HOXA5-Induced Apoptosis in Breast Cancer Cells Is Mediated by Caspases 2 and 8

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Received 2 June 2003/Returned for modification 10 July 2003/Accepted 6 October 2003

HOXA5 is a transcriptional factor whose expression is lost in more than 60% of breast carcinomas. Our previous work demonstrated that the overexpression of HOXA5 in MCF7 cells resulted in cell death through a p53-dependent apoptotic pathway. To determine whether p53-independent apoptotic pathways are involved in HOXA5-induced cell death, we engineered a p53-mutant breast cancer cell line, Hs578T, to inducibly express HOXA5. Induction of HOXA5 expression led to cell death with features typical of apoptosis within 24 h, and the expression levels of mutant p53 and its target genes either decreased or remained unchanged. To decipher apoptotic pathways, the HOXA5-expressing cells were treated with a variety of apoptotic inhibitors. Besides a general caspase inhibitor, caspase 2- and 8-specific inhibitors largely abolished HOXA5-induced apoptosis, whereas caspase 1-, 3-, 6-, and 9-specific inhibitors had no significant effects. Western blot analysis further confirmed that caspases 2 and 8 were activated after the induction of HOXA5 expression. Further, several small interfering RNAs which specifically silenced caspase 2 and caspase 8 expression significantly blocked HOXA5-induced apoptosis. HOXA5 expression could also sensitize cells to tumor necrosis factor alpha-induced apoptosis by at least 100-fold. These results indicate that expression of HOXA5 can induce apoptosis through an apoptotic mechanism mediated by caspases 2 and 8.

HOX genes are well known for their control of anterior-posterior patterning during the embryonic development of worms, flies, mice, and humans. Recently, accumulating evidence suggests that HOX genes, when dysregulated, play important roles in oncogenesis (1, 14, 15). Many HOX genes are aberrantly expressed in breast cancer cell lines and primary breast carcinomas, although their roles in tumorigenesis are far from clear (31). In 1994, Friedman et al. reported that *Hoxa1* was detected only in neoplastic cells but not in normal mouse mammary gland throughout the development cycles (19). Later, many HOX gene transcripts including *HOXA1*, *HOXA4*, *HOXB6*, *HOXC6*, *HOXB7*, and *HOXA10* were detected in MCF7, a human breast cancer cell line (11–13). In primary breast carcinomas, *HOXC6*, *HOXB3*, and *HOXB4* were detectable immunochemically in over 90% of neoplastic cells (5), while a more detailed study that used reverse transcription-PCR showed the altered expression of a number of HOX genes in breast cancers compared to normal breast tissue (7). The tumor-specific overexpression of some HOX genes in breast cancer implies an oncogenic action for HOX genes in these cells. Consistent with this notion, overexpression of *HOXB7* in SKBR3 cells promoted cell proliferation, growth factor, and anchorage-independent cell growth; more importantly, *HOXB7*-transfected cells form tumors in nude mice (8–10). More recently, both in vitro and in vivo experiments have shown that *HOXA1* causes neoplastic transformation in normal breast epithelial cells, MCF10A (51). In contrast to the overexpression of several HOX genes observed in breast can-

cer cells, we found that *HOXA5* expression is lost in human breast cancer (41). This loss occurs largely due to promoter hypermethylation in more than 60% of breast cancer cell lines and carcinomas, suggesting that *HOXA5* may act like a tumor suppressor gene instead of an oncogene. In line with this hypothesis, we found that *HOXA5* directly bound to and trans-activated both the mouse and human p53-promoters (41). Also, overexpression of *HOXA5* in the breast cancer cell line MCF7 led to cell death through a p53-dependent apoptotic pathway (41). The connection of HOX genes to apoptosis opened a very interesting research field, considering the overwhelming evidence for deregulated HOX genes in many different cancers and the critical role of apoptosis in both development and tumorigenesis. Thus far, very few studies have reported that HOX genes exert their functions through regulating apoptosis (34, 40).

Apoptosis is largely executed by caspases, a family of proteases that disassembles a cell (2, 16, 28, 43). The caspase cascade can be initiated either from mitochondria (the intrinsic pathway) or through cell death receptors (the extrinsic pathway), depending on the cytotoxic stimulus. The stimuli that are collectively referred to as cytotoxic stress, such as UV rays and chemotherapeutic drugs, activate caspase by initiating signaling pathways that lead to the permeabilization of the mitochondrial membrane and release of cell death-promoting proteins. One of these released proteins is cytochrome c, which in a complex with the cytoplasmic protein Apaf-1 activates caspase 9. Caspase 9 in turn activates caspase 3, the protease that cleaves the majority of caspase substrates during apoptosis. Mitochondria also release an apoptosis-inducing factor and endonuclease G, which appear to kill cells independently of caspases (33, 39). Another way to activate caspases, used by cytokines such as tumor necrosis factor alpha (TNF- α), is to

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assemble receptor complexes that recruit initiator caspases such as caspase 2, caspase 8 and/or caspase 10, thereby inducing their autocatalytic processing. These activated initiator caspases then activate other downstream effector caspases including caspase 3, caspase 6, and caspase 7, leading to apoptosis (43). The activated initiator caspases in the extrinsic pathway can also cleave Bid, and a proteolytic fragment of Bid can translocate into and permeabilize mitochondria. In this case, the intrinsic pathway serves as a signal amplifier. In addition, DNA damage signals in the intrinsic pathway can first lead to activation of caspase 2 which can also cleave Bid and lead to translocation of the cytoplasmic Bcl-2 family member Bax to mitochondria, thereby accelerating cell disassembly as described above (20, 29).

