Induction of Programmed Death/Apoptosis in Androgen-dependent Mouse Mammary Tumor Cell Line (Shionogi Carcinoma 115) by Androgen Withdrawal

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Shionogi Carcinoma 115 (SC 115) cells are a cloned cell line derived from androgen-dependent mouse mammary tumor. They can grow in serum-free culture if a physiological level of androgen is present in the medium, but can not proliferate in culture without testosterone. In the present study, the mechanism of cell death in SC 115 cells after androgen withdrawal was examined. Based upon the temporal sequence of DNA fragmentation, morphologic changes and loss of cell viability, androgen withdrawal induces programmed cell death (apoptosis) of SC 115 cells in serum-free culture. Northern blot analysis was used to identify a series of genes whose expression per cell is enhanced during the recruitment of cells from a nonproliferative (i.e. G₀) state into G₁ (i.e., cyclins D1 and C), from G₁ into the S phase of the cell cycle (i.e., cdk2), and during the programmed cell death pathway (i.e. testosterone repressed prostatic message-2 (TRPM-2), transforming growth factor- β 1 (TGF- β 1) and glucose regulated 78 kilodalton protein (GRP-78)). Expression of TRPM-2, TGF- β 1, GRP-78, and calmodulin genes increases, but that of cyclins C and D1, and cdk2 genes decreases during programmed cell death of SC 115 cells. These results demonstrate that androgen-dependent SC 115 cells undergo programmed cell death induced by androgen withdrawal, and that this death does not require proliferation or progression into G₁ of the proliferative cell cycle. SC 115 cells should be a good model for investigating programmed death of hormone-dependent cancer.

Key words: Shionogi Carcinoma 115 — Apoptosis — Androgen — Cell cycle

Normal prostate is androgen-responsive due to the presence of androgen-dependent cells within the gland. Following androgen withdrawal, the prostate rapidly regresses because of the activation of the process of cellular suicide, termed programmed cell death (apoptosis), which is normally repressed by androgen within these androgen-dependent prostatic glandular cells.¹⁻⁴⁾ This death occurs in prostatic glandular cells out of the cell cycle, as indicated by flow cytometric and ³H-thymidine labeling experiments.⁵⁾ Recently Colombel *et al.* have suggested that programmed death of prostatic glandular cells induced by androgen withdrawal involves a re-entry of the cells into a defective proliferation cell cycle and that enhanced p53 expression is involved in this abortive cycle, as they detected an increase in the incorporation of nucleotide precursors into genomic DNA in prostatic glandular cells after castration.⁶⁾ However, several reports have provided evidence demonstrating that prostatic glandular cells fail to enter the S phase or G₁ phase of the proliferative cell cycle after androgen withdrawal.^{7,8)} Programmed cell death of rat prostate induced by androgen withdrawal does not require the cells to enter the cell cycle, and these cells die out of cycle in G₀.^{7,8)} During the death process, when double-stranded DNA fragmentation occurs, a futile cycle of DNA repair is induced in which nucleotide precursors are incorpo-

rated into the DNA. This DNA repair is not required since blocking of this nucleotide incorporation does not prevent cell death induced by androgen withdrawal.⁷⁾

Shionogi Carcinoma 115 (SC 115) is an androgen-dependent mouse mammary tumor, which was established in 1964.⁹⁾ SC 115 contains androgen receptors and its growth is regulated by androgen.^{10,11)} SC 115 cells have been established with retention of androgen responsiveness in our laboratory.¹²⁻¹⁴⁾ They can be grown in serum-free culture and their growth is controlled by testosterone.¹²⁻¹⁴⁾ Thus, it is of interest to examine the mechanisms of growth and death of SC 115 cells, especially to examine the effect of gene products, due to the simplicity of this culture system. SC 115 cells secrete the eighth member of the fibroblast growth factor family, androgen-induced growth factor, when cultured with testosterone, and this factor may act in an autocrine fashion.¹⁵⁾ However, whether these cells die through an apoptotic or a necrotic pathway in cultures without testosterone has not been resolved. In the present studies, to clarify the mechanism of cell death especially in relation to the cell cycle, the kinetics of SC 115 cells after androgen withdrawal were examined. The temporal change in expression of a series of genes was also studied. These genes included apoptosis-associated genes (i.e. testosterone repressed prostatic message-2 (TRPM-2), transforming growth factor- β 1 (TGF- β 1), tissue transglutaminase, α -prothymosin, glucose regulated 78 kilo-

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dalton protein (GRP-78), and calmodulin genes) and cell cycle-associated genes (i.e. cyclin family genes).

