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ORIGINAL ARTICLE

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Intracellular signaling in the induction of apoptosis in a human breast cancer cell line by water extract of Mekabu

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Abstract
Background. We previously reported that water extract of Mekabu, a kind of seaweed, induced apoptosis in a human breast cancer cell line. In the present study we investigated intracellular signaling in apoptosis, with a focus on caspases.
Methods. Mekabu extract, obtained with ultrapure water, was used to induce apoptosis in a human breast cancer cell line, MDA-MB231, and DNA fractionation was investigated by flow cytometry and electrophoresis. In addition, using the caspase detection kit Caspa Tag, activation of caspases 3, 6, 8, and 9 was observed under a fluorescence microscope. Furthermore, using antibodies to caspases 3, 8, 9, and Bid, we conducted a protein analysis by Western blotting to determine the activation of these substances.
Results. Obvious ladder formation demonstrating DNA fractionation was seen, confirming that Mekabu extract induced apoptosis. In the fluorescence microscope observations, activation of caspases 3, 6, and 8, but not caspase 9, was seen. Activated caspases 3 and 8 were detected in the Western blotting analysis, but no proteins of activated caspase 9 or Bid were detected.
Conclusion. Mekabu extract activates caspases 3, 6, and 8 and contributes to intracellular signaling to induce apoptosis in a human breast cancer cell line. This signaling is not via the mitochondria.

Key words Water extract of Mekabu · Breast cancer · Apoptosis · Caspase · Bid

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Introduction

Breast cancer patients have frequent complications with thyroid disorder, and we have reported several experiments focusing on the relation between iodine and breast cancer.[1–5] Based on these results, we have conducted experiments to show the effects of Wakame and Mekabu, types of seaweed with a high iodine content, on cell proliferation in a human breast cancer cell line (in vitro) and on the induction of rat breast cancer by 7,12 dimethylbenz(a)anthracene (DMBA) (in vivo). The results showed that both Wakame and Mekabu inhibited the proliferation of human breast cancer cells by inducing apoptosis in them.[4,5] Furthermore, water extract of Mekabu showed a strong preventive effect against the development of breast cancer induced by DMBA in rats.[5] In that experimental series, we found that water extract of Mekabu acted to suppress breast cancer carcinogenesis in rats, without toxicity. Converting from rat to human body weight, the daily intake required for this effect would be about 5 g/day in humans. However, the mechanism of action remains unclear, and in the present experiment, using a human breast cancer cell line, we focused on intracellular signaling, mainly by caspases, in apoptosis induced by a water extract of Mekabu.

Materials and methods

The human breast cancer cell line used in this study was MDA-MB231 (American Type Culture Collection, Bethasda, MD, USA). MDA-MB231 was obtained from breast cancer in a 51-year-old white woman. This cell line is characterized by negativity for hormone receptors,[6] the expression of membrane surface recptors for epidermal growth factor (EGF) and transforming growth factor (TGF)α, and Fas/CD95 positivity.[7] The Mekabu extract that was added to the culture medium was prepared according to the following method. Pulverized dried Mekabu

powder (1 g) was dissolved in 50 ml of ultrapure water, and extracted by leaving at rest for 12 h at 4°C. After centrifugation, the supernatant was collected and filtered through GDIX Syringe Filters (Whatman, Clifton, NJ, USA). The liquid component was freeze-dried to obtain 250 mg of Mekabu water extract freeze-dried power (MWEFDP). The culture medium used was MEM with Earle's balanced salts (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Life Technologies) plus 2 mml of glutamine. The following experiments were performed using the above system.

Detection of DNA fragmentation by flow cytometry

MDA-MB231 cells (6×10^5/ml) were spread on a 6-cm dish and precultured for 12 h. The cells were then cultured for 96 h in two groups. One group had an addition of 2 mg/ml MWEFDP dissolved in culture medium, and the other did not. In the basic experiments we wanted to determine the smallest amount of MWEFDP that would induce a sufficient level of apoptosis. We found this to be 2 mg/ml MWEFDP; beyond this concentration we saw no considerable difference in the results. In terms of the weight of Mekabu this is equal to 1/125 g, which is a reasonable concentration considering application to humans. The supernatant was combined with trypsinized cell, and washed in cold phosphate buffered Saline (PBS) (−). Cells were fixed for 10 min on ice in 500 µl cold ethanol, and after a washing in PBS, were processed for 30 min in RNase 80 mg/ml (Sigma, St. Louis, MO, USA) at 37°C. After two washings with PBS (−), the cells were suspended in 450 µl PBS (−). Propidium iodide (0.2 mg/ml; Sigma) was added and the cells were stained for 30 min in darkness on ice, after which flow cytometry (EPICSXL, Coulter, Hialeah, FL, USA) was performed.