In this study, we investigated whether p53 activation is indispensable for HOXA5-induced apoptosis and whether HOXA5 can induce apoptosis through alternative pathways. We studied HOXA5-induced apoptosis more closely by using another breast cancer cell line, Hs578T, containing mutant p53 (18, 36, 37). In addition, no endogenous expression of HOXA5 is detectable in these cells. All our attempts to make stable HOXA5-expressing clones in this cell line failed, suggesting that HOXA5 induces apoptosis in this p53-mutant cell line as well. To circumvent this problem, we constructed a *tet*-off inducible HOXA5 cell line. In this cell culture system, expression of *HOXA5* was tightly controlled by the presence of doxycycline in the medium; removing doxycycline resulted in the rapid expression of *HOXA5* and dramatic cell death within 24 h. Upon investigating the mode of death in this system, we found that Hs578T cells, like MCF7 cells, die by apoptosis. However, p53 does not appear to be involved in the death of these cells. Instead, these cells apparently undergo death through the activation of caspase 2 and caspase 8. This conclusion was further confirmed by synergistic activation of apoptosis by HOXA5 and TNF- α , since, similar to HOXA5, TNF- α also activated caspase 2 and caspase 8.

MATERIALS AND METHODS

Cell culture and reagents. Hs578T cells were purchased from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium complemented with 10% fetal bovine serum. The following caspase inhibitors and substrates were purchased from Calbiochem (La Jolla, Calif.): a general caspase inhibitor, Z-Val-Ala-Asp(OMe)-CH2F (Z-VAD-FMK); a caspase 1 inhibitor, Z-Tyr-Val-Ala-Asp(OMe)-CH2F (Z-YVAD-FMK); a caspase 2 inhibitor, Z-Val-Asp(OMe)-Val-Ala-Asp(OMe)-CH2F (Z-VDVAD-FMK); caspase-3 inhibitor, Z-Asp(OCH3)-Glu(OCH3)-Val-Asp(OCH3)-FMK (Z-DEVD-FMK); a caspase 6 inhibitor, Z-Val-Glu(OMe)-Ile-Asp(OMe)-CH2F (Z-VEID-FMK); a caspase 8 inhibitor, Z-Ile-Glu(OMe)-Thr-Asp(OMe)-CH2F (Z-IETD-FMK); a caspase 9 inhibitor, Z-Leu-Glu(OMe)-His-Asp(OMe)-CH2F (Z-LEHD-FMK); a caspase 2 colorimetric substrate, Ac-Val-Asp-Val-Ala-Asp-pNA (Ac-VDVAD-pNA); and a caspase 8 colorimetric substrate, Ac-Ile-Glu-Thr-Asp-pNA (Ac-IETD-pNA). The caspase 3 activity kit was purchased from Sigma (St. Louis, Mo.). Antibodies to caspase 2 (C-terminal), p53, p21, Bax, MDM2, and β -actin were purchased from Santa Cruz (Santa Cruz, Calif.). Phycoerythrin (PE)-conjugated antibodies to TNF- α , TNF- α receptor-1 (TNFR1), Fas ligand, and Fas were purchased from Santa Cruz. The neutralizing antibody to TNFR1 was purchased from Calbiochem. TNF- α recombinant protein was purchased from Roche (Indianapolis, Ind.). The caspase 2 (N-terminal) and caspase 8 antibodies were purchased from BD Biosciences (San Diego, Calif.). HOXA5 antibody was provided by Zymed (South San Francisco, Calif.). Bongkreik acid (BA) and 3,4-dihydro-5-[4-(1-piperidinyl)butoxyl]-1(2H)-isoquinolinone (DPQ) were purchased from Sigma. The small interfering RNAs (siRNAs) to lamin, caspase 1, and caspase 2 were purchased from Dharmacon (Lafayette, Colo.). The caspase 8 siRNAs (target 1,5'-CTG GAT TTG CTG ATT ACC T-3'; target

2, 5'-GAG CCT GCT GAA GAT AAT C-3'; target 3, CCT CAA ACG AGA TAT ATC C-3'; and target 4, 5'-CCT CGG GGA TAC TGT CTG A-3') were synthesized in the A4 option by Dharmacon Research.

Establishment of HOXA5-inducible cell lines. We transfected breast cancer cell line Hs578T with tTA-IRES-Neo (a gift from Bert Vogelstein) (tTA, *tet* activator; IRES, internal ribosome entry site; *neo*, G418 resistance gene) which express tTA in a *tet*-off manner and selected cells with 800 μ g of G418 per ml for 2 to 3 weeks (49). Single clones were isolated. pBI-GL (CLONTECH), a reporter plasmid which expresses luciferase and β -galactosidase in a tTA-dependent fashion, was transfected into these G418-resistant clones for testing tTA response. The highly tTA-responsive clones were selected for establishing a HOXA5-inducible cell line. The HOXA5 cDNA from pIND-HOXA5 (7) was inserted into the sites of another tTA-responsive vector, pBI-MCS-EGFP (a gift from Bert Vogelstein), which carries a green fluorescent protein (GFP) gene, for easy selection of single clones later. The generated HOXA5-expression plasmid (pBI-HOXA5) was cotransfected into the above *tet*-off clones with pTK-hygro (CLONTECH). Single colonies were obtained by limiting dilution or ring cloning with 400 μ g of G418 per ml and 250 μ g of hygromycin B per ml (Sigma) in the presence of 20 ng of doxycycline per ml for 2 to 3 weeks. Clones that have low background GFP and homogeneous GFP induction were selected. The expression of HOXA5 was further confirmed by performing Western blot analysis.

Western blot analysis. Twenty micrograms of protein was fractionated in a 4 to 12% NuPAGE gel (Invitrogen) and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 100 ml of Tris-buffered saline (10 mM Tris-base [pH 7.5], 0.9% NaCl) containing 5% dry milk and 0.1% Tween-20 for 1 h on the shaker at room temperature or overnight in a cold room. The membrane was rinsed once with TBS before being incubated with an appropriate dilution of the primary antibody in TBS containing 5% milk and 0.02% Tween-20 on the shaker for 1 h. The primary antibody-bound membrane was washed with TBS containing 0.1% Tween-20 four times and then incubated with the secondary antibody (anti-rabbit or anti-mouse from Amersham ECL kit) of at a dilution of about 1:1000 for 1 to 1.5 h on the shaker. The filter was developed by using the ECL-Plus reagent (Amersham). To prepare blots for reuse, they were stripped with 0.2 M glycine-HCl, pH 2.5, for 10 to 15 min and then neutralized with 0.1 M Tris-HCl, pH 8.0, for 45 min on a shaker, with the buffer changed once after 25 min.

