁷ M E₂ (Sigma, St. Louis, MO), or 10⁻⁷ M Tm (Sigma), the cells were examined on an inverted microscope and photographed (original magnification, ×400). For visualization of the nuclei, L929MfasTmR cells were plated on Lab-Tek chamber slides (Nunc, Naperville, IL) in the absence or presence of 10⁻⁷ M Tm. After 4 h of culture, cells were stained with 10⁻⁶ M Hoechst 33258 dye (Sigma) at 37°C for 2 h, and observed under a fluorescence microscope (original magnification, ×400).

DNA fragmentation assay Parent L929, L929MfasER, and L929MfasTmR (5×10⁵ cells) were exposed to no ligand, 10⁻³ M E₂, or 10⁻³ M Tm at 37°C for 4 h. Low-molecular-weight DNA was extracted with an ApopLadder Ex kit (TakaRa, Otsu) and electrophoresed on a 2% agarose gel to detect fragmented DNA.

Ligand dose-cytotoxicity assay Ligand dose-cytotoxicity assay was conducted in 96-well microtiter plates as described.24) In brief, 2×10⁴ parent L929, L929MfasER and L929MfasTmR cells were preincubated in 100 μl for 3 h and incubated with various concentrations (0, 10⁻¹²–10⁻⁶ M) of E₂ or Tm at 37°C for 16 h. The challenged L929 cells were stained with crystal violet, and dye uptake was quantitated by measuring A590.

RESULTS

Construction and expression of the MfasTmR fusion protein The structure of MfasTmR, the fusion protein used in this study, is depicted in Fig. 1. The MfasTmR cDNA encodes the transmembrane through cytoplasmic portion of mouse Fas (amino acids 135–305) and the carboxyl-half of murine TmR (amino acids 287–599).16,25) The intracellular region of Fas contains the “death domain” of about 70 amino acids, which is necessary and sufficient for apoptotic signal transduction.26) The carboxyl-half of murine TmR contains an LBD with a single amino acid change from glycine to arginine at position...
Fig. 1. Schematic structures of Fas, mutant estrogen receptor G525R (TmR), and MfasTmR fusion protein. The amino acid numbers of the original Fas and TmR are shown above and below the schematics, respectively. The death domain (DD; hatched box) covers amino acids 201 to 286 in mouse Fas. TmR contains a single amino acid substitution of arginine for glycine at position 525. TM (black box), transmembrane domain of Fas. LBD (shaded box), ligand-binding domain of TmR.

Fig. 2. Western blot analysis of L929MfasTmR cells with an anti-ER antibody. Lane −, untransfected L929 cells; lane +, L929MfasTmR-transfected L929 cells. The arrowhead indicates MfasTmR protein.

Fig. 3. Morphological changes in L929MfasTmR cells on 17β-estradiol (E2) and 4-hydroxytamoxifen (Tm) treatment. Untreated (A, 0 h; D, 6 h; G, 24 h) and 10⁻⁷ M E₂-treated (B, 0 h; E, 6 h; H, 24 h) L929MfasTmR cells grew well, whereas 10⁻⁷ M Tm-treated cells shrank markedly and started to detach from the culture dishes after 6 h (panel F), and died within 24 h (panel I). Panel C, L929MfasTmR cells before Tm treatment.
As a result, TmR is unable to bind estrogen yet retains affinity for Tm. L929 fibroblasts were lipofected with pMX/MfasER-IRESneo or pMX/MfasTmR-IRESneo and the G418-resistant colonies were isolated. A subset of each colony was tested for responsiveness to E₂ or Tm. Representative populations well responsive to E₂ and Tm were designated as L929MfasER and L929MfasTmR, respectively. Expression of MfasTmR protein was determined by western blot analysis, as shown in Fig. 2. MfasTmR was detected as a 57 kDa protein with an anti-ER antibody (right lane), which was not present in the untransfected L929 cells (left lane).

Selective induction of apoptosis in MfasTmR-expressing L929 cells by 4-hydroxytamoxifen

We have previously shown that MfasER induced apoptosis under the control of estrogen. We challenged L929MfasTmR cells with E₂ or Tm, to determine whether MfasTmR is selectively activated by Tm to induce apoptosis. As shown in Fig. 3, 10⁻⁷ M E₂ had no effect on L929MfasTmR cells (panels B, E and H), and these cells grew like the untreated cells (panels A, D and G). When incubated with 10⁻⁷ M Tm, however, L929MfasTmR cells showed marked cell death (panels C, F and I). During a few hours of incubation with Tm, L929MfasTmR cells began to display morphological changes such as nuclear and cytoplasmic shrinkage, formation of apoptotic bodies, and detachment from the culture dishes. These phenomena are characteristic of apoptosis and were even more remarkable after 6 h of Tm treatment; most cells died within 24 h.