Detection of DNA fragmentation by agarose gel electrophoresis

MDA-MB231 cells (6×10^5) were spread on a 6-cm dish and precultured for 12 h. MWEFDP (2 mg/ml) was dissolved in the culture medium and the cells were cultured for 0 and 96 h. Induction potency was stronger with culture time in the order of 24 h < 48 h < 96 h. Thus, the strongest effect occurred with a culture of 96 h. DNA was detected using an Apoptosis Ladder Detection Kit (Wako, Osaka, Japan), and Photographed with a UV transilluminator.

Detection of caspase activity

MDA-MB231 cells (6×10^5) were spread on a 6-cm dish and precultured for 12 h. MWEFDP (2 mg/ml) was dissolved in the culture medium and the cells were cultured for 0, 24, and 48 h. Trypsinized cells were combined with the supernatant, and washed in cold PBS (−). Fluorescence staining was conducted using a Caspa Tag Caspase-3 (DEDV) Kit (Intergen, Manhattanville, NY, USA), a Caspa Tag Caspase-6 (VEID) Kit (Intergen), a Caspa Tag Caspase-8 (IETD) Kit (Intergen), and a Caspa Tag Caspase-9 (LEHD) Kit (Intergen). The cells were dropped on slides and observed under a fluorescence microscope.

Detection of caspase activity by Western blotting

MDA-MB231 cells (6×10^5) were spread on a 6-cm dish and precultured for 12 h. MWEFDP (2 mg/ml) was dissolved in the cultured medium and the cells were cultured for 0, 24, and 48 h. Trypsinized cells were combined with the supernatant, and washed in cold PBS (−). Cells was suspended in 200 µl cold Cell Lysis Buffer (MBL, Nagoya, Japan) and extracted for 20 min on ice. An equivalent amount of sample buffer was added (×2: 62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate [SDS], 10% glycerol and 2% 2-mercapto ethanol), and processed for 5 min at 100°C. SDS-polyacry lacride gel electrophoresis (PAGE) was conducted with a 15% gel, and the cells were transferred electrically to a polyvinylidene difluoride (PVDF) membrane. After blocking for 1 h in 10% skim milk at room temperature, primary antibodies of mouse anti-caspase 3 (MBL), mouse anti-caspase 8 (MBL), mouse anti-caspase 9 (MBL), and goat anti-Bid (MBL) (1 mg/ml each) were placed on the membrane and reacted for 12 h at 4°C. After a washing in PBS, horseradish peroxide (HRP)-labeled anti-mouse IgG antibody (MBL), or HRP-labeled anti-goat IgG antibody (MBL), both at a 10000-fold dilution, were added to the membrane and reacted for 30 min at room temperature. After another washing in PBS, caspase activity was detected on X-ray film, using a scientific emission system (ECL plus; Amersham Biosciences, Piscataway, NJ, USA).

Results

Demonstration of apoptosis inducibility

A morphological analysis of apoptosis under phase-contrast microscopy revealed the formation of apoptotic bodies (Fig. 1A). Nucleic acids were stained with Hoechst 33342 and observed under a fluorescence microscope, and nuclear aggregation and fragmentation were seen after 96 h of culture; these features were not seen at 0 h of culture (Fig. 1B). The fragmentation rate of cells was calculated using flow cytometry, and it was shown that, compared to 0.5% at 0 h of culture, the rate had increased to 62.5% after 96 h of culture, indicating strong induction of apoptosis (Fig. 1C).

DNA ladder formation was investigated by electrophoresis using agarose gel, and after culture for 96 h, ladders were detected showing nuclear DNA fragmentation in nucleosome units (Fig. 2).

Thus, MWEFDP has a strong ability to induce apoptosis in a human breast cancer cell line. These results confirm the findings of one of our earlier reports.