The procaspase 2 and its p33 fragment were detected by using an N-terminal specific monoclonal antibody (BD Biosciences) and the p14 fragment was detected by using a C-terminal polyclonal caspase 2 antibody (Santa Cruz).

DNA fragmentation analysis. A total of 5×10^6 cells were harvested and washed with phosphate-buffered saline (PBS) three times. Equal numbers of cells were resuspended in 500 μ l of lysing buffer (5 mM Tris, 20 mM EDTA, 0.5% Triton X-100) and incubated on ice for 20 min. The samples were centrifuged at 27,000 $\times$ g ($\sim$ 14,000 rpm) for 20 min. The supernatant was saved and the protein was removed by extracting with 0.5 ml of phenol-chloroform-isoamyl (25:24:1; stored at 4°C) once. The DNA fragments were then precipitated with 2 volumes of ice-cold 100% ethanol and resuspended in 0.5 ml of $0.1 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) buffer containing 100 U of DNase-free RNase A per ml at 37°C for at least 30 min. The DNA fragments were precipitated again with 100% ethanol and separated on a 1% agarose gel.

Cell cycle distribution analysis. A total of 5×10^5 cells were harvested and washed twice with 2 ml of immunofluorescent assay media ($1 \times$ PBS, 4% fetal bovine serum, 1 mg of sodium azide/ml). The cells were fixed with 70% methanol on ice for 5 min. The fixed cells were collected by centrifuging at 1,400 rpm ($\sim$ 2,250 $\times$ g) for 7 min and were treated with 250 μ l of RNase solution (100 μ g of RNase/ml in PBS) at 37°C for 15 min. PBS (250 μ l) containing 100 μ g of propidium iodide/ml was directly added into the samples. The cells were incubated on ice for 1 h and analyzed on a Becton-Dickson FACScan flow cytometer by using the CellQuest software.

Annexin V staining assay. The PE-conjugated annexin V was purchased from BD Biosciences. The staining assay was performed according to the manufacturer's instructions. Briefly, the cells were harvested and washed once with cold PBS. The cell pellet was resuspended in 100 μ l of binding buffer containing 5 μ l of annexin V-PE on ice for 30 min. Binding buffer (500 μ l) was added to the sample and centrifuged to remove the unbound annexin V. The samples were then resuspended in 500 μ l of binding buffer for flow cytometry analysis.

In vitro caspase activity assay. A total of 10^6 cells were harvested and washed once with cold PBS. The cells were resuspended in cell lysis buffer (50 mM HEPES [pH 7.4], 5 mM 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, 5 mM dithiothreitol) and incubated on ice for 15 min. The sample was then centrifuged at 14,000 rpm for 10 min. The supernatant was either analyzed immediately or saved at -80°C in aliquots. Equal amounts of protein measured by bicinchoninic acid assay (Pierce) were used in a colorimetric assay for detec-

FIG. 1. Establishment of *tet*-off inducible HOXA5 cell line. (A) The constructs used to transfect breast cancer cell line Hs578T cells. (B) Screening the stable clones with GFP-inducible expression. Doxycycline (DOX) is the analog of tetracycline. Removal of DOX from the culture medium induced the expression of both enhanced GFP (EGFP) and HOXA5. (C) The time course of induction of HOXA5 expression in HOXA5-inducible clone 10 by Western blot analysis. V_0 and V_{24} represent the cellular lysate which was extracted from vector-transfected cells at 0 and 24 h postinduction.

tion of caspase 2-, caspase 3-, and caspase 8-like activity according to the manufacturer's instructions (Sigma). The substrates for caspase 2-, caspase 3-, and caspase 8-like activity were Ac-VDVAD-pNA, Ac-DEVD-pNA, and Ac-IETD-pNA, respectively. The optical density at 405 nm (OD_{405}) was measured by using a spectrometer. The same experiments were repeated three times, and average data are shown in the figures.

siRNA transfection. The siRNA transfections were done as described (29). In brief, 10^5 cells were cultured on a 6-well plate in 2 ml of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 500 μ g of G418/ml, 50 μ g of hygromycin/ml, and 20 ng of doxycycline/ml for 24 h. Ten microliters of 20 μ M siRNA (final concentration, 100 nM) was mixed with 10 μ l of oligofectamine reagent (Invitrogen) for 20 min at room temperature. Meanwhile, cells were rinsed with 2 ml of PBS, and 1 ml of serum-free medium was added per well. The siRNA mixture was then added by drops onto the cells while the plates were gently agitated. After 3 h, 1 ml of medium containing 20% serum was added to the cells. At 48 h posttransfection, half of the cells were harvested for Western blot analysis, and the other half was induced for another 16 h for apoptosis analysis.

RESULTS

Establishment of a *tet*-off inducible HOXA5 cell line. We previously used an ecdysone-inducible system to express the HOXA5 gene in MCF7 breast cancer cells (41). However, the results were not satisfactory due to the leakiness of the system. To overcome this problem, a recently modified *tet*-off system was generated to tightly control HOXA5 gene expression in the Hs578T breast cancer cell line. As the HOXA5 and enhanced GFP were bidirectionally controlled by the *tet*-response promoter (Fig. 1A), we selected a few clones which displayed strong GFP fluorescence after removal of doxycycline from the culture medium while no signal or weak GFP signals were detectable in the presence of doxycycline (Fig. 1B). The inducible expression of HOXA5 in these clones was examined by

Western blotting. HOXA5 was highly expressed after 3 h of induction, while no HOXA5 expression was detectable in either the uninduced cells or vector-transfected cells (Fig. 1C).