To illustrate the changes of nuclei on apoptosis, L929MfasTmR cells were stained with Hoechst 33258 dye before and after Tm treatment. Fluorescence micrographs revealed highly condensed nuclei in Tm-treated L929MfasTmR cells (Fig. 4B), corresponding to the apoptotic changes in the cytoplasm.

DNA fragmentation in 4-hydroxytamoxifen-treated L929MfasTmR cells

Tm-specific induction of apoptosis in L929MfasTmR was further confirmed by DNA fragmentation analysis. Parent L929, L929MfasER and L929MfasTmR cells were exposed to 10⁻⁷ M E₂ or 10⁻⁷ M Tm, and low-molecular-weight DNA preparations were examined. At as early as one hour of incubation, DNA fragmentation was detected in Tm-treated L929MfasTmR and E₂-treated L929MfasER cells. DNA ladders in those cells were prominent after 4 h (Fig. 5, lanes 3 and 4). No DNA fragmentation was observed in parent L929 cells with E₂ or Tm (Fig. 5, lanes 1 and 2). This assay demonstrated the specificity of MfasER and MfasTmR for their ligands to induce apoptosis.

Dose-response of L929MfasER and L929MfasTmR cells to estrogen agonists

Finally, we examined the dose-response of wild-type and the transfected L929 cells to estrogen agonists. Cell viability was measured after 16 h of incubation with various concentrations of Tm or E₂ (Fig. 6). Neither ligand had an effect on the viability of parent L929 cells, indicating very low background apoptosis in the untransfected cells (Fig. 6A). As for L929MfasTmR, the cells were susceptible to Tm but refractory to E₂. That is, while more than 90% of L929MfasTmR cells were killed by Tm at 10⁻⁸ M or higher concentrations, most cells remained intact up to 10⁻⁷ M E₂ and only 30% of the cells were apoptotic with 10⁻⁶ M E₂ (Fig. 6B). In contrast, apoptosis was observed in a fraction of L929MfasER cells at 10⁻¹⁰ M E₂ and was fully induced at 10⁻⁹ M or greater, whereas the same cells showed no apoptosis with Tm in the range tested in this study (up to 10⁻⁶ M) (Fig. 6C). These results demonstrated that MfasTmR was specifically controlled by Tm to induce apoptosis, being 100- to 1,000-fold less sensitive to E₂ than MfasER.

Fig. 4. Fluorescence microphotographs of L929MfasTmR cells stained with Hoechst 33258 after Tm treatment. (A) L929MfasTmR cells without Tm. (B) L929MfasTmR cells incubated with 10⁻⁷ M Tm for 4 h. Characteristic condensed nuclei were observed, indicating apoptosis in these cells.
Fig. 5. DNA fragmentation in E2- or Tm-treated L929 cells. Low-molecular-weight DNA extracted from wild-type L929 (lanes 2–4), L929MfasTmR (lanes 6–8), and L929MfasER (lanes 10–12) cells on a 2% agarose gel. Lanes 1, 5, 9: DNA molecular weight markers (φX174/HaeIII digest). Lanes 2, 6, 10: DNA from untreated cells. Lanes 3, 7, 11: DNA from E2-treated cells. Lanes 4, 8, 12: DNA from Tm-treated cells.

Fig. 6. Dose-cytotoxicity of estrogen agonists on L929 cells. (A) Parent L929 cells with Tm (open circles) and E2 (closed circles). (B) L929MfasTmR cells with Tm (open circles) and E2 (closed circles). (C) L929MfasER cells with Tm (open circles) and E2 (closed circles). Parent and derivative L929 cells were incubated with various concentrations (0, 10^{-12}–10^{-6} M) of Tm or E2 for 16 h and stained with crystal violet. The percentages of viable cells were calculated as described in “Materials and Methods,” and are presented as the mean±SD of triplicate determinations.
DISCUSSION

In the present study, we evaluated the apoptosis-inducing properties of MfasTmR, of which the LBD was derived from a Tm-specific mutant receptor. Tm induced prompt and extensive apoptosis in L929MfasTmR cells at 10^{-6} M or greater concentrations, while E2 induced negligible apoptosis in these cells at up to 10^{-7} M. Only the highest dose of E2 (10^{-6} M) induced cell death (at a low level) in L929MfasTmR. In contrast, E2 elicited apoptosis in some L929MfasER cells at a concentration as low as 10^{-10} M, and was fully active at 10^{-7} M or greater doses. Although the mean E2 concentration in human peripheral tissues via endogenous estrogen, which would limit the clinical application of the prototype MfasER/E2 system.

One alternative to Fas is TNF receptor 1, which associates with TRADD and RIP adapter proteins instead of FADD. Developing inducible apoptosis systems with such FADD-independent receptors may expand the potential applications of suicide genes in cancer treatment.

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