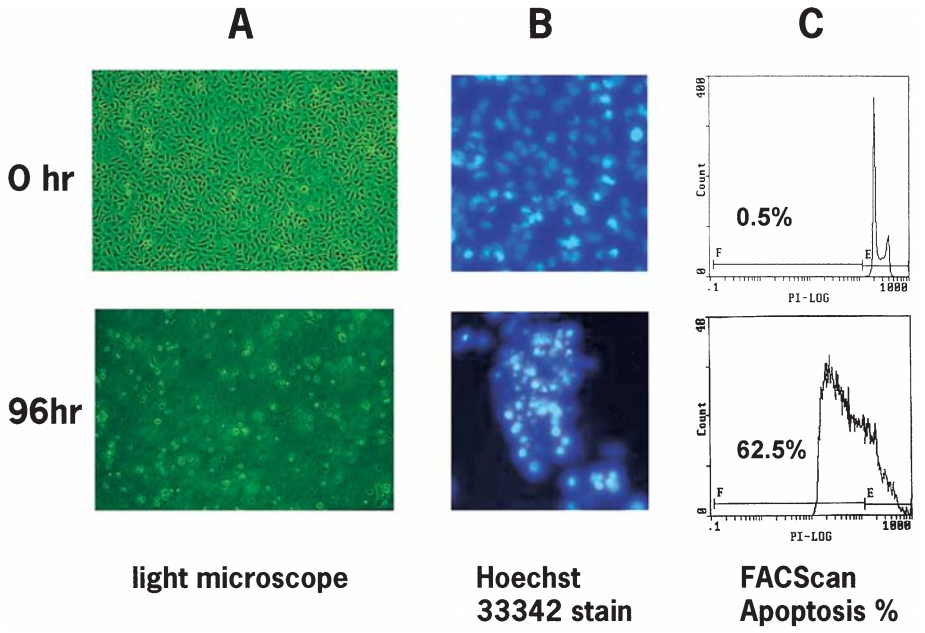

Fig. 1. A The formation of apoptotic bodies was seen after 96 h culture, these bodies were not seen at 0 h. **B** There were no morphological changes of the nuclei at 0 h, but nuclear aggregation was seen after 96 h. **C** The DNA fragmentation rate was calculated to be 0.5% at 0 h, rising to 62.5% after 96 h

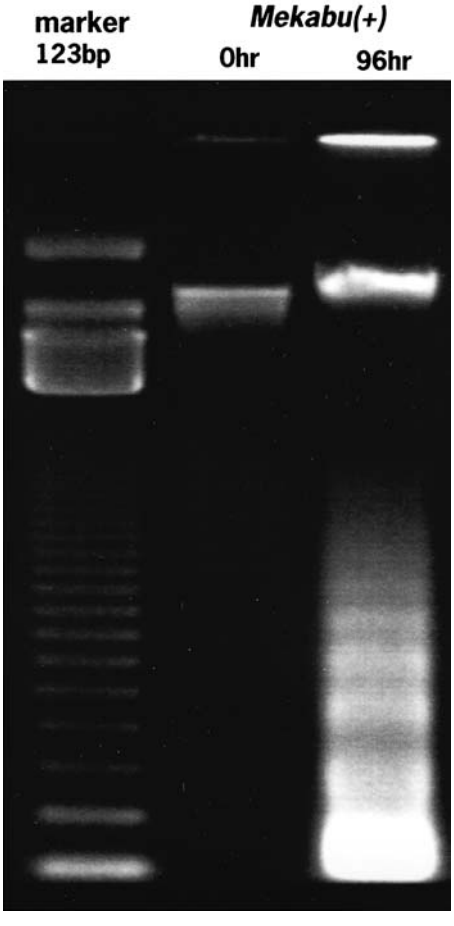

Fig. 2. In the Mekabu-added group, there was no DNA fragmentation at 0 h of culture, but a marked appearance after 96 h, and ladders were formed

