Augmented Release of Matrix Metalloproteinase-9 by PKC Activation in Organotypic Cultures of Human Breast Cancer and Adjacent Normal Breast Tissue and Fibroadenoma

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Abstract

The organotypic culture technique and quantitative gelatin zymography were used to determine the expression of matrix metalloproteinase (MMP)-9 and MMP-2 in human breast cancer and adjacent normal breast tissue and fibroadenoma. MMP-9 and MMP2 were constitutively expressed in all cultures. The release of these two enzymes in breast cancer was higher than that in adjacent normal breast tissue and fibroadenoma. Administration of 12-o-tetradecanoyl- phorbol-13-acetate (TPA) increased the release of MMP-9 but not of MMP-2. This response was inhibited by protein kinase C (PKC) inhibitor (H7), transcription inhibitor (actinomycin D) and translation inhibitor (cycloheximide). Moreover, the increased level of MMP-9 by TPA in breast cancer was also higher than that in adjacent normal breast tissue and fibroadenoma. These phenomena were also observed in the DAG-treated culture. These findings suggested that the MMP-9 expression in the breast cancer tissue may be more sensitive for the PKC activation.

Key Words: breast cancer, matrix metalloproteinase-9, 12-o-tetradecanoyl-phorbol-13- acetate, protein kinase C

Introduction

Tumor invasion has been attributed to the destruction of extracelluar matrix and basement membrane by proteases (17). Matrix metalloproteinases (MMPs), seryl proteases, and cathepsins are important proteases participating in this destructive process (29). Among the MMPs, MMP-2 and MMP-9 may take part in tissue regeneration and angiogenesis. Although these two MMP members have

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Table 1. Clinical characteristics of patients with breast cancer[a] or fibroadenoma (FA).

Patient ID	Age	Histology	Stage	LNM	ER	PR
1	35	IDC	II	–	+	–
2	46	IDC	II	–	–	–
3	51	IDC	III	+	-+	
4	47	IDC	III	–	–	–
5	41	IDC	III	+	–	–
6	72	IDC	III	+	+	+
7	38	IDC	II	+	+	–
8	51	IDC	III	+	+	+
9	52	IDC	III	+	+	+
10	49	IDC	II	-	+	+
11	44	IDC	III	+	–	–
12	46	IDC	II	–	–	–
13	31	FA				
14	39	FA				
15	22	FA				
16	21	FA				
17	31	FA				

[a]Grade I is least differentiated; grade II intermediate; and grade III most differentiated. LNM = lymph node metastases; ER = estrogen receptor; PR = progesterone receptor; +, positive; –, negative; IDC = infiltrating ductal carcinoma. The level of 10 fmol receptor/mg or greater is considered clinically positive.

similar amino acid sequences, they have significant differences in their tertiary structures and regulatory factors. They may even play an important role in tumor progression (3, 23).

MMP-9 is secreted by normal and cancer cells (16, 18, 30). It has also been reported to play an important role in trophoblast implantation (13), inflammation (7), bone resorption (21), arthritis (1), asthma (9) and cancer metastasis (11). This MMP member is related to the tumor progression of various tissues (4, 5, 6, 10, 27). Recently, it has been demonstrated that the expression of MMP-9 may be induced by 12-o-tetradecanoyl-phorbol-13-acetate (TPA) in breast cancer cell lines (12, 14, 28). This overexpression may lead to a significant increase in the potential of tumor invasion. Since TPA is an activator of protein kinase C (PKC), MMP-9 may be induced through the PKC pathway. However, the activation of PKC has also been attributed to the influences of MMP-2 (8) or MMP-3 (stromelysin) (20). At the cellular level, the overexpression of MMP-2 may catalyze the conversion of proMMP-9 to active MMP-9, which in turn participates in tumor progression (8). However, the mechanism of the induction of MMP-9 in human breast cancer at the tissue level remains unclear. In this study, we used the organotypic culture technique and quantitative gelatin zymography to evaluate the effects of Con A, activators and inhibitors of PKC and inhibitors of transcription and translation on the expressions of MMP-2 and MMP-9 in malignant breast carcinoma, fibroadenoma and the adjacent normal breast tissues.

Materials and Methods

Specimens

Surgical specimens of breast cancer, fibroadenoma and the adjacent normal breast tissues were obtained from mastectomies in the operating rooms of the Department of Surgery, Chung Shan Medical University Hospital, Taichung, and the Department of Surgery, Changhua Christian Hospital, Changhua, Taiwan. These specimens were immediately placed in test tubes containing Dulbeccoe's modified Eagle medium (DMEM, Gibco/BRL, USA) and sent to our laboratory within 1 h. Demographic and clinical information of the subjects are shown in Table 1.

