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PA-MSHA Inhibits Proliferation and Induces Apoptosis Through the Up-Regulation and Activation of Caspases in the Human Breast Cancer Cell Lines

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ABSTRACT

To investigate the effects of PA-MSHA (*Pseudomonas aeruginosa*-mannose sensitive hemagglutinin) on inhibiting proliferation of breast cancer cell lines and to explore its mechanisms of action in human breast cancer cells. MCF-10A, MCF-7, MDA-MB-468, and MDA-MB-231HM cells were treated with PA-MSHA or PA (Heat-killed *P. aeruginosa*) at different concentrations and different times. Changes of cell super-microstructure were observed by transmission electron microscopy. Cell cycle distribution and apoptosis induced by PA-MSHA were measured by flow cytometry (FCM) with PI staining, ANNEXIN V-FITC staining and Hoechst33258 staining under fluorescence microscopy. Western blot was used to evaluate the expression level of apoptosis-related molecules. A time-dependent and concentration-dependent cytotoxic effect of PA-MSHA was observed in MDA-MB-468 and MDA-MB-231HM cells but not in MCF-10A or MCF-7 cells. The advent of PA-MSHA changed cell morphology, that is to say, increases in autophagosomes, and vacuoles in the cytoplasm could also be observed. FCM with PI staining, ANNEXIN V-FITC and Hoechst33258 staining showed that the different concentrations of PA-MSHA could all induce the apoptosis and G_0–G_1 cell cycle arrest of breast cancer cells. Cleaved caspase 3, 8, 9, and Fas protein expression levels were strongly associated with an increase in apoptosis of the breast cancer cells. There was a direct relationship with increased concentrations of PA-MSHA but not of PA. Completely different from PA, PA-MSHA may impart antiproliferative effects against breast cancer cells by inducing apoptosis mediated by at least a death receptor-related cell apoptosis signal pathway, and affecting the cell cycle regulation machinery. J. Cell. Biochem. 108: 195–206, 2009.

KEY WORDS: PA-MSHA VACCINE; BREAST TUMOR; APOPTOSIS; CASPASE

As a metropolis with rapid social and economical development over the past three decades, Shanghai has a breast cancer incidence that surpasses all other cancer registries in China [Fan et al., 2009]. Although there has been a slight decrease in mortality in breast cancer patients [Schiffman et al., 2002] and adjuvant chemotherapy is effective in improving patient survival and treatment of the primary tumor, it is not uncommon for even early-stage breast cancer to metastasize. In addition, chemotherapy always destroys the immune system of the patient, resulting in serious problems. Therefore, there is a clear need for newer effective agents which possess both immunoregulatory and anticancer effects for patients with breast cancer. Novel therapeutic strategies are constantly being pursued. However, angiogenesis inhibitors and monoclonal antibodies [Bange et al., 2001] that act as anticancer chemotherapeutics cannot act as systemic adjuvant reagents in cancer immunotherapy. On the other hand, vaccines and breast cancer treatment approaches using dendritic cells [Tangri et al., 2001], live or attenuated pathogenic bacteria or their products are only able to act as systemic adjuvant reagents in cancer immunotherapy, and do not have anticancer cytotoxic activity

Zhe-Bin Liu and Yi-Feng Hou contributed equally to this work.

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[da Rocha et al., 2001; Chakrabarty, 2003]. Therefore, innovative treatment approaches are urgently needed.

Heat-killed *Pseudomonas aeruginosa* (PA), a gram-negative bacterium, has been successfully used as a protective vaccine; the *P. aeruginosa* vaccine is widely used for anti-infection and anti-inflammation purposes and even in anti-tumor therapies as an immune modulator [Lee et al., 2000a,b]. Recently, a vaccine using *P. aeruginosa*-mannose sensitive hemagglutinin (PA-MSHA) has been shown to increase antigen presenting function by activating the proliferation and differentiation of dendritic cells (DC cells) by the body [Mu, 1986]. The PA-MSHA strain is a kind of peritrichous *P. aeruginosa* strain with MSHA fimbriae established by Professor Xi-ya Mu, a Chinese microbiologist. He adopted biological engineering technology to make the non-MSHA heat-inactivated *P. aeruginosa* strain have many tenuous and upright MSHA fimbriae around the mycelium (Fig. 1). Besides the MSHA fimbriae, there are some other differences between PA and PA-MSHA as shown in Table I. Furthermore, PA-MSHA possesses cytotoxic qualities due to the addition of MSHA, which have been shown to have anticarcinogenic activity against human hepatocarcinoma cells and gastric cancer cells [Cao et al., 2008; Ling et al., 2008]. These findings suggest the use of PA-MSHA may be beneficial in breast cancer chemotherapy and so represents a possible tool in adjuvant therapy modalities.

Our study was designed to investigate the chemotherapeutic potential of PA-MSHA against breast carcinoma. We used both estrogen receptor (ER) negative and positive human breast cancer cell lines to characterize the anti-tumor activities of PA-MSHA in vitro. PA-MSHA significantly increased apoptosis in ER negative breast cancer cells, as visualized by phase contrast microscopy and evaluated by the annexin V assay. Western blotting and immunostaining revealed up-regulation of the cleaved apoptotic caspases and Fas protein expression. We hypothesized that PA-MSHA would have an antiproliferative effect on breast cancer cells via induction of the extrinsic caspase-mediated and intrinsic apoptosis pathways. Our results support the potential for the clinical application of a PA-MHSA-based vaccine in future treatments of breast cancer. Taking into account the new options offered to us by the biotherapy of cancer-related diseases, we can give an affirmative answer to the above question—PA-MSHA is capable of anticarcinogenic activity without losing its immune-modulatory effect.

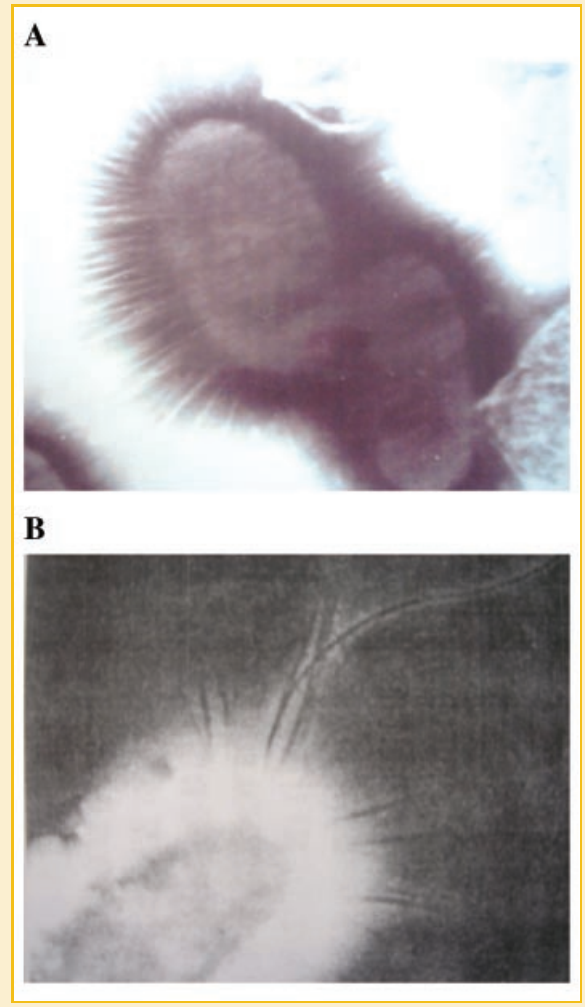

Fig. 1. A: PA-MSHA photographed by electron microscopy. B: PA photographed by electron microscopy. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

MATERIALS AND METHODS

CELL LINES, MATERIALS, AND ANTIBODIES

Non-tumorigenic breast epithelial cells (MCF-10A) and human breast cancer cell lines (MCF-7, MDA-MB-468, and MDA-MB-231HM) were used in this study. The MDA-MB-231HM cell line has a high potential for lung metastasis and was established from MDA-MB-231 using an in vivo stepwise selection scheme in our laboratory [Li et al., 2006]. The MCF-10A, MCF-7, and MDA-MB-468 cell lines was obtained from the American Type Culture Collection (ATTC, Manassas, VA), and grown in complete growth medium (as recommended by the manufacturer) supplemented with 10% fetal bovine serum (FBS) (GIBCO) and maintained in a humidified 5% CO_2 atmosphere at 37°C.