MATERIALS AND METHODS

Cell line The cloning of SC 115 cells has been described previously.¹²⁻¹⁴ SC 115 cells were maintained in minimum essential medium/Ham's F-12 (1/1, v/v) containing 10% fetal bovine serum (0.05% dextran-coated 0.5% charcoal-treated) and 10^{-8} M testosterone (in ethanol at a final concentration of 0.01%) equilibrated with 5% CO₂/95% air at 37°C. To examine the effect of testosterone, cells were cultured in medium with or without 10^{-8} M testosterone as described previously.¹⁶ Determination of the number of viable cells at various times after treatment was performed using trypan blue exclusion.¹⁶

For morphological examination, cells were washed with phosphate-buffered saline (PBS), fixed in 3:1 (v:v) methanol: acetic acid, rehydrated with PBS, stained with 1 µg/ml bisbenzimidazole (Sigma, St. Louis, MO), and examined by fluorescence microscopy using a Nikon universal microscope (Tokyo) as described previously.¹⁷

Qualitative and quantitative analyses of DNA fragmentation For qualitative analysis of DNA fragmentation, total cell pellets were obtained by combining the unattached cells isolated by centrifugation from the media with attached cells isolated by trypsinization. Cells were lysed in 0.5% Triton X-100, 10 mM Tris-HCl, pH 7.5, 20 mM EDTA for 20 min at 4°C. After centrifugation at 27,000×g for 20 min, supernatants containing fragmented DNA were extracted with phenol-chloroform-isoamyl alcohol and DNA was precipitated in ethanol with 0.3 M sodium acetate. Each sample extracted from approximately 1×10^6 cells was electrophoresed on a 1.5% agarose gel. After electrophoresis, the gel was incubated in RNase A (50 µg/ml) for 3 h at 37°C with mild agitation and stained with ethidium bromide (0.5 µg/ml) for 20 min at room temperature. The gel was destained with distilled water and photographed. Double-stranded DNA fragmentation was quantitated according to the method described previously.³

RNA isolation and Northern analysis Total RNA was prepared by the acid guanidium thiocyanate-phenol-chloroform method.¹⁸ Aliquots of RNA were electrophoresed on 1% agarose gel and transferred onto nylon filters by blotting in 20×SSC (1×SSC is 0.15 M NaCl and 0.015 M sodium citrate). The probes used for TRPM-2, TGF-β1, γ-actin, calmodulin, GRP-78, tissue transglutaminase, and α-prothymosin were described previously.¹⁹⁻²² Probes were labeled with [³²P]dCTP using a multiprimer DNA labeling kit (Takara, Kyoto). Hybridizations were performed in 0.5 M sodium phosphate, pH 7.2, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA, and 1% bovine serum albumin for 16–20 h at

65°C. The filters were washed first with 0.5 M sodium phosphate, pH 7.2, 1% SDS, 1 mM EDTA for 1 h at 65°C, then with 0.25 M sodium phosphate, pH 7.2, 1% SDS, 1 mM EDTA (30 min at 65°C) and finally with 0.1 M sodium phosphate, pH 7.2, 0.1% SDS, 1 mM EDTA (30 min at 65°C). Autoradiographs were quantitated with a scanning densitometer and results were normalized on a per cell basis and given as relative expression of untreated cells as described previously.¹⁹

RESULTS

Effect of testosterone on viability, morphology, and DNA fragmentation of SC 115 cells Treatment of SC 115 cells in serum-free culture with 10^{-8} M testosterone resulted in continuous growth throughout the experimental period as determined by the trypan blue exclusion method. On the other hand, cultures without testosterone caused a decline in viable cell number after 72–96 h of treatment (Fig. 1). At 96 h, more than 90% of cells cultured with or without testosterone continued to exclude trypan blue, indicating that androgen withdrawal is not associated with loss of membrane integrity at this time point.

Morphological analysis revealed nuclei with chromatin condensation, which is characteristic of apoptosis, in cells cultured without testosterone, but not in those cultured with androgen (Fig. 2). In untreated SC 115 cells at time 0, less than 1% of the cells had nuclear chromatin condensation. At 216 h, $15.9 \pm 2.0\%$ of the cells showed

Fig. 1. Growth curve of SC 115 cells cultured in serum-free media with or without testosterone as determined by cell counting. Medium was replaced every 48 h. Points, mean of 3 values; bars, SD.