Organotypic Culture

Five pieces of 4×4-mm^2 specimen were placed into each well of 24-well culture plates containing 2 ml DMEM. The tissues were cultured in a 5% CO_2 incubator at 37°C for 1 h. After washing twice with DMEM, the tissues were separately treated with Con A (20 μg/ml), PKC activators (100 ng/ml TPA or 100 μg/ml DAG), PKC inhibitors (100 μM H7) and their combinations (100 μM H7 and 100 ng/ml TPA or 100 μM H7 and 100 μg/ml DAG). The tissues were also treated with transcription or translation inhibitors (10 μM actinomycin D, 10 μM mitomycin C or 10 μM cycloheximide) and their combinations with 100 ng/ml TPA in 2 ml DMEM. A well of tissues with 2 ml DMEM was cultured as a control. These tissues were then cultured in a 5% CO_2 incubator at 37°C for 24 h. The culture media were collected into vials and stored at -70°C. The tissues was dried and weighed and then stored at -70°C.

Tissue Extraction

Extracts were obtained by homogenizing the tissues in a PBS buffer (0.14 M NaCl, 3 mM KCl, 1.4 mM KH_2PO_4, 14 mM K_2HPO_4) (1 mg tissue/10 μl PBS). The homogenates were placed on ice for 10 min and centrifuged at 10,000 xg for 30 min. The supernatant was collected and designated as the cytosol fraction. The precipitate was homogenized again in a SDS buffer (20 mM Tris-HCl, pH 6.8; 1% SDS; 10% glycerol; 0.01% bromophenol blue) (1 mg tissue/10 μl) and centrifuged at 10,000 xg for 30 min. The supernatant was collected and designated as the particulate fraction. These fractions were then stored at -70°C.

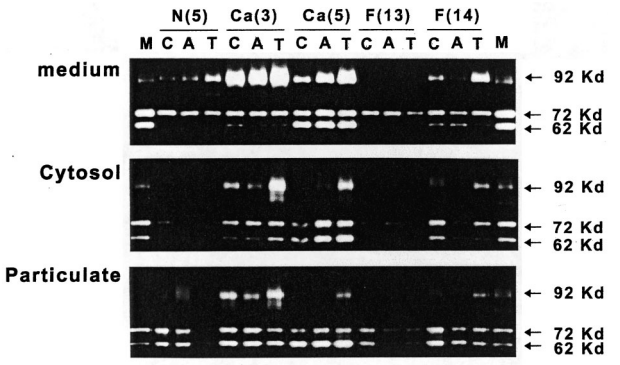

Fig. 1. Effect of TPA (T) and Con A (A) on the gelatinase expression in organotypic cultures of human breast cancer tissue (Ca), normal breast tissue (N) and fibroadenoma (F). T he samples of the cultured medium and the cytosolic and particulate fractions were processed for zymography as described in the "Materials and Methods" section. The indications of 92-Kd, 72-Kd and 62-kd were marked according to previous study. C, control; M, marker. The number in the parentheses is the patient number.

Gelatin Zymography Protease Assay

After calibrating the cultured media with the PBS buffer to contain the secretion from 20 mg of tissue, gelatin zymography was performed by loading 10 µl of the calibrated cultured media, the cytosol and particulate fractions on 0.1% gelatin- and 8% SDS-PAGE, and ran by electrophoresis at 150 V for 2.5 h. Enzymes on the gels were renatured by washing twice in a 2.5% Triton X-100 solution with shaking for 30 min. The gels were then incubated in 200 ml of a reaction buffer (40 mM Tris-HCl, pH 8.0; 10 mM $CaCl_2$, 0.01% NaN_3) at 37°C for 16 h before staining with 0.25% Coomassie brilliant blue R-250 for 30 min. Quantitative analysis was preformed after discoloring the stain in a discoloring solution (875 ml dH_2O, 50 ml methanol, and 75 ml acetic acid).

Quantitative Analysis of Zymography

Expressions of proMMP-9 (92 Kd), proMMP-2 (72 Kd) and active MMP-2 (62 Kd) gelatinases in different fractions of the organotypic cultures were determined by the AlphaImager 2000 destimetor (Manufacturer, City). The results of MMPs expression were calculated as percentages of a randomly chosen human breast cancer biopsy as a marker and analyzed by the Student's *t* test. Associations betweenMMP-9 expression and clinical factors were compared by the Mann-Whitney U test. $P < 0.05$ was considered statistically significant.

Results

Effects of Con A and TPA

To determine the optimal concentrations of Con A and TPA, different concentrations of Con A (10, 20, 30, 40, or 50 µg/ml) and TPA (12.5, 25, 50, 100, or 200 ng/ml) have been evaluated. The concentrations of 20 µg/ml for Con A and 100 ng/ml for TPA showed maximal values and no toxic reactions were observed in the organotypic cultures (data not shown).

Table 2. The effect of TPA on the secretion (% of marker) of MMP-9 in organotypic cultures of human breast cancer and adjacent normal breast tissue.