TABLE I. Characteristics of PA-MSHA Compared With PA

Characteristics of the bacteria	PA-MSHA	PA	Criterion (method of detection)
Morphology	A kind of peritrichous *P. Aeruginosa* with MSHA fimbriae (many tenuous and upright fimbriae around the mycelium)	There are no peritrichous MSHA fimbriae	Electron microscopy
Mannose hemagglutination test	Positive	Negative	Mu [1985]
Erythrocyte adhesion test on plate	Positive	Negative	Light microscopy (100×)
Direct agglutination test of yeast	Positive	Negative	Light microscopy (1,000×)

Inactivated *P. aeruginosa* (PA) was obtained from the China General Microbiological Culture Collection Center (No. 10118-4) and was used as a negative control for PA-MSHA throughout the study. PA-MSHA used in this study was donated by Professor Xi-ya Mu and kindly provided by Wanter Biopharma Company (Beijing, China). PA-MSHA is a mutant type of PA characterized by its mannose-sensitive hemagglutination pilus. After wild-type PA strain was fully attenuated by culturing with antibiotics for over 200 passages, the genomic DNA isolated from the attenuated PA strain was then repeatedly digested and ligated by endonucleases and DNA ligase. PA-MSHA was finally obtained from the attenuated PA strain transformed with such recombinant DNA. The PA-MSHA used in this study was scale-cultured at 37°C for 24 h, inactivated by chemical method and purified by centrifugation. In this study, the following primary antibodies used were all from Cell Signaling Technology (Danvers, MA): anti-caspase 3 (#9662), anti-cleaved caspase 3 (Asp175) (#9661), anti-caspase 8 (1C12) (#9746), anti-cleaved caspase 8 (Asp384) (11G10) (#9748), anti-caspase 9 (#9502), anti-cleaved caspase 9 (Asp315) (#9505), anti-Fas (#4233), and anti-beta-actin (#4967).

CELL PROLIFERATION

Cytotoxic effects of PA-MSHA or PA on the cells were evaluated by Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies Inc., Gaithersburg, MD) assays. Cells were seeded in 96-well plates (1×10^4 cells/well) in a concentration- or time-dependent way. The following day, the medium was changed and fresh medium containing 1% FBS and several indicated concentrations of PA-MSHA or PA (10, 5, 2.5, 1.25, 0.625, 0.313, or 0.156×10^9 bacterial per ml) was added; the cells were then incubated at 37°C for another 0, 1, 2, 3, 4, and 5 days. In the PA-MSHA treatment groups, 0.313×10^9/ml PA was added as a negative control. The culture medium was exchanged for fresh 1% FBS-medium with vehicle or PA-MSHA or PA after incubation for 48 h. The inhibition of cell growth was determined 24 h later by a reduction assay as follows: 10 μl of CCK-8 was added per well, cells were incubated for an additional 4 h, and absorbance at 450 nm was recorded using a 96-well plate reader (Sunrise Microplate Reader, Tecan US, Inc., Charlotte, NC).

EFFECT OF PA-MSHA ON MORPHOLOGICAL CHANGES

MDA-MB-231HM and MDA-MB-468 cells were incubated with 3.6×10^8/ml PA as a negative control and increasing concentrations of PA-MSHA (1.8×10^8/ml, 3.6×10^8/ml, and 6×10^8/ml) for a fixed time of 24 h. The appearance of morphological differentiation was assessed and observed under a phase contrast inverted light microscope and photographed with a Nikon F-601 AF Camera.

MORPHOLOGICAL MEASUREMENT OF APOPTOSIS

The morphological changes of apoptosis were assayed under a fluorescence microscope following staining with Hoechst 33258. The cells were treated with the appropriate drugs (PA of 3.6×10^8/ml, PA-MSHA of 1.8×10^8/ml and 3.6×10^8/ml) for 24 h and stained with 5 mg/L Hoechst 33258 (Sigma) for 30 min at 37°C, then visualized under a fluorescence microscope with standard excitation filters. Apoptotic cells were defined as cells showing nuclear and cytoplasmic shrinkage, chromatin condensation and apoptotic bodies. At least 300 cells were counted and the percentage of apoptotic cells (Apoptotic Index) was calculated at 400× magnification.

TRANSMISSION ELECTRON MICROSCOPY

MDA-MB-231HM cells (2×10^5) were plated in six-well plates and allowed to attach overnight. The cells were then treated with either 3.6×10^8/ml PA (control) for 24 h or 3.6×10^8/ml PA-MSHA for 6, 12, or 24 h at 37°C. Transmission electron microscopy to determine the effect of PA-MSHA treatment on the ultrastructure of MDA-MB-231HM cells was done essentially as described by Watkins and Cullen [1987]. Ultrathin sections (65 nm) were examined on a JEM-100CX transmission electron microscope (JEOL, Japan) at 10,000× or 50,000× magnification.

FLOW CYTOMETRY WITH ANNEXIN V-FITC AND PI STAINING

Cells were pretreated with the indicated concentration of PA (3.6×10^8/ml) and PA-MSHA (1.2, 1.8, 3.6, or 6×10^8/ml) for 12 h and single-cell suspensions containing at least 1×10^6 cells were made. Cell cycle and apoptotic analyses were done by flow cytometry as described previously, using a FACScalibur system (Becton Dickinson Biosciences, San Diego, CA) [Burdette et al., 2005; Wang et al., 2008]. Apoptotic cells were analyzed by quadrant statistics on the propidium iodide-negative and annexin V-positive cells. Data for the cell cycle analysis were done using ModFit LT™ software (Verity Software House, Inc., Topsham, ME) to determine the proportion of cells in the G_0/G_1, S, and G_2/M fractions of the cell cycle. Means ± SD were calculated for the cell populations from triplicate data.