Induction of HOXA5 expression resulted in cell death through apoptosis. At 16 to 24 h postinduction, the HOXA5-expressing cells began to round up and gradually die, while the vector-transfected cells grew equally well in the presence or absence of doxycycline (Fig. 2A). Results of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) cell viability assay showed that more than 90% of the cells died after 72 h of induction. Meanwhile, the removal of doxycycline had no effect on the growth rate of vector-transfected cells (Fig. 2B). Several vector- and HOXA5-transfected inducible clones were selected and examined (data not shown). All of them displayed similar results by MTT assay, indicating that cell death induced in these cells is HOXA5-specific.

To determine whether HOXA5 induced cell death through the apoptotic pathway, we utilized one of these HOXA5-inducible clones (clone 10) to perform three different tests. First, we performed a DNA fragmentation assay. At 9 h postinduction, no DNA fragments were observed; however, a large number of DNA fragments were visible 24 h after the induction of HOXA5 expression (Fig. 3A). Next, we quantitatively measured HOXA5-induced apoptosis with an annexin V staining assay and, lastly, we used a cell cycle distribution assay (Fig. 3B and C). At 24 h postinduction, we observed that 35.5% of the cells were annexin V positive; by 48 h, the percentage of annexin V-positive cells increased to 56.5%. The sub- G_0/G_1 cell population in the cell cycle analysis represented the apoptotic cells. Similar to the annexin V assay, this assay showed that

FIG. 2. Induction of HOXA5 expression resulted in cell death. (A) HOXA5- and vector-inducible clones were cultured in the presence or absence of doxycycline (DOX) for 24 h. Cells were photographed under a microscope (magnification, $\times 10$). (B) The relative number of viable cells was measured by MTT assay. HOXA5- and vector-inducible cells were cultured in the presence or absence of DOX. Cell viability was measured every 24 h. The y axis represents the relative number of viable cells compared to the number at day 0 when the DOX was first removed from the medium. Since the uninduced cells and vector-transfected cells continued to grow, there were more cells after 24 to 96 h of induction.

23.5 and 55.7% of the cells were apoptotic after the induction of HOXA5 expression for 24 h and 48 h, respectively. In both the annexin V staining assay and the cell cycle analysis, the detectable apoptotic population in vector-transfected cells did not change significantly although annexin V could nonspecifically bind to part of the vector-transfected cells.

HOXA5-induced apoptosis in Hs578T cells is p53 independent. In MCF7 cells which contain a wild-type p53 gene, HOXA5 has been shown to transactivate the promoter of p53 and cause apoptosis (41). Previous studies have shown that in Hs578T cells an amino acid substitution of V for F at position 157 within the p53 DNA binding domain resulted in loss of its transcriptional activity (37). After γ -irradiation, no increase in the expression levels of the p53 putative target genes was observed, further indicating that the mutant p53 in Hs578T cells is not functional (37). To study whether p53 is involved in HOXA5-induced apoptosis in Hs578T cells, we first examined the expression status of p53 and its target genes after induction of HOXA5 expression (Fig. 4). Contrary to expectation, we found that the expression levels of p53 and MDM2 slightly decreased in induced cells compared to uninduced cells after the removal of doxycycline from the culture medium, and by 48 h postinduction there was a dramatic reduction in p53 levels. MDM2 was so weakly expressed in Hs578T cells that the specific band became visible only after overnight exposure of the film. The expression levels of Bax, a proapoptotic gene downstream of p53, remained unchanged. The slightly decreased expression of p53 and its target genes indicated that p53 was not activated, at least at the transcriptional level, after HOXA5 induction. Although we did not completely rule out

the possibility that p53 was involved in HOXA5-induced apoptosis, it is unlikely that it plays a major role in this case.

HOXA5-induced apoptosis is caspase dependent. Poly-(ADP-ribose) polymerase (PARP) cleavage was observed in HOXA5-induced apoptosis in MCF7 cells (41). Presumably, PARP was cleaved by activated caspases. To determine whether HOXA5-induced apoptosis in Hs578T cells was caspase dependent, HOXA5-induced cells were treated with a general caspase inhibitor, Z-VAD-FMK. Apoptosis was inhibited by Z-VAD-FMK in a dose-dependent manner (Fig. 5A and B), suggesting that the caspase cascade was activated by HOXA5. In the presence of 100 μ M Z-VAD-FMK, HOXA5-induced apoptosis was completely abolished (Fig. 5A). At 24 h postinduction, cell viability was increased from 30% in the absence of Z-VAD-FMK to 90% in the presence of 100 μ M Z-VAD-FMK. However, the cells gradually died after 24 h, possibly due to the switch from apoptosis to necrosis when caspase activity was inhibited, as many other studies have shown (30, 42). In addition, the same concentration of Z-VAD-FMK had no effect on the growth of uninduced cells, indicating that the cell death was not caused by the toxicity of Z-VAD-FMK (Fig. 5B).

In an attempt to find out how the caspase cascade was initiated, we tested a variety of known apoptotic inhibitors including BA (a mitochondrial permeability transition pore [PTP] inhibitor), DPQ (a PARP inhibitor), and several individual caspase inhibitors as well. BA has been shown to inhibit the mitochondrial PTP (6), and DPQ is a PARP inhibitor which blocks the PARP-mediated activation of the apoptosis-inducing factor (6, 50). Treatment with BA and DPQ had no

FIG. 3. HOXA5 induces cell death through apoptosis. (A) DNA fragmentation analysis. DNA fragments were extracted from HOXA5-inducible cells harvested at 0, 9, and 24 h postinduction and then fractionated on a 1% agarose gel. (B) Flow cytometry analysis after annexin V staining. The vector- and HOXA5-transfected cells were harvested and counted at 0, 24, and 48 h postinduction. Cells (4×10^5) from each sample were stained with PE-conjugated annexin V for 30 min and then analyzed by flow cytometry. Annexin V-positive cells were shown in the gated box and the percentages were displayed. FSC, forward scatter. (C) Flow cytometry analysis of cell cycle distribution after HOXA5-induction. HOXA5-inducible cells were harvested at 0, 12, 24, 48 h postinduction and subjected to flow cytometry analysis. The sub-G₀/G₁ cell population (M1) represents the apoptotic cells. The sub-G₀/G₁ percentages were also displayed.