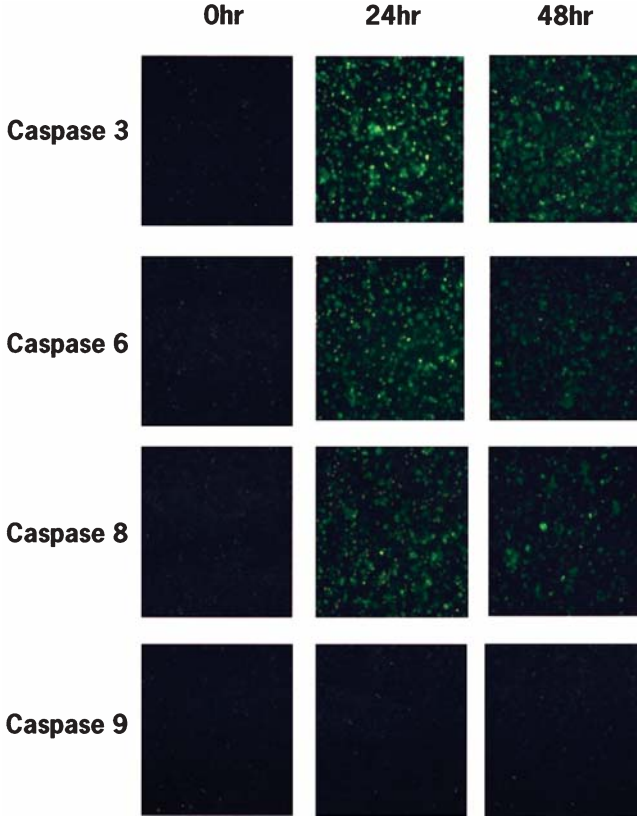

Fig. 3. Fluorescence coloring of caspases 3, 6, and 8, clearly indicating activation, was seen at 24 h. Continued activation was seen at 48 h, although this was of lesser intensity. This fluorescence coloring was not seen at 0 h of culture. Activation of caspase 9 was not seen at either 24 or 48 h

Fluorescence microscopy

Using fluorescence microscopy, we investigated the temporal changes in the activity of caspases 3, 6, 8, and 9 in the signaling system in apoptosis induced by MWEFDP, and found no activity in any of the caspases at 0 h of culture. Activation of caspases 3, 6, and 8, but not caspase 9, was seen after 24 h of culture. Similarly, after 48 h of culture, activated caspases 3, 6, and 8 were detected, but there was no activated caspase 9 at all (Fig. 3).

Western blotting

Western blotting with antibodies was used to analyze the proteins associated with caspase activation. After 24 h, 32-kDa procaspase 3 had disappeared and changed to 17-kDa active caspase 3, demonstrating caspase 3 activation. Caspase 8 activation was demonstrated by the appearance of a band in the 40-kDa region, showing the presence of the active form. For caspase 9 there was no change in the 48-kDa band at either 0 or 24 h, and no band was seen at 17-kDa that would indicate active caspase 9. Bid activation was examined for the purpose of determining whether or not there was any role played by the mitochondria, which are part of a major signaling pathway for the induction of apoptosis. A comparison of findings at 0 and 24 h revealed no difference in the density of the 22-kDa band, and no band was seen at 15-kDa that would indicate activated Bid (Fig. 4).

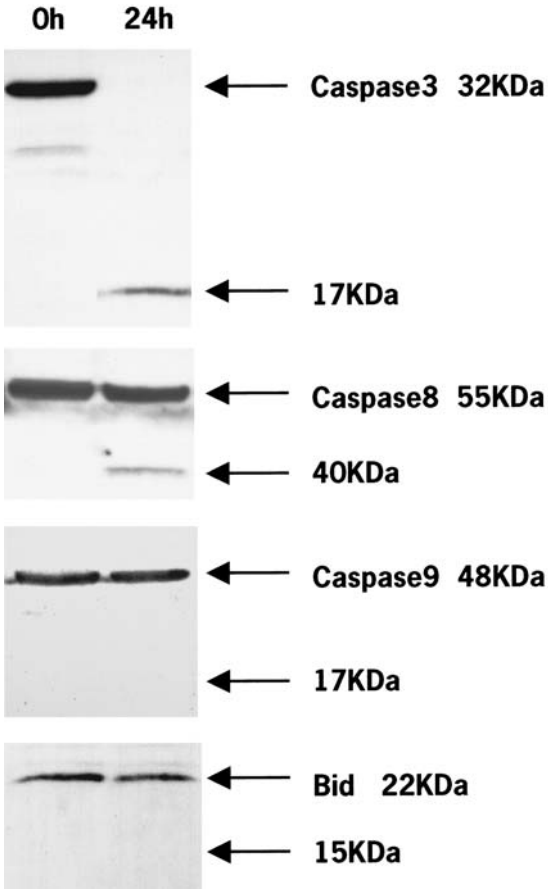

Fig. 4. The procaspase bands seen at 32-kDa at 0 h of culture had disappeared after 24 h of culture, and all had changed to a 17-kDa active type. Procaspase 8 presented bands near 55 kDa, and after 24 h of culture some had changed to a 40-kDa active type. Caspase 9 was seen only as a 48-kDa band at both 0 and 24 h of culture. No band was seen in the vicinity that would show the 17-kDa active type. After 24 h of culture, no band was seen in the vicinity of 15-kDa, which would show active Bid

Discussion

The strong induction of apoptosis in a human breast cancer cell line by Mekabu after 96 h of culture (Fig. 1) was demonstrated morphologically by DNA fragmentation through ladder formation (Fig. 2). This agrees with previously reported experimental results.[4,5]

In the present experiment, we investigated the signaling in apoptosis induction, with a focus on caspases, using Western blotting and fluorescence microscope observations.