WESTERN BLOT

Cells were lysed following the standardized protocol. Equal amounts of protein lysate (80 μg) concentrations were electrophoresed in 15% SDS–PAGE for cleaved caspase 3 and cleaved caspase 8 or 10% SDS–PAGE for Fas, caspase 3, caspase 8, caspase 9 and cleaved caspase 9 detection, followed by electroblotting onto polyvinylidene fluoride (PVDF) (Millipore Immobion™, Bedford, MA), for 1 h at 100 V. Membranes were blocked for 1 h at room temperature or overnight at 4°C in 5% nonfat milk in TBS with 0.1% Tween. The blot was incubated with the first antibody and then incubated with a horseradish-peroxidase conjugated second antibody (DAKO) diluted 1:1,000. Chemiluminescent detection of antibody binding was accomplished using an enhanced Chemiluminescence (ECL) Detection Kit (Amersham Pharmacia Biotech, Uppsala, Sweden) and images were captured by the FUJIFILM LAS-1000+ system (Fujifilm, Tokyo, Japan). To ensure that equal amounts of proteins were loaded, the blot was reprobed with rabbit anti-β-actin monoclonal antibodies (1:1,000). A densitometry analysis was performed by the software Quantity One (Bio Rad, Hercules). Relative expression level of protein was normalized by dividing the level of target proteins by the level of β-actin for each sample.

STATISTICAL ANALYSIS

Statistical analysis was performed using the software of Statistical Package for the Social Sciences (SPSS) Version 15 for Windows

(SPSS Inc., Chicago, IL). Student's *t* tests were used to determine the statistical significance of the differences between the experimental groups. A *P*-value of less than 0.05 was considered significant. Graphs were created with Excel software (Microsoft Office for Windows 2003).

RESULTS

PA-MSHA INHIBITS GROWTH OF BREAST CANCER CELL LINES AND IC_{50} VALUES OF PA-MSHA IN DIFFERENT BREAST CANCER CELL LINES

Changes in cell number caused by PA-MSHA were assessed every 24 h using a nonradioactive CCK-8 cell proliferation assay (Fig. 2). Fifty percent inhibitory concentrations (IC_{50}) are shown in Table II. As shown in Figure 2A, exposure of tumor cells to PA-MSHA for up to 120 h had a cumulative effect on MDA-MB-231HM (a,b) and MDA-MB-468 (c,d) cell proliferation in a concentration- and time-dependent manner for the same time and concentration range. However, exogenously added PA-MSHA did not inhibit the immortalized nontransformed human mammary epithelial cell line (MCF-10A) proliferation (Fig. 2B,a,b) and also showed very low inhibition effect on the ER positive cell line (MCF-7) (Fig. 2B,c,d), indicating that MCF-10A and MCF-7 cells were relatively resistant to PA-MSHA induced apoptosis with at least 70% viability compared with control cells. As a negative control for PA-MSHA, exogenously added PA (Fig. 2C) caused very little cell growth inhibition on MDA-MB-231HM (a,b) and MDA-MB-468 (c,d) cells following the same incubation concentration and periods.

PA-MSHA CAUSES A REDISTRIBUTION OF THE CELL CYCLE

Because PA-MSHA slowed proliferation of cells, we investigated the mechanism by which it exerted these growth-regulatory effects. Cells treated with either PA or PA-MSHA for 12 h were stained with PI and analyzed by flow cytometry. Challenging MDA-MB-231HM (Fig. 3A) and MDA-MB-468 (Fig. 3B) with rising concentrations of PA-MSHA (1.8, 3.6, and 6×10^8/ml) dose dependently arrested MDA-MB-231HM and MDA-MB-468 cells in the G_0/G_1 phase of the cell cycle, thereby decreasing the proportion of cells in the S phase. In both cell lines, an additional

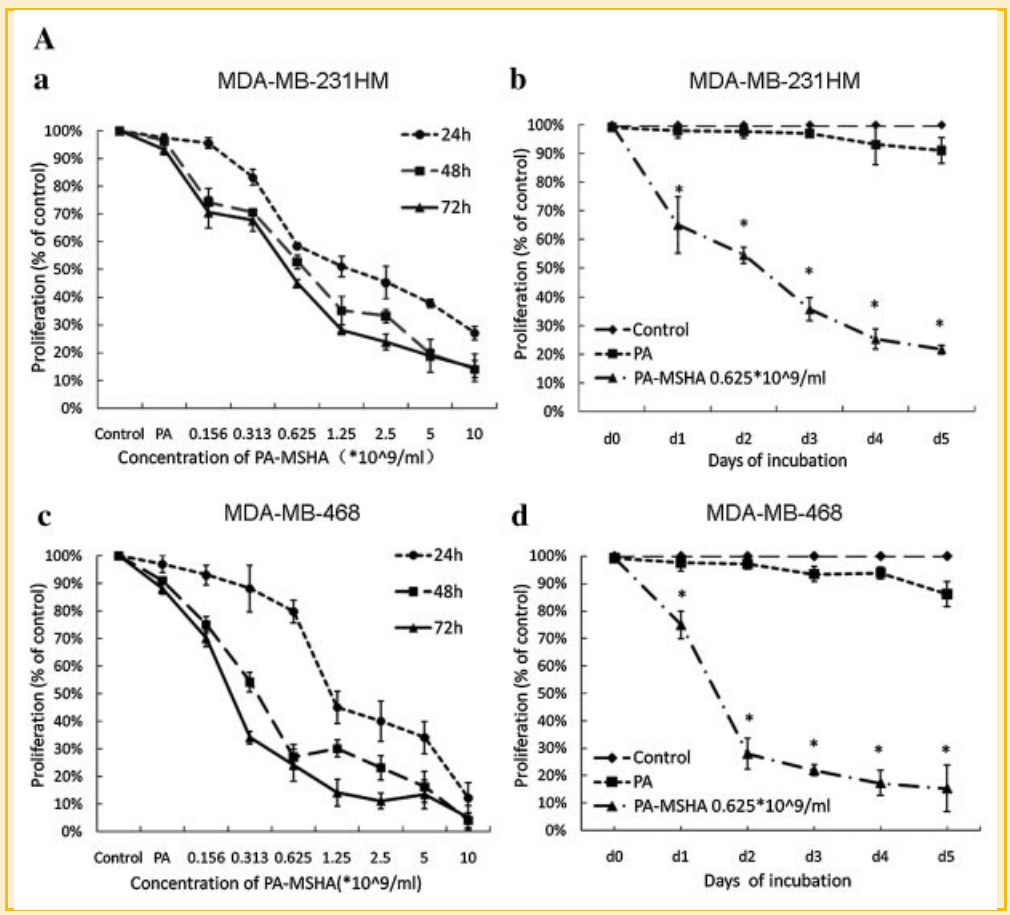

Fig. 2. Effect of PA-MSHA or PA on cell proliferation. Values are given as a percentage of untreated control cells. The data are presented as the averages for triplicate results from a representative experiment; bars, SD. A,B: Dose-dependent (a,c) and time-dependent (b,d) effect of PA-MSHA on MDA-MB-231HM (A,a,b), MDA-MB-468 (A,c,d), MCF-10A (B,a,b) and MCF-7 (B,c,d) cell proliferation. C: Dose-dependent (a,c) and time-dependent (b,d) effect of PA on MDA-MB-231HM (a,b) and MDA-MB-468 (c,d) $^*P < 0.05$ for MDA-231HM or MDA-MB-468 cells treated with PA-MSHA versus control.