effect on HOXA5-induced apoptosis (Fig. 6A). Among the caspase inhibitors tested were caspase 1 inhibitor Z-YVAD-FMK, caspase 2 inhibitor Z-VDVAD-FMK, caspase 3 inhibitor Z-DEVD-FMK, caspase 6 inhibitor Z-VEID-FMK, caspase 8 inhibitor Z-IETD-FMK, and caspase 9 inhibitor (Z-LEHD-FMK). Among these, Z-VDVAD-FMK and Z-IETD-FMK effectively inhibited HOXA5-induced apoptosis in Hs578T cells, suggesting that caspase 2 and caspase 8 were likely to be involved (Fig. 6A). This conclusion was further confirmed by the results of the Western blot analysis. As shown in Fig. 6B, the protein level of procaspase 2 dramatically decreased with the induction of HOXA5 expression. The cleavage of procaspase 2 was also evidenced by the appearance of

small fragments (33 and 14 kDa). The N-terminal-specific antibody could detect both the procaspase 2 and the 33-kDa fragment, while the smaller fragment (14 kDa) was only detected by the C-terminal-specific antibody (Fig. 6B). The protein level of procaspase 8 showed reduction with slower kinetics. The cleaved fragment was not detectable until 16 h of induction (Fig. 6C).

In line with the observation of the cleavage of procaspase 2 and procaspase 8, caspase 2- and 8-like activities were also detectable by an *in vitro* assay at 4 and 6 h postinduction, respectively (Fig. 6D). The caspase 2-like activity continued increasing with the induction time up to 24 h. In contrast, the caspase 8-like activity reached the peak at 6 h postinduction

FIG. 4. HOXA5-induced apoptosis is p53-independent. HOXA5-inducible cells were harvested at 0, 3, 6, 9, 24, and 48 h postinduction. Twenty micrograms of the whole-cell lysate were used for the Western blot analysis of the expression of p53 and its target genes, MDM2 and BAX. V, vector-transfected cell lysate.

and then remained at a relatively stable level for the rest of the induction time (Fig. 6D). The activation of caspases 2 and 8 also led to the activation of the downstream caspase cascade since the caspase 3-like activities showed continual increases at

4 h postinduction (Fig. 6D). The caspase 2 and caspase 8 inhibitors could not only inhibit their own activity but also abolished the activities of downstream caspases such as caspase 3-like activities, confirming that caspase 2 and/or caspase 8 initiated the caspase cascade (Fig. 6E). Notably, the other caspase inhibitors such as the caspase 1 and 9 inhibitors that failed to block apoptosis also had no effect on the downstream caspase 3-like activities (Fig. 6E).

The failure to inhibit apoptosis by several individual caspases does not exclude the possibility that such caspases are involved in HOXA5-induced apoptosis. The caspase 3 inhibitor could not inhibit apoptosis, but caspase 3-like activity increased by three- to fivefold after HOXA5-induction for 24 h (Fig. 6D and E). Also, the failure to block apoptosis was not necessarily due to the inability of individual caspases to inhibit the corresponding caspase activities. As shown in Fig. 6E, the caspase 3 inhibitor efficiently blocked the caspase 3-like activities. Due to the existence of a complicated caspase activation network, the involvement or contribution of each of these caspases in the HOXA5-activated caspase cascade required further examination in detail.

Requirement of caspase 2 and caspase 8 activation in HOXA5-induced apoptosis. The above results indicated that caspase 2 was one of the first activated caspases. To further confirm the requirement of caspase 2 activation in HOXA5-induced apoptosis, we used a siRNA to caspase 2 that has been

FIG. 5. Z-VAD-FMK inhibits HOXA5-induced apoptosis. (A) Inhibition of HOXA5-induced apoptosis by different concentrations of Z-VAD-FMK. After the removal of doxycycline (DOX) from the culture medium, HOXA5-inducible cells were treated for 24 h with Z-VAD-FMK at concentrations of 10 μ M, 25 μ M, 50 μ M, and 100 μ M. The apoptotic cell death (sub-G₀/G₁) was measured by flow cytometry analysis as described. (B) HOXA5-inducible cells were cultured in the presence or absence of DOX and 100 μ M Z-VAD-FMK for 24 h. (C) The relative number of viable cells was measured by MTT assay. At 24 h after growing under uninduced conditions (+DOX), the cells were treated with the following combination of DOX and ZVAD: (i) +DOX + ZVAD, (ii) +DOX - ZVAD, (iii) -DOX + ZVAD, and (iv) -DOX - ZVAD. The relative number of viable cells was measured every 24 h and compared to the number at 0 h. The data shown here represent the average values of two independent experiments with triplicate wells for each time point.

shown to specifically and efficiently silence the expression of caspase 2 in other studies (29). As expected, transfection of the siRNA to caspase 2 into the HOXA5-inducible cells substantially knocked down the expression level of caspase 2. In contrast, the control siRNA to lamin and caspase 1 had no effect on the expression of caspase 2 (Fig. 7A). In addition, the siRNA to caspase 2 did not affect expression of other caspases such as caspase 8 (Fig. 7A).

To examine the effects of the siRNA to caspase 2 on HOXA5-induced apoptosis, the siRNA-transfected cells were induced for HOXA5 expression for an additional 16 h. The apoptotic cell percentages were measured by flow cytometry analysis. Compared to the control cells that were transfected with the siRNA to lamin, apoptosis in caspase 2 siRNA-transfected cells decreased about 50%, while the apoptosis percentage in caspase1 siRNA-transfected cells did not change significantly (Fig. 7B). This finding provided further confirmation that caspase 2 activation was required for HOXA5-mediated apoptosis in Hs578T cells.

Similarly, we tested four caspase 8 siRNAs for their gene silencing abilities and inhibition of HOXA5-induced apoptosis. We found that three out of the four caspase 8 siRNA (numbers 1, 2, and 3) substantially knocked down the expression level of caspase 8, while the number 4 siRNA had no dramatic effect (Fig. 7C). Consistent with the gene silencing function of these siRNAs, transfection of the first three siRNAs produced more dramatic inhibition of HOXA5-induced apoptosis than transfection of the number 4 siRNA (Fig. 7D). Thus, caspase 8 is also involved in HOXA5-induced apoptosis in this system.