In the apoptotic cascade, caspase 8 acts as the initiator in the caspase family, while caspases 3 and 6 are classified as effectors. Procaspase 8 is shown in a 55-kDa band, but it is degraded and transformed to caspase 8, with the appearance of a 40-kDa band. Western blotting showed that the 55-kDa band was obviously fainter at 24 h than at 0 h of culture, suggesting the existence of activated caspase 8 at 24 h (Fig. 4).

Caspase 8 is activated by the limited degradation of Bid, and cytochrome C within the mitochondria is released from the mitochondria into the cell. Apaf-1 in the cytoplasm binds with d-ATP, and serves to activate procaspase 9.[8—10]

Meanwhile, caspase 3 is an inactive precursor (procaspase 3) with a molecular weight of 32-kDa that is broken down into 20-kDa and 12-kDa subunits by upstream caspases 8 and 9.[11] The 20-kDa subunit is further broken down into a 17-kDa fragment and activated. The active form of caspase 3 is a quarter made up of two 17-kDa subunits and two 12-kDa subunits.

In our examination of caspase 3 activation by Western blotting, the inactive 3-kDa pro-caspase 3 had disappeared after 24 h of culture. The detection of a band around 17-kDa demonstrated that procaspase 3 had changed to active caspase 3 (Fig. 4).[12]

The extent to which caspases 3, 6, 8, and 9 are associated with apoptosis induction was investigated using a fluorescent substrate, and only caspase 9 showed no activation under fluorescence microscopy. This suggests a low probability of apoptosis signaling through the mitochondria in this experimental system (Fig. 3).

To ascertain that the signaling pathway was not via the mitochondria, we investigated whether Bid and caspase 9 activation could be seen in Western blotting. The results showed activation of neither (Fig. 4).

Thus, the intracellular signaling pathway in the apoptosis due to water extract of Mekabu was found to be not via the mitochondria, but, rather, to be based on caspase activation in a route from caspase 8 to caspases 3 and 6.

Despite the DNA ladder result that showed the induction of apoptosis to peak at 96h of culture, the caspase cascade was already activated at 48h of culture. Possible reasons for this time lag include the continuation of caspase activation by water extract of Mekabu for longer than expected, or the involvement of some other factor between the intracellular signaling produced by water extract of Mekabu and the expression of apoptosis.

The main components of Mekabu are proteins, carbohydrates, and ash. It also contains minerals such as iodine and vitamins such as carotene. In addition, a well-known special component is fucoidan. We have previously reported that iodine itself has a suppressive effect on mammary tumor growth,[3] but Mekabu contains a high concentration of iodine that is about four times that in Wakame.

We were unable to find any reports on whether fucoidan induces apoptosis in breast cancer cells. However, it is known to induce apoptosis via Fas in colon cancer cells and leukemia cells.[13] There are also reports that carotene and other vitamin groups have an inhibitory effect on carcinogenesis.

Considering that water extract of Mekabu contains many of these active substances, its strong apoptosis induction potency may be due to not only iodine and fucoidan but also to other additional substances.

The water extract of Mekabu we used in this series of studies showed nearly equal apoptosis induction potency in human breast cancer cell lines other than MDA-MB231; namely, MCF-7 (estrogen receptor-positive) and T-47D (estrogen receptor-positive). Conversely, it had almost no effect on a cell line of normal human breast cells, MCF-10A.[5] Water extract of Mekabu is just a simple water extract of the natural foodstuff Mekabu, with no use of chemical agents whatsoever. This extract is, therefore, completely safe for humans and can also be obtained inexpensively, making it a very attractive substance.

Further investigation is necessary to resolve the questions of the effective dose and form of ingestion of Mekabu when applied to humans, as well as the question of how much of the ingested amount is absorbed in the intestinal tract, and how it acts in humans.

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