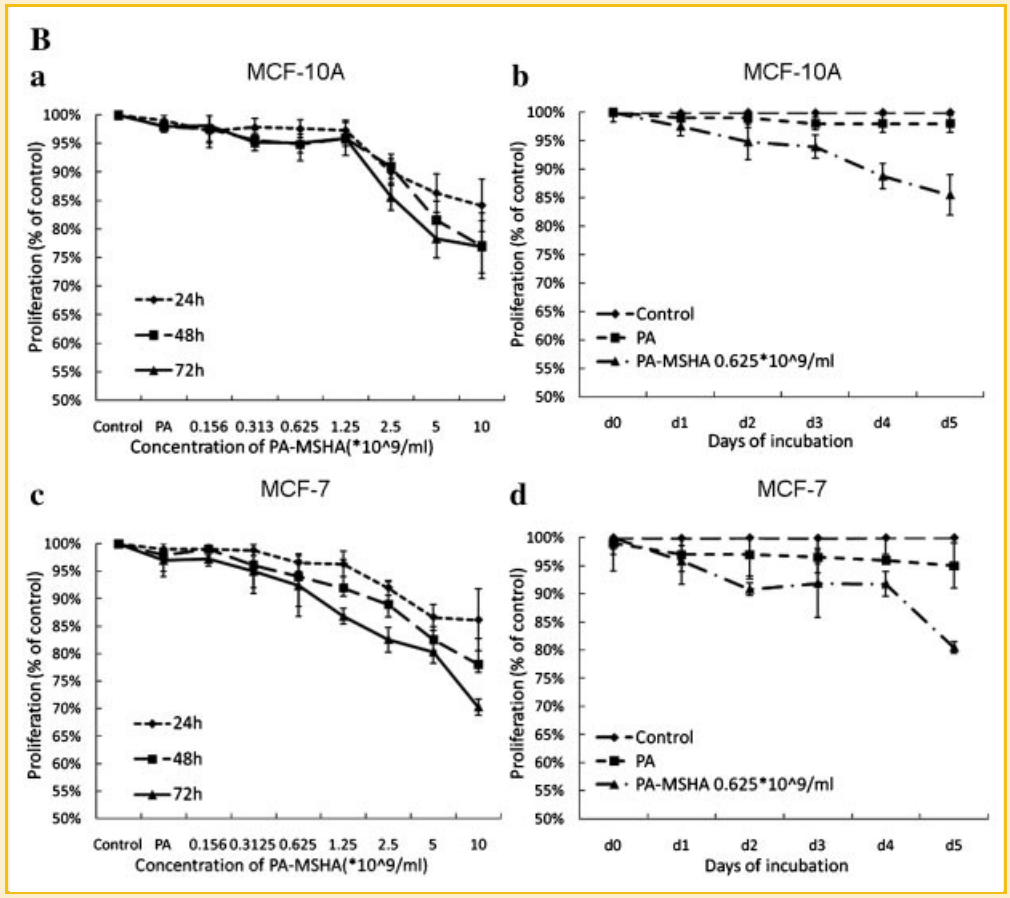

Fig. 2. (*Continued*)

accumulation in the G_2/M phase was observed. However, in MDA-MB-231HM cells, G_2/M phase arrest was observed with PA-MSHA concentrations under 3.6×10^8/ml.

CELL APOPTOTIC MORPHOLOGICAL CHANGE INDUCED BY PA-MSHA

When viewed with a phase-contrast microscope (Fig. 4A), the MDA-MB-231HM and MDA-MB-468 cells treated with PA remained flat with a uniform polygonal shape and exhibited typical growth patterns (a,e). When treated with different concentrations of PA-MSHA, cells became irregular and detached (b,f). Some of the cells lost their adherence and cellular alterations were found including shrinkage, rounding, detachment, membrane blebbing, destruction of cell membrane, reduction in the number of pseudopodium and microvilli, and segregation of cellular structure; these changes were noted in the presence of 3.6×10^8/ml PA-MSHA (c,g). At 6×10^8/ml PA-MSHA, the cells lost their surface morphology and there was a dramatic increase in the number of apoptotic cells rounding-up, detaching from the substrate and subsequently dying (d,h). In order to get some more detailed morphological changes, we used transmission electron microscopy (Fig. 4B). Compared with cells treated with PA, MDA-MB-231HM cells treated with PA-MSHA for 12 h exhibited much less cytoplasmic organelles. Most of the cells contained intracellular bacteria within vacuoles (long arrows). Furthermore, the transmission electron microscopy data also showed an increase in autophagocytic vacuoles in the PA-MSHA treated cells (short arrows). After 24 h, changes noted included shrinkage of total cell volume and numerous dilatation and cytoplasmic vacuolization of the endoplasmic reticulum (bold arrows), all characteristic of cells undergoing apoptosis. After cells were incubated for 48 h, most cells were dead with disruption of cytoplasmic architecture and formation of cytoplasmic "blebs" (arrowheads). The effect of PA-MSHA on the morphology of breast cancer cells was further tested in the MDA-MB-231HM and MDA-MB-468 cell lines using the fluorescent nuclear DNA stain Hoechst-33258. Treatment of cells with 1.8×10^8/ml and 3.6×10^8/ml PA-MSHA for at least 24 h resulted in morphological changes distinguished by their characteristic patterns of apoptosis, including nuclear condensation, cytoplasmic rounding and nuclear fragmentation resulting in groups of isolated pieces of condensed chromatin (Fig. 4C).

MEASUREMENT OF APOPTOSIS BY ANNEXIN V ASSAY

Cultures of both MDA-MB-231HM and MDA-MB-468 cells growing in medium with PA contain a low number of apoptotic cells (Fig. 4D,a,e). In the presence of PA-MSHA (1.8×10^8/ml), a slightly

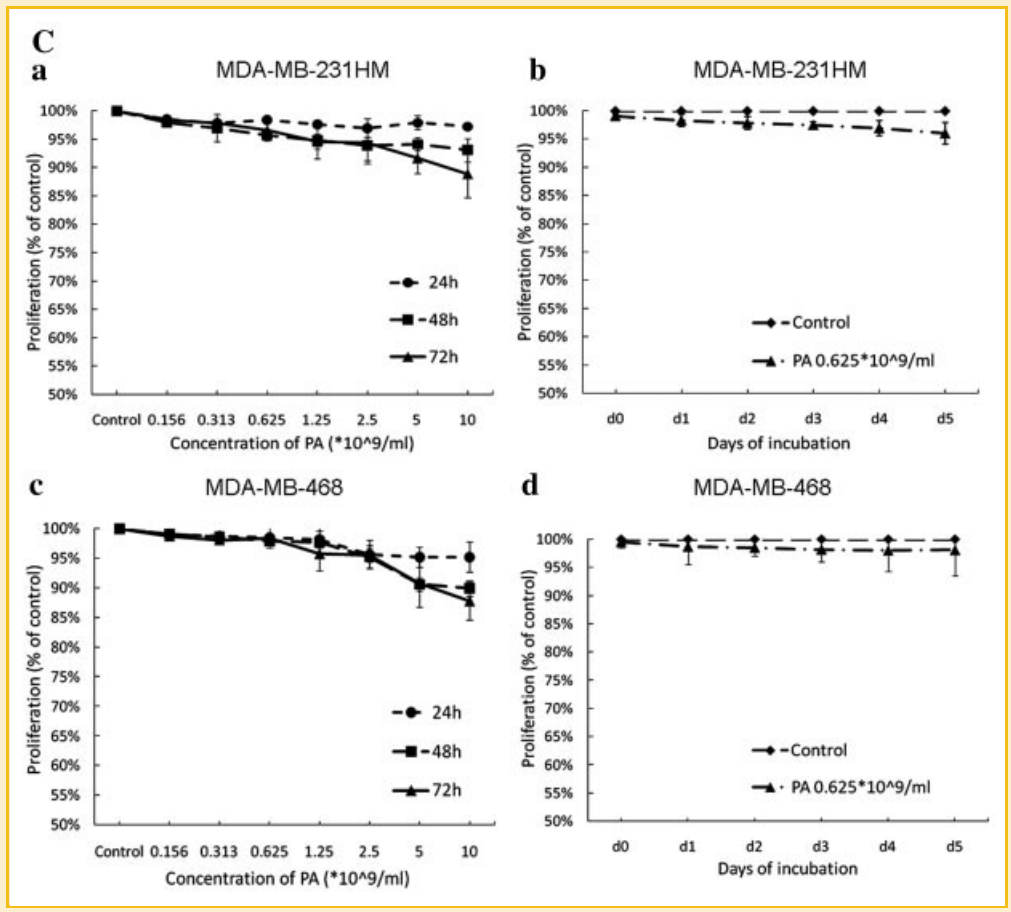

Fig. 2. (*Continued*)