Expression of HOXA5-sensitized cells to TNF- α -induced apoptosis. Caspase 2 and caspase 8 activation after HOXA5 induction is reminiscent of events accompanying TNFR-mediated apoptosis. Therefore, we considered the possibility that the death receptor-mediated pathway was activated by HOXA5. However, we found that the expression levels of TNFR1, TNF- α , Fas, and Fas ligand remained unchanged after HOXA5 induction (data not shown). Further, treatment with a neutralizing antibody to TNFR1 had no effect on HOXA5-induced cell death (data not shown). These negative findings suggested that HOXA5 might not directly activate death receptors or their ligands. Instead, it is likely that an unknown factor, which can transmit apoptotic signals downstream of the death receptor to the caspase cascade, is activated by HOXA5. If this hypothesis is valid, the expression of HOXA5 would sensitize cells to TNF- α -induced apoptosis. Uninduced Hs578T cells were resistant to TNF- α -induced apoptosis at a concentration of 100 ng/ml in the absence of

cycloheximide. However, they were sensitive to TNF- α -induced apoptosis in the presence of cycloheximide (Fig. 8A). Very similar to the cycloheximide treatment, the removal of doxycycline from the culture medium and induction of HOXA5 expression not only induced apoptosis but also potentiated TNF- α -induced cell death (Fig. 8B). TNF- α in as low a concentration as 1 ng/ml was sufficient to cause a nearly 40% increase in the percentage of HOXA5-induced apoptotic cell death. The cell death percentage induced by HOXA5 and TNF- α together was greater than the additive values of individual treatments, suggesting a synergistic action between HOXA5 and TNF- α on cell death induction (Fig. 8B).

To test whether the exact same pathway was activated by TNF- α and HOXA5, we used the different caspase inhibitors to block apoptosis. Compared to the inhibition effects of individual caspase inhibitors on HOXA5-induced apoptosis (Fig. 6A), several caspase inhibitors shared similarities and displayed some minor differences in the efficiency of inhibition on the apoptosis induced by TNF- α in the presence of cycloheximide. The caspase 8 inhibitor almost completely blocked both HOXA5- and TNF- α -mediated apoptosis (Fig. 8C). The caspase 2 inhibitor completely blocked HOXA5-induced apoptosis and significantly, but not completely, inhibited TNF- α -induced apoptosis. The caspase 3 inhibitor that failed to block HOXA5-induced apoptosis effectively (Fig. 6A) inhibited TNF- α -mediated apoptosis (Fig. 8C). The synergistic action on apoptosis induced by HOXA5 and TNF- α was almost completely blocked by the caspase 2 and caspase 8 inhibitors and moderately inhibited by the caspase 3 inhibitor (Fig. 8D). This minor difference might reflect the fact that HOXA5 and TNF- α have different preferences in the activation of initiator caspase(s).

DISCUSSION

Previous work has shown that HOXA5 induced apoptosis in breast cancer cells through a p53-dependent pathway (41). In the present study, we found that HOXA5-induced apoptosis in Hs578T cells is mediated through a novel caspase-dependent but p53-independent pathway. Specifically, we present several lines of evidence to show that during apoptosis, both caspase 2 and caspase 8 activation occurs downstream of HOXA5 induction. Our experiments have demonstrated the following: (i) the inhibition of HOXA5-induced apoptosis by caspase 2 and caspase 8 inhibitors and their corresponding siRNAs, (ii) procaspase 2 and 8 cleavages after HOXA5-induction, and (iii) a synergistic activation of apoptosis by HOXA5 and TNF- α .

FIG. 6. Induction of HOXA5-expression resulted in the activation of caspase 2 and caspase 8. (A) The effects of different caspase inhibitors on HOXA5-induced apoptosis. Casp1in, Z-YVAD-FMK; Casp2in, Z-VAVAD-FMK; Casp3in, Z-DEVD-FMK; Casp6in, Z-VEID-FMK; Casp8in, Z-IETD-FMK; Casp9in, Z-LEHD-FMK. BA was used at a concentration of 50 μ M, and DPQ was used at a concentration of 30 μ M. All other inhibitors were used at a concentration of 100 μ M. The HOXA5-induced cell death without inhibitor treatment was referred to as 100% of apoptosis. (B) Time-dependent cleavage of procaspase (Pro-Casp) 2 after HOXA5 induction. (C) Time-dependent cleavage of procaspase 8 after HOXA5 induction. (D) Time course induction of caspase (Casp) 2-like, caspase 3-like, and caspase 8-like activities. Proteins were extracted at the times indicated, and caspase activities were measured as the absorbance of OD₄₀₅ with the substrates for caspase 2 (Ac-VAVAD-pNA), caspase 3 (Ac-DEVD-pNA), and caspase 8 (Ac-IETD-pNA). The OD₄₀₅s were normalized to the protein amounts. (E) Effects of caspase inhibitors on the caspase 2-, 3-, and 8-like activities by in vitro assay. The cell lysates were prepared from uninduced and induced cells and from induced cells treated with 100 μ M Casp1in, Casp2in, Casp3in, Casp8in, Casp9in, and ZVAD (Z-VAD-FMK) for 24 h. Each lysate was split into three parts used for the assay of caspase 2-, 3-, and 8-like activities.