Fig. 2. Effect of androgen withdrawal on morphology of SC 115 cells. A and B, cell morphology was determined by fluorescence microscopy after staining with bisbenzimidazole. Cells were cultured in serum-free media with (A) or without (B) testosterone for 96 h. Arrow; apoptotic cell (C), percentage of apoptotic SC 115 cells cultured with or without testosterone. Points, combined data from 3 separate experiments; bars, SD. At some points, SD values are too small to depict.

a change in morphology when cultured without testosterone, while only $3.9 \pm 0.7\%$ of the cells had apoptotic morphology when cultured with testosterone ($P < 0.01$).

The ability to induce double-stranded DNA fragmentation of the genomic DNA is a stereotypic hallmark of programmed cell death.^{3,16} For qualitative analysis of the genomic DNA fragmentation, low-molecular-weight DNA was extracted from cells cultured with or without testosterone and analyzed by gel electrophoresis (Fig. 3). A characteristic nucleosomal size ladder was detectable as early as 72 h post treatment without testosterone, but was not detectable in the culture with testosterone. Such a nucleosomal ladder indicating cleavage of genomic

Fig. 3. Electrophoretic analysis of DNA isolated from cells cultured without (lane 1) or with (lane 2) testosterone for 72 h. Lane 1 shows typical ladder formation.

DNA at internucleosomal loci is consistent with the death of the SC 115 cells occurring via apoptosis.

In untreated SC 115 cells at time 0, $2.0 \pm 0.3\%$ of genomic DNA was fragmented (Fig. 4) as measured by quantitative analysis. At 240 h, $31.0 \pm 3.0\%$ of genomic DNA was fragmented in the absence of testosterone, whereas $13 \pm 1.0\%$ of genomic DNA was fragmented in cells cultured with testosterone ($P < 0.01$). In the first 48 h of treatment with or without testosterone, little additional DNA fragmentation occurred. After this 72–96 h lag period, however, DNA fragmentation increased substantially with $>30\%$ of the DNA being fragmented by 240 h during culture without testosterone.

Gene expression during the programmed death of SC 115 cells induced by androgen withdrawal Since androgen withdrawal induces programmed cell death of SC 115 via a common process of DNA fragmentation, morphologic change, and apoptosis, expression of various genes was examined during the dying process. For these studies, total RNA was isolated from SC 115 cells at 0–96 h following androgen withdrawal in the serum-free culture and assayed for the level of expression of a series of genes by Northern analysis. The genes analyzed were chosen

Fig. 4. Percent double-stranded DNA fragmentation of SC 115 cells cultured in serum-free media with or without testosterone. High- and low-molecular-weight DNAs were measured by the diphenylamine method. Points, mean of combined data from 3 separate experiments; bars, SD.

based upon previous studies of gene expression during the programmed death of androgen-dependent normal prostatic glandular cells induced by androgen withdrawal.¹⁹⁾ The time period chosen for these studies was based upon the data in Fig. 4, which demonstrated that after 96 h of treatment substantial amounts of genomic DNA were fragmented.

The results were expressed on a per cell basis and normalized to untreated SC 115 cells (Figs. 5 and 6). Recent studies have demonstrated that mRNA levels for TRPM-2 and TGF- β 1 increase in a variety of cells undergoing programmed cell death.^{19, 23, 24)} In the present study, 5-fold induction of TRPM-2 and 2-fold induction of TGF- β 1 occurred after 2 days of androgen withdrawal. Enhanced expression of GRP-78 (3-fold increase) was also induced in SC 115 cells after 2 days of culture without testosterone. When the cells were cultured with 10^{-8} M testosterone, expression of these genes per cell did not change throughout the experimental period (data not shown). Expressions of calmodulin and α -prothymosin were also increased by 5- and 2-fold, respectively, after 2 days (calmodulin) or 1 day (α -prothymosin) during the cell death induced by testosterone withdrawal in SC 115 cells.

To determine whether SC 115 cells die out of the cell cycle or not, the expression of a series of G₁-specific genes was examined. Expression of these genes was not changed or was slightly enhanced (i.e., <2-fold). There was a greater than 50% decrease (*cdk2*) and 75% decrease (cyclins C and D1) in the expressions of cyclins C

Fig. 5. Northern blot analyses of TRPM-2, α -prothymosin, GRP-78, tissue transglutaminase, and γ -actin gene expression in SC 115 cells following androgen withdrawal.