elevated number of apoptotic cells were detected (Fig. 4D,b,f, E). The apoptotic cell number increased 8- to 10-fold after treatment with high concentration (3.6×10^8/ml and 6×10^8/ml) of PA-MSHA (Fig. 4D,c,d,g,h,E). But in MCF-7 cells, the apoptotic cell numbers did not change a lot even after treatment of the highest concentration of PA-MSHA (Fig. 4D,i–l,E).

PA-MSHA INDUCED APOPTOSIS VIA CASPASE CASCADE PROTEINS

In order to further investigate the mechanisms behind PA-MSHA-induced apoptosis, MDA-MB-231HM and MDA-MB-468 cells were treated with PA-MSHA to check for the activation of caspase associated proteins. The lysates were analyzed using an antibody directed against the caspases 3, 8, and 9, and the cleaved caspase form of caspase protein and Fas protein. As we expected, by 12 h post-PA-MSHA, neither MDA-MB-231HM nor MDA-MB-468 cells revealed further activation of all caspases (data not shown). However, when MDA-MB-231HM and MDA-MB-468 cells were exposed to PA-MSHA for more than 24 h, there was a dose-dependent loss of procaspase-3, -8, and -9, and also a concentration-dependent increase in Fas protein and cleaved caspase protein (Fig. 5A,B), indicating the proteolytic processing of the proenzyme to its active enzyme subunits. However, when MDA-MB-231HM and MDA-MB-468 cells were exposed to PA, there was no dose-dependent loss of procaspase-3, -8, and -9, nor was there a concentration-dependent increase in cleaved caspase protein at 24 h (Fig. 5C,D).

DISCUSSION

Anaerobic bacteria have been employed in treating cancer for a long time—almost 150 years [Samuilov, 2003; Punj et al., 2004b]. The use of live microorganisms has been validated for inhibiting tumor growth involving breast tumor cells [Samuilov, 2003], but it is always associated with infectious or other side effects related to the

TABLE II. IC_{50} Values of PA-MSHA in Different Breast Cancer Cell Lines

	IC_{50} ($\times 10^9$/ml)		
Cell lines	24 h	48 h	72 h
MDA-MB-231HM	2.2	0.75	0.54
MDA-MB-468	1.8	0.41	0.20
MCF-10A	20.7	14.4	4.3
MCF-7	15.5	10.8	3.7

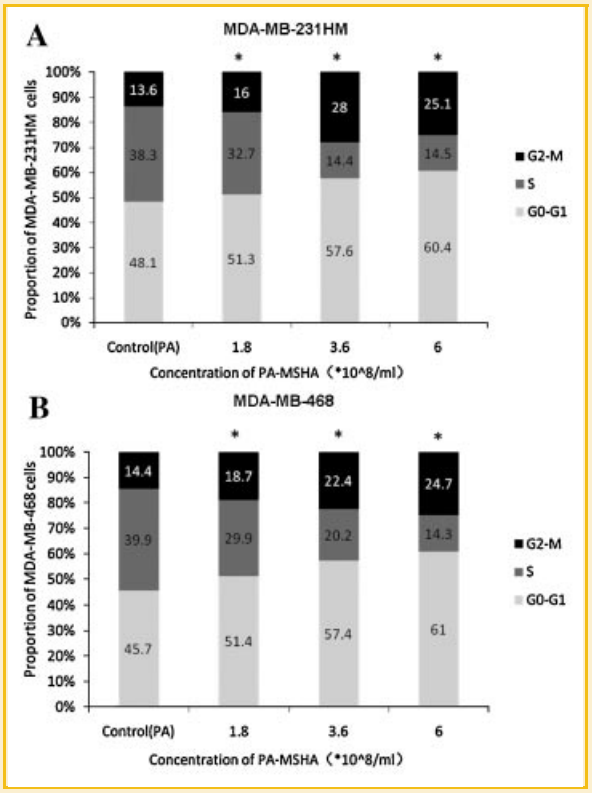

Fig. 3. PA-MSHA redistributed cell cycles. Cell-cycle distribution of MDA-MB-231HM (A) and MDA-MB-468 cells (B) in the three phases of the cell cycle are represented by percentages and representative pictures under these treatment conditions. $^{*}P < 0.05$ for cells treated with PA-MSHA versus control in all G_0–G_1, S and G_2–M phases.

microorganisms [Dang et al., 2001; Chakrabarty, 2003]. Although a bacterial constituent or metabolite such as a *P. aeruginosa* cupredoxin, azurin [Punj et al., 2004a] or exoenzyme S (ExoS) [Alaoui-El-Azher et al., 2006] can act as an anticancer chemotherapeutic, it cannot act as a systemic adjuvant reagent in cancer immunotherapy. In previous publications we have learned that heat-killed *P. aeruginosa* is able to act as a systemic adjuvant reagent in cancer immunotherapy, but does not possess anticancer cytotoxic activity [Mathe et al., 1977]. Therefore, we need to identify a new type of microorganism associated not only with powerful anticancer activity and minimal side effects but also with great immunoregulatory potency. As a result of our pursuit, we have identified, isolated, and purified a biologically engineered bacterial product (PA-MSHA) that not only has potential powerful anticancer cytotoxic activity to arrest tumor growth in vitro and in vivo in nude mice, but also an immunotherapy effect [Mu, 1986]. Establishing PA-MSHA takes the naturally found non-peritrichous, non-MSHA *P. aeruginosa* toxic strain and, through several generations, lowers its toxicity and induces many tenuous and upright MSHA fimbriae around the mycelium using biological engineering technology (Fig. 1A). The critical effective pharmacological site of PA-MSHA is located in the fimbrium and is characterized by a mannose sensitive binding protein that can specifically conjugate with rich mannose on the surface of tumor cells of the high mannose type. Such kind of specific combination is capable of inhibiting tumor cell proliferation, apoptosis and the morphological changes of super microstructure. The MSHA fimbriae are the most important reason why PA alone has no antiproliferative or apoptotic effects. Aside from the MSHA fimbriae, there are some other differences between PA and PA-MSHA. As shown in Table I, the results of the mannose hemagglutination test, plate erythrocyte adhesion test and direct agglutination test of yeast are all positive in PA-MSHA but negative in PA.