FIG. 7. Requirement of caspase 2 for HOXA5-induced apoptosis. (A) Specific inhibition of caspase 2 expression by siRNA. HOXA5-inducible cells were transfected with the siRNAs to lamin, caspase 1 (Casp1), and caspase 2 (Casp2). At 48 h posttransfection, whole-cell lysate was prepared and used to determine the expression levels of procaspase 2 (Pro-Casp2) and procaspase 8 (Pro-Casp8; a control caspase) by Western blot analysis. (B) The siRNA to caspase 2 inhibited HOXA5-induced apoptosis. The siRNAs were transfected into the Hs578T-HOXA5 cells in duplicate. At 48 h posttransfection, doxycycline was removed (–DOX) from the medium of one set of transfected cells and left (+DOX) in the medium of another set of cells. After the expression of *HOXA5* was induced for an additional 16 h, the apoptotic cell percentages (sub- G_0/G_1 populations) were measured by flow cytometry analysis. (C) Specific inhibition of caspase 8 (Casp8) expression by siRNA. HOXA5-inducible cells were transfected with the siRNA to caspase 8. Four caspase siRNAs whose target sequences were shown in the Materials and Methods section were transfected as described above. (D) The siRNA to caspase 8 inhibited HOXA5-induced apoptosis.

Caspase 2 was the first identified mammalian apoptotic caspase (48). The exact role of caspase 2 in apoptosis is still controversial. Some studies showed that caspase 2, acting as an effector caspase, was cleaved by caspase 8 and caspase 3 but was not required for apoptosis induction (24, 32, 38). In other systems, caspase 2 acts as an initiator caspase and its activation has also been reported to be required for, and to have occurred upstream of, caspase 3 activation in apoptosis induced by etoposide, γ -irradiation, serum withdrawal, and treatment with atractyloside (23, 24, 26, 45, 46). Although there were no overt phenotypes in caspase 2-deficient mice (4), more recent studies

showed that caspase 2, as an initiator caspase, plays a critical role in stress or DNA damage-induced apoptosis (29). Since caspase 2 poorly activated other caspases *in vitro* (47), caspase 2 induced apoptosis presumably through the mitochondrial pathway. Indeed, caspase 2 activation has been shown to be required for translocation of the death protein Bax to the mitochondria as well as for release of the mitochondrial proteins cytochrome *c* and Smac/Diablo, early steps in the apoptotic program (23, 29). In our study, the caspase 2 inhibitor efficiently blocked caspase 2 activity as well as downstream caspase 3-like activity, whereas the caspase 3 inhibitor pre-

FIG. 8. Synergic activation of apoptosis by HOXA5 and TNF- α . (A) Induction of apoptosis in Hs578T cells by TNF- α required another costimulus such as cycloheximide (CHX). The cells were treated with different concentrations of TNF- α in the presence or absence of cycloheximide (1 μ g/ml) for 24 h and then measured for apoptosis by flow cytometry analysis. (B) Induction of HOXA5 expression potentiated cells to apoptosis induced by TNF- α . Cells cultured under induced (without doxycycline [-DOX]) or uninduced (+DOX) conditions were treated with same concentrations of TNF- α for 24 h and then measured for apoptosis. (C) Inhibition of TNF- α -induced apoptosis by caspase inhibitors. TNF- α (50 nM) and 1 μ g of cycloheximide/ml were used to induce apoptosis for 24 h. The inhibitors (each at a concentration of 100 μ M) described in the legend of Fig. 6 were used. C2, caspase 2 inhibitor; C3, caspase 3 inhibitor; C8, caspase 8 inhibitor; ZV, Z-VAD-FMK. (D) Inhibition of HOXA5- and TNF- α -induced apoptosis by caspase inhibitors.

vented caspase 3, but not caspase 2, activation. More importantly, the caspase 2 inhibitor could eliminate HOXA5-induced apoptosis, while the caspase 3 inhibitor had no effects on apoptosis. These findings on apoptosis inhibition suggest that caspase 2 acts as an initiator caspase in our system.

It remains unknown whether caspase 2 is the first caspase activated by HOXA5 since both caspase 2 and caspase 8 inhibitors can block HOXA5-induced apoptosis. It is likely that caspase 2 and caspase 8 were activated sequentially but not independently. The Western blot and in vitro activity assay results showed that caspase 8 activation is weaker and later than caspase 2 activation (Fig. 6B, C, and D). It seems likely that in this system caspase 2 directly activated caspase 8. But this sequential activation order seems to conflict with previously published data showing that caspase 8 can directly cleave and activate procaspase 2 in vitro but not vice versa (47). Caspase 2 substrates remain largely unknown and none of the known caspases can be efficiently cleaved by caspase 2 in vitro; it is unclear whether caspase 2 can directly cleave caspase 8 and other caspases in the cellular context. If not, caspase 2 may activate the caspase cascade through the mitochondrial pathway as shown in other systems, and caspase 8 activation shown by the Western blot analysis (Fig. 6C) may represent the feedback activation by other caspases.

The question raised here is how a transcriptional factor like HOXA5 initiated caspase activation. Actually, caspase activa-

tion mediated by transcriptional factors is not unprecedented (27). One previous study has shown that a transcriptional factor, STAT-1, is required for caspase 2 activation under certain conditions (27). p53 has been shown to transactivate caspase 6 and thereby increase the apoptotic sensitivities (35). In this study, HOXA5 did not directly activate the transcription of caspase 2 and caspase 8 at the transcriptional level because, upon induction of HOXA5 expression, the mRNA levels of caspase 2 and caspase 8 remained unchanged (data not shown) and the procaspase 2 protein levels decreased, presumably due to cleavage (Fig. 6B). p53 plays an important role in DNA damage-induced apoptosis, and its promoter has been shown to be directly activated by HOXA5 (41). p53 thus represents a perfect candidate gene which could relay the apoptotic signal from HOXA5 expression to caspase activation. However, in this paper we have shown that the expression levels of p53 and its target genes slightly decreased or remained unchanged. This discrepancy may partially be due to fact that p53 expression is regulated by many factors at several levels. The expression level of p53 will be determined by the availabilities and activities of all of these regulators. In addition, Hs578T cells carry a mutant p53 (with the amino acid substitution of D for E) (37). The obvious change in p53 expression levels was first observed at 24 h postinduction when more than 20% of the cells had died through apoptosis. Therefore, p53 appeared not to play an important role in mediating HOXA5-induced apo-