Fig. 6. Northern blot analyses of calmodulin, TGF- β 1, cyclin C, cyclin D1, and *cdk2* gene expression in SC 115 cells following androgen withdrawal.

and D1, and cdk2 between 3 and 4 days after androgen withdrawal. These results suggest that androgen withdrawal induces androgen-dependent SC 115 cells to leave the proliferative cell cycle and die out of the cell cycle.

DISCUSSION

SC 115 cells are a cloned cell line derived from androgen-dependent mouse mammary tumor and can be grown in serum-free culture in the presence of androgen.¹²⁻¹⁴ Although the mechanism of cell growth of SC 115 cells has been well studied,¹⁰⁻¹⁵ the mechanism of cell death remains obscure. As a model of cell death in androgen-dependent cells, rat prostatic glandular cells have been studied in many laboratories.^{1-4, 25-29} The rapid involution of the prostate induced by androgen withdrawal is due to the death of prostate glandular cells occurring via the process of programmed cell death.¹⁻⁴ This programmed death pathway involves an energy-dependent cascade in which the cells undergo an epigenetic reprogramming, which leads to double-strand fragmentation of their DNA followed by cellular fragmentation into apoptotic bodies.^{3, 4} This process involves a decreased production of secretory proteins coupled with an increase of a variety of new gene products,^{23, 25-29} leading to activation of a Ca^{2+} -dependent endonuclease which fragments the DNA in the nucleus into a characteristic nucleosomal size ladder associated with programmed cell death.³⁰ Following DNA fragmentation, the cell itself is fragmented into apoptotic bodies which are phagocytosized either by neighboring epithelial cells or by fixed macrophages.^{4, 30} Based upon the temporal sequence of DNA fragmentation, morphologic changes and loss of cell viability in the present study, androgen withdrawal induces the programmed cell death of SC 115 cells in serum-free culture, and this process is almost identical to that in the rat prostate gland.

During the active process of programmed cell death in the prostate following castration, certain genes are rapidly turned off while other genes are rapidly induced. The result of this epigenetic reprogramming is that during the death process, there is a change in the profile of proteins that are synthesized,²⁵ which is coupled with inhibition of glandular cell proliferation.^{3, 4} As in prostatic glandular cells, certain genes (i.e., TRPM-2, TGF- β 1, GRP-78, α -prothymosin, and calmodulin genes) are induced in SC 115 cells during androgen withdrawal, resulting in programmed cell death. TRPM-2 and TGF- β 1 mRNA expression is upregulated in a variety of cells undergoing apoptosis.^{23, 24, 26, 31} There are indications that enhanced

expression of TGF- β 1 mRNA and protein as well as the receptor for TGF- β 1 following castration are somehow involved in the elevation in the intracellular free Ca^{2+} level.^{23, 32, 33} α -Prothymosin is a nuclear protein and is thought to be involved in opening up genomic DNA in the linker region between nucleosomes.³⁴ Calmodulin is a multifunctional calcium-binding protein involved in calcium-dependent regulation of more than a dozen enzymes.³⁵ Calmodulin has been implicated in glucocorticoid-mediated proliferation-independent apoptosis of lymphocytes.³⁶ The present study demonstrates that elevated expression of calmodulin per cell occurs during the apoptotic process, suggesting that Ca^{2+} - Mg^{2+} -dependent endonuclease plays an important role in this process. GRP-78 shares sequence homology with heat shock protein and has a protective function on cellular processes.³⁷ All these genes are associated with programmed cell death and are upregulated during the death process in SC 115 cells.

The fact that no enhancement of the expression of several of the genes required for entrance into the G_1 -phase of the proliferative cell cycle (i.e. G_1 cyclins) occurs during androgen withdrawal-induced programmed cell death of SC 115 cells demonstrates that neither entrance into the S-phase nor progression through a defective proliferative cell cycle is involved in androgen withdrawal-induced programmed death of these cells.³⁸ In prostatic glandular cells, flow cytometric and ^3H -thymidine labeling experiments have demonstrated that there is a decrease in percent of prostatic glandular cells entering the S phase following androgen withdrawal.^{7, 8} Recently we have shown that prostatic glandular cells undergo apoptosis in G_0 without recruitment into the G_1 phase of a defective cell cycle because expressions of all cyclins, p53, WAF1/CIP1 genes decrease and retinoblastoma protein phosphorylation does not proceed.⁸ These results demonstrate that androgen-dependent SC 115 cells undergo programmed death induced by androgen withdrawal, and that this death does not require proliferation or progression into G_1 of the proliferative cell cycle. SC 115 cells are a good model for investigating programmed death of hormone-dependent cancer.

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