The immunoregulatory effect of PA-MSHA has been validated. PA-MSHA has been shown to be effective as a biological agent in improving the immune response of patients with several cancer types and some other diseases including several cancer types [Li et al., 2000; Sun et al., 2000], lymphocyte proliferation and NK cell [Zhang and Jin, 1994], severe traumas [Shan et al., 2007], infection [Cheng et al., 2000], chronic diseases such as hepatic fibrosis [Jia et al., 1999], MSHA has also been shown to be effective as a vaccine in plasma phospholipids metabolic profiling and in the ratio of Th2/Th1 cells within immune organs of mice with IgA nephropathy [Jia et al., 2007]. Aside from its immunoregulatory effect, we now discover that PA-MSHA has an anticancer cytotoxic effect. Only two studies have validated its anticancer cytotoxicity. One demonstrated that PA-MSHA inhibits the growth of human hepatocarcinoma cells via mannose-mediated apoptosis [Cao et al., 2008]. The other in vitro study has also reported that the inactivated PA-MSHA can bind specifically to the tumor cell surface of gastric cancer cells MKN45, and through the fimbriae inhibit the growth of gastric cancer cells [Ling et al., 2008]. Our study is the first systematic effort to assess the cytotoxic effects of PA-MSHA on breast cancer cells. Antiproliferative assays of the breast tumor-derived cell lines were done in the present study. In Table II, the IC_{50} doses for MDA-MB-231HM and MDA-MB-468 cell lines were below 2.5×10^9/ml. This IC_{50} dose range is reasonable, meaning that the tumor cells are sensitive to this agent (PA-MSHA). The IC_{50} doses for the other two cell lines (MCF-7 and MCF-10A), however, were clearly significantly higher than has been demonstrated for sensitive cell lines. In sensitive cell lines, a very low concentration of PA-MSHA can cause cell death. However, in the relatively resistant cell line (MCF-7) and the nontumorigenic human mammary epithelial cell line (MCF-10A), even 10-fold higher concentrations of PA-MSHA failed to induce a greater degree of cytotoxity. The data presented in our study demonstrated that PA-MSHA exerted direct and selective antiproliferative effects on breast cancer cells without affecting the normal mammalian epithelial cells at similar concentrations. PA-MSHA preferentially inhibited the proliferation of estrogen receptor (ER) negative cell lines MDA-MB-231HM and MDA-MB-468; these cell lines are all ER negative and EGFR/Her-1 positive, PA-MSHA was relatively resistant to the ER positive cell line (MCF-7). In our study, this finding may help us to identify more therapeutic targets and clarify the mechanism of the antiproliferative effect of PA-MSHA. However, more experiments are needed to validate these hypotheses.

Based on the results of PA-MSHA cytotoxicity, we have done further studies to examine the morphological changes of cells via light, electron and fluorescence microscopy. The apparent PA-MSHA-treated cells have disruption of cell adhesion, thus causing

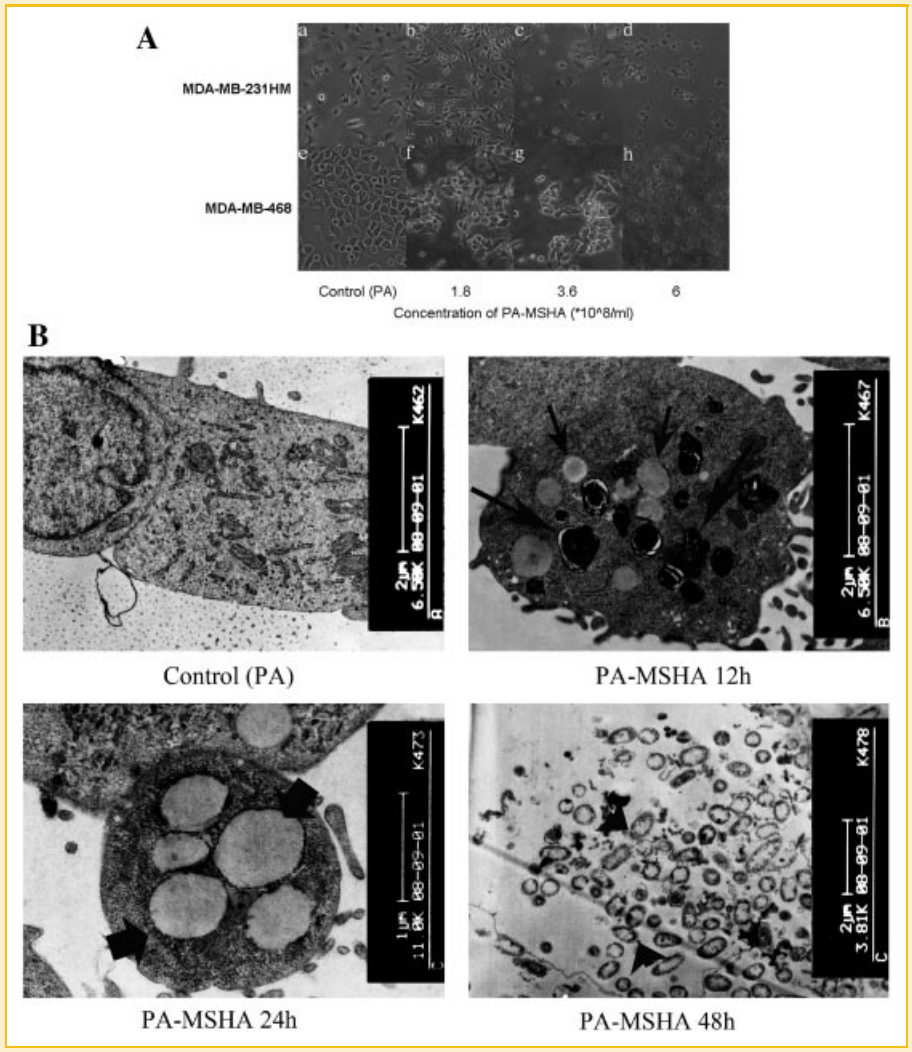

Fig. 4. A: Phase contrast microscopic pictures showing the morphology of MDA-MB-231HM (a–d) and MDA-MB-468 (e–h) cells treated with the indicated concentrations of PA-MSHA for 24 h. Cells were photographed under a microscope (magnification, 200×). B: PA-MSHA induced ultrastructural morphological change of MDA-MB-231HM cells under the transmission electron microscope. Cells were treated with 1.8×10^8/ml PA or with 3.6×10^8/ml PA-MSHA for 12, 24, and 48 h, respectively. Bars were as shown in the pictures. C: Nuclear staining of MDA-MB-231HM and MDA-MB-468 cells with Hoechst 33258. Upper panel: control Cells with intact nuclear morphology. Middle and lower panel: Cells treated with PA-MSHA for 24 h with typical punctuated nuclear morphology of apoptotic cells (original magnification, 400×). D: the apoptotic fraction of cells detected by annexin V staining (*x*-axis)/propidium iodide (*y*-axis) staining after different treatments. The ratio of apoptotic cells which should be the annexin V positive/PI negative fraction was measured in MDA-MB-231HM (a–d), MDA-MB-468 (e–h) in serum-free medium with PA or in increased concentrations of PA-MSHA as indicated with serum-free medium for 24 h. Results are representative of three independent experiments. E: The percentages are displayed showing the annexin V-positive/PI negative fraction. Columns are expressed as mean ± SD of three independent experiments. $^*P < 0.05$ for PA-MSHA versus control in MDA-MB-231HM cells. $^\#P < 0.05$ for PA-MSHA versus control in MDA-MB-468 cells.

morphological changes and the detachment of cells from the substratum and their neighbors. Furthermore, we did more experiments to help us confirm the PA-MSHA-induced anticancer effect. With Annexin-V/PI staining (FITC+/PI−), PA-MSHA-induced apoptotic changes were observed in MDA-MB-231HM and MDA-MB-468 cells by FACS analysis; these changes represent early markers of apoptosis. In the cell-cycle analysis, a significant increase of the cell population in the G_0/G_1 phase was observed at increased concentration of PA-MSHA. PA-MSHA activated caspase-3, the major effector caspase. Characteristic apoptosis was observed by all of the above apoptosis-related experiments. Taken together, these results suggest that PA-MSHA is able to decrease the viability of breast cancer cells through the induction of caspase-dependent apoptosis.