ptosis in Hs578T cells. We would like to propose a few possible mechanisms through which these genes downstream of HOXA5 may directly or indirectly activate the caspase cascade. First, this molecule may be an unidentified caspase or proteinase which may directly cleave procaspase 2 or 8. Second, there may be adaptor molecules which interact with caspase 2 or 8 analogous to the activation of caspases 2 and 8 through binding to the death domain (DD)-containing adaptor molecules FADD and RAIDD/CRADD (17). Finally, it was recently demonstrated that procaspase 2 is present in the intermembrane space of liver mitochondria and T-cell hybridoma mitochondria but is released in an activated form after PTP opening (45). The HOXA5-downstream genes may directly cause PTP opening and provide support for an autocatalytic mechanism of caspase 2 activation. Considering that the PTP inhibitor BA had no effect on HOXA5-induced apoptosis, the last possibility is less likely. The above theories are based on the transcriptional activation ability of HOXA5. We must also include the possibility that HOXA5 directly induces apoptosis through a protein-to-protein interaction in a transcription-independent manner. In order to identify the mediator of HOXA5-induced apoptosis, we have performed microarray analyses to obtain the gene profiles of induced and uninduced cells (our unpublished data). The microarray results allowed us to identify several candidate genes which have been shown to be involved in apoptosis. Further evaluation and functional studies of these genes are in progress.

The finding that HOXA5 expression sensitizes cells to TNF- α -mediated apoptosis provided other clues about the mechanisms of HOXA5-induced apoptosis. Upon binding to TNF- α , TNFR1 can form a death-inducing signal complex which leads to activation of caspase 8 and thereafter turns on the caspase cascade (21, 22). In addition, TNFR1 can recruit another receptor interactive protein (RIP) which stimulates pathways leading to activation of mitogen-activated protein (MAP) kinase and NF κ B. Although the role of MAP kinase in apoptosis is controversial, NF κ B has been shown to be a repressor of apoptosis (21, 22). More intriguingly, RIP can also recruit caspase 2 by interacting with another adaptor protein, RIP-associated ICH-1/CED-3 homologous protein (RAIDD) (17). A decision between life and death after TNF- α treatment is made based on the availability and active status of all these apoptotic activators and repressors. We did not observe apparent cell death even though the cells were treated with a high concentration (100 ng/ml) of TNF- α , indicating that Hs578T cells have balanced death-promoting and survival pathways. Interestingly, TNF- α at a concentration of 1 ng/ml was sufficient to induce further apoptosis in Hs578T-HOXA5 cells in the induced condition. An obvious explanation is that HOXA5 expression had activated caspase 2 and caspase 8, which had tipped the balance toward death. However, we could not rule out the possibility that HOXA5 had blocked the NF κ B and MAP kinase-mediated survival pathway as well.

Despite the similarity between the HOXA5- and TNF- α -mediated apoptotic pathways, they displayed some minor differences. That the caspase 8 inhibitor blocked both HOXA5- and TNF- α -mediated apoptosis indicated that caspase 8-like activity is required for both pathways. However, the caspase 2 inhibitor completely blocked HOXA5-mediated apoptosis but less efficiently blocked TNF- α -mediated apoptosis. In both

caspase 2-deficient mice and a caspase 2 siRNA knockdown cell line, TNF- α -mediated apoptosis was not significantly impaired, presumably because the caspase 8 pathway is still functional. In the Hs578T-HOXA5 cells, both caspase 2- and caspase 8-like activities are required for HOXA5-induced apoptosis. The individual contributions of caspase 2 and caspase 8 during HOXA5-induced apoptosis have not been completely resolved in this study.

Since all of our studies were performed under nonphysiological conditions, it remains unknown whether this HOXA5-induced p53-independent apoptotic pathway is cell-line-specific or an intrinsic signaling pathway which occurs in vivo. To address this issue, we need to extend our studies to more cell lines or primary tumors. We have shown that HOXA5 expression increased the apoptotic sensitivity to TNF- α . If these results could be confirmed under physiological conditions, one can postulate that the loss of HOXA5 can protect breast cancer cells from immune surveillance, especially to escape apoptosis induced by a cytokine (like TNF- α). We are currently testing whether the loss of HOXA5 expression renders tumor cells resistant to drugs and whether HOXA5 reexpression will sensitize tumor cells to apoptotic stimuli including chemotherapeutic drugs, proapoptotic gene expression, etc. If HOXA5 acts as apoptotic sensitizer, we can develop novel therapeutic strategies for drug-resistant tumors.

Besides its role in tumorigenesis, HOXA5 also plays a very important role in development, such as regulating hematopoietic lineage determination and maturation. *Hoxa5*-knockout animals displayed severe defects in the development of axial skeleton, the pectoral girdle, and the respiratory tract, which led to a high rate of perinatal lethality (3, 25). It remains an open question whether HOXA5-mediated apoptosis is involved in these functions. In fact, recently, HOX gene-mediated apoptosis was found to play an important role in development. For example, a Hox-like protein, Dfd in *Drosophila*, is a direct transcriptional activator of *rpr* in the anterior maxillary segment. In *Drosophila*, reaper (Rpr) is one of the three death-promoting proteins that induced most of the embryonic apoptosis (34). *Dfd* null mutants had abnormalities in the shape and the locations of the mandibular and maxillary lobes, partially due to an apparent excess of cells in the ventral part of the maxillary and mandibular segments. Another example is that of *Hoxa13* heterozygous mutant mice, where there is no interdigital apoptosis and no digit separation in 14-day-old embryos (40, 44). It remains to be seen whether *Hoxa13* and other Hox genes are direct regulators of apoptotic genes. Therefore Hox-regulated apoptosis is likely to be a general mechanism used to generate and maintain metameric patterns during animal development. In cancer cells, it appears that dysregulation of HOX gene expression may tip the balance of homeostasis against apoptosis and towards proliferation.

ACKNOWLEDGMENTS

We greatly appreciate help from Alan Rein in generating the HOXA5 polyclonal antibody. We are grateful to Atul Bedi for critically reading the text.

This work is supported by a Susan Komen fellowship (PDF0100603) to H.C. and an NIH Specialized Programs of Research Excellence grant P50CA88843 to S.S.

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