The observed induction of apoptosis and cell cycle arrest by PA-MSHA in breast cancer cells is important because dysregulation of normal cell cycle- and apoptotic-machinery plays a critical role in the development of a neoplasm. The fact that PA-MSHA may induce cell cycle arrest and apoptosis strongly suggests that either it binds on the surface of MDA-MB-231HM and MDA-MB-468 cells with specific receptors to modulate a signaling pathway, or it enters the cytosol of the cells to directly induce cell cycle arrest or apoptosis. It

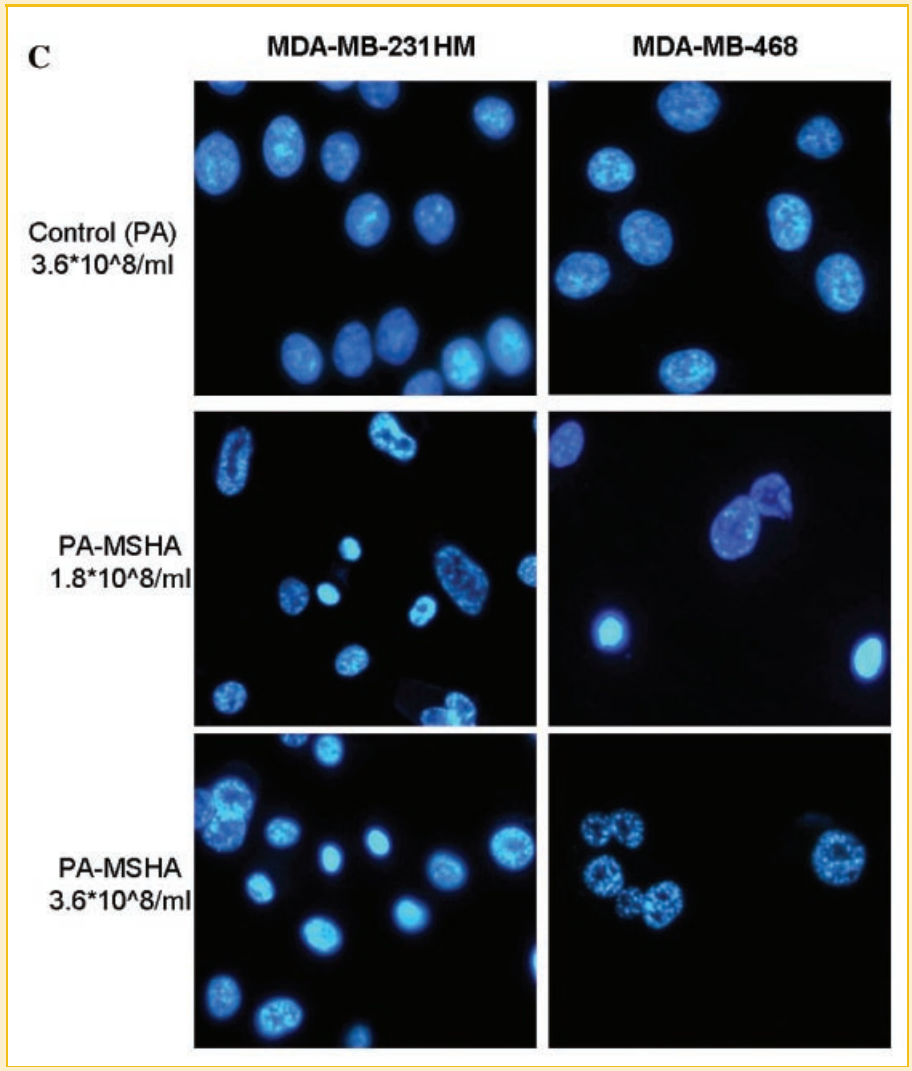

Fig. 4. (*Continued*)

was also of interest to determine if the binding and/or entry of PA-MSHA is a process involving receptor binding. In our subsequent studies, experiments showed that PA-MSHA may inhibit the cell proliferation, survival, and transformed phenotype via the Her-1/EGFR signal pathway, promoted probably in a mannose-mediated pathway. However, further in-depth and detailed experiments are needed to verify this theory.

Caspases have been shown to be activated during apoptosis in many cells and play critical roles in both the initiation and execution of apoptosis. It has been previously shown that live *P. aeruginosa* can induce apoptosis via both the extrinsic and intrinsic apoptosis pathways [Jendrossek et al., 2001; Cannon et al., 2003], yet apoptosis is not induced by heat-killed *P. aeruginosa* [Jenkins et al., 2004; Punj et al., 2004a]. Currently, there exist two recognized points at which caspases are activated to initiate apoptosis. In the extrinsic pathway, the initiator caspase-8 is activated by adapter-mediated recruitment to the receptor's cytosolic face following Fas ligation [Muzio et al., 1998]. Alternatively, in the intrinsic pathway, the initiator caspase-9 is activated following release of mitochondrial components to form the Apaf complex [Liu et al., 1996]. Our data suggest that PA-MSHA acted by triggering both the intrinsic and the extrinsic apoptosis pathways; however, we do not know yet which way was dominant. Although the importance of the mitochondrial pathway in death receptor-triggered apoptosis is unknown, we are confident that mitochondria play a central role in the apoptotic process. Both the intrinsic pathway and the extrinsic pathway can converge at the mitochondrial level and trigger mitochondrial membrane permeabilization. Moreover, we have mentioned that PA-MSHA is a mannose sensitive bacterial vaccine and was sensitive to the ER negative and EGFR/Her-1 positive cell lines. The EGFR pathway also cross reacts with the proapoptotic caspase cascade [Datta et al., 1997]. Therefore, our data suggest that PA-MSHA-induced apoptosis of human breast cancer cells is mediated via caspase activation triggered by both the death receptor signaling pathway and some other mitochondrial or EGFR associated pathways. Nevertheless, the exact mechanism(s) by

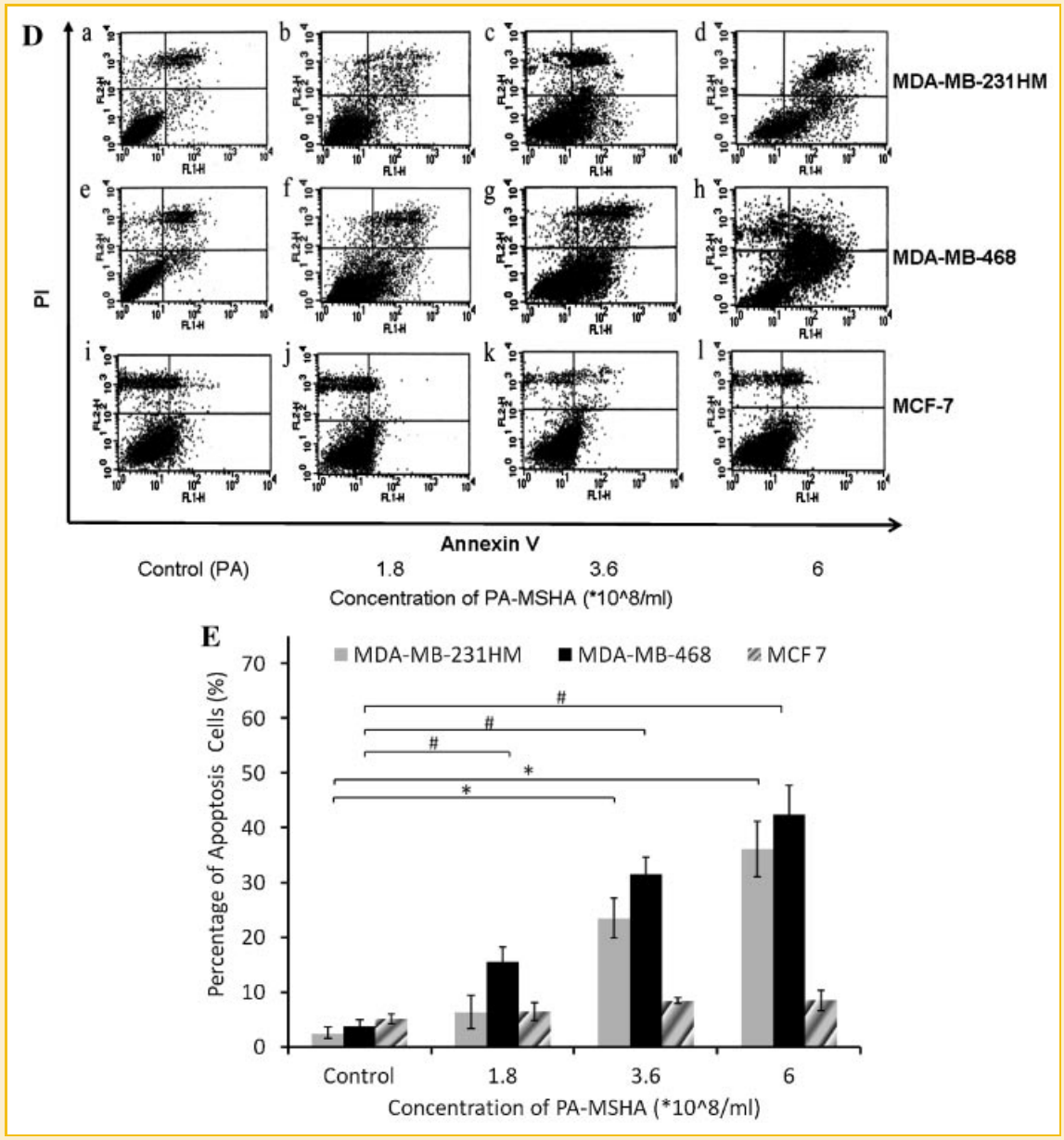

Fig. 4. (*Continued*)

which PA-MSHA affects these elements leading to cell cycle arrest and apoptosis is unclear, and needs more detailed and in-depth basic studies.

Taken together, our data demonstrate for the first time that PA-MSHA may impart cancer antiproliferative/chemotherapeutic effects against breast cancer by inducing apoptosis mediated via modulating caspase family proteins and affecting cell cycle regulation machinery. It has generally been believed that bacteria activate the immune system and then allow for cancer regression. The experiments with PA-MSHA, however, indicated that besides an activated immune system, cytotoxicity may also contribute to cancer regression. We believe that PA-MSHA, either alone or in combination with standard therapy, could be a novel strategy for the management of breast cancer. However, further studies are needed to validate our findings in appropriate animal models.

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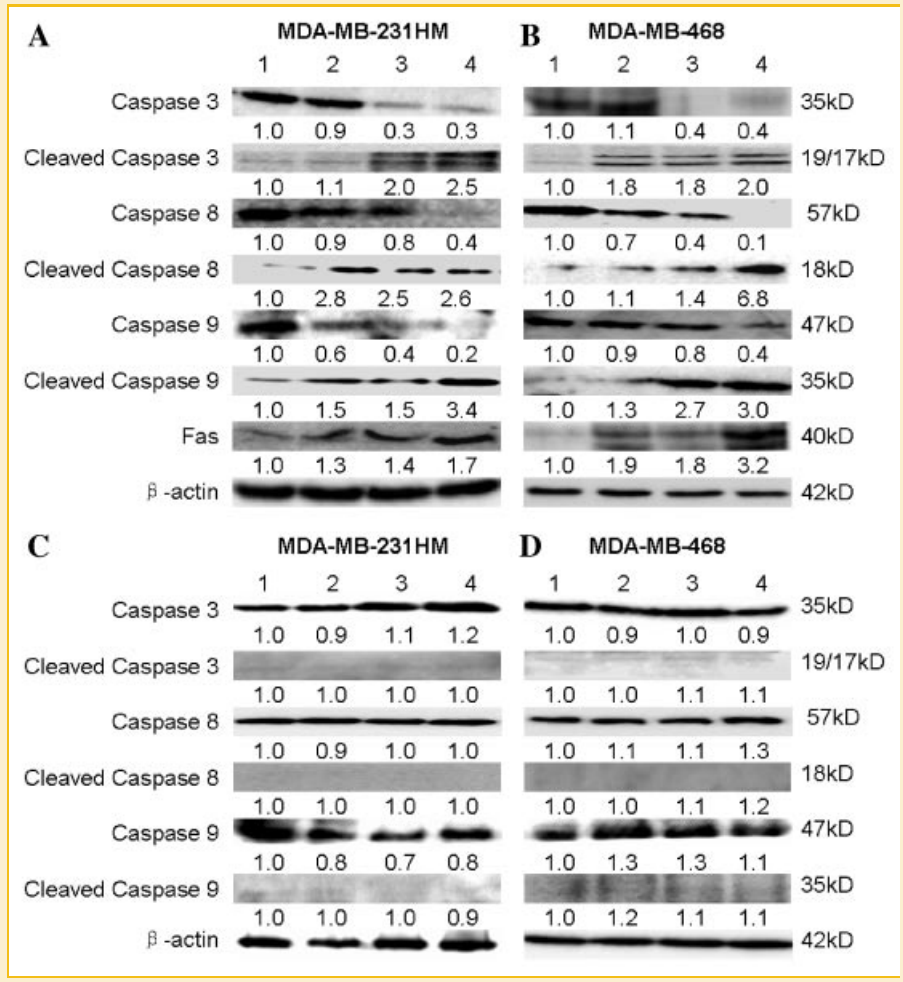

Fig. 5. The cells were treated with PA-MSHA at the indicated concentrations for 24 h. MDA-MB-231HM cells (A,C) and MDA-MB-468 cells (B,D) were treated with PA-MSHA (A,B) or PA (C,D) for 24 h. Each antibody was used in at least three independent experiments. Protein loading was normalized based on β-actin. Lane 1: Control; Lane 2: PA-MSHA or PA of 1.8×10^8/ml; Lane 3: PA-MSHA or PA of 3.6×10^8/ml; Lane 4: PA-MSHA or PA of 6×10^8/ml. The densitometric quantitation on caspases and Fas, relative to control group, was done on the bands of Western blotting and shown below each lane.

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