	Cancer		Normal	
Patient ID	Ethanol	TPA	Ethanol	TPA
1	1208	1716	28	362
2	866	1659	17	207
3	979	1656	ND[a]	ND
4	361	1044	31	356
5	144	1008	65	120
6	462	898	ND	ND
7	311	864	23	47
8	580	836	62	118
9	31	714	23	77
10	58	664	17	428
11	289	494	80	386
12	275	459	46	69
Mean±SEM	463±113	1001±134[b]	39±8	217±50[c]

[a]ND, not done.
[b]$P < 0.01$ vs. ethanol-treated cancer group.
[c]$P < 0.01$ vs. ethanol-treated normal group.

The release of the proMMP-9 was observed in all cultures of human breast cancer and fibroadenoma and normal breast tissue, and was elevated in all the TPA-treated cultures. However, the breast cancer culture had a significant higher release of proMMP-9 not only in the control but also in treatment with Con A or TPA (Fig. 1 and 2). The increased level of this enzyme by treatment with TPA in breast cancer (537.3±68.3) was 3 fold higher than that in adjacent normal breast tissue (177.9±50.9) (Table 2). Moreover, the TPA-treated breast cancer tissue both in the cytosolic and particulate fractions had a significantly higher expression of proMMP-9 (Fig. 1 and 2). Besides, the expressions of proMMP-2 and active MMP-2 only elevated in the all fractions from human breast cancer tissue, but no differences among the control, TPA-treated and Con A-treated cultures (data not shown).

Effects of TPA, DAG and H7

In both human breast cancer and the adjacent normal breast tissues, the expression of proMMP-9 was significantly higher in the TPA-treated culture than the control (Fig. 3 and 4). The augmented expression of proMMP-9 was significantly inhibited

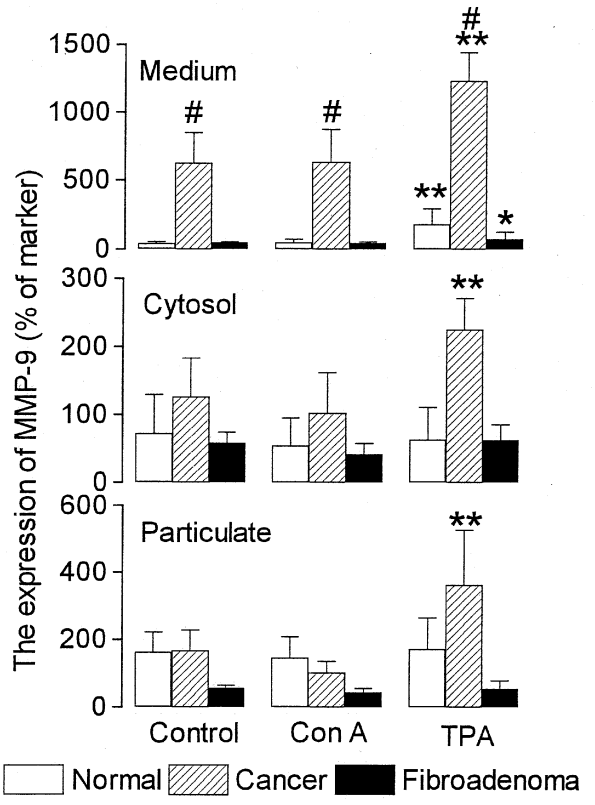

Fig. 2. The quantitative data of the effect of TPA and Con A on the MMP-9 expression in organotypic cultures of human breast cancer tissue, normal breast tissue and fibroadenoma. Data are expressed as mean±SEM. *, $P < 0.05$ vs. ethanol-treated control. **, $P < 0.01$ vs. ethanol-treated control. #, $P < 0.05$ vs. normal breast tissue. Represents the results of cancer samples from patient ID 1, 3, 5, 6 and 7. Represents the results of fibroadenoma samples from patient ID 13-17. Represents the result of normal samples from patient ID 1, 5 and 7. Refer to legend of figure 1 for details.

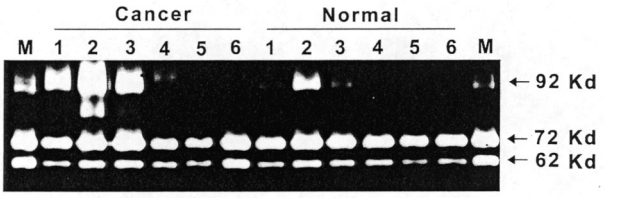

Fig. 3. Effect of PKC activators and/or inhibitor on the gelatinase expression in organotypic cultures of human breast cancer tissue and normal breast tissue. The result is from patient 2. Each lane represents as follow: Lane 1, control; lane 2, TPA; lane 3, DAG; lane 4, H7; lane 5, TPA plus H7; lane 6, DAG plus H7. Refer to legend of figure 1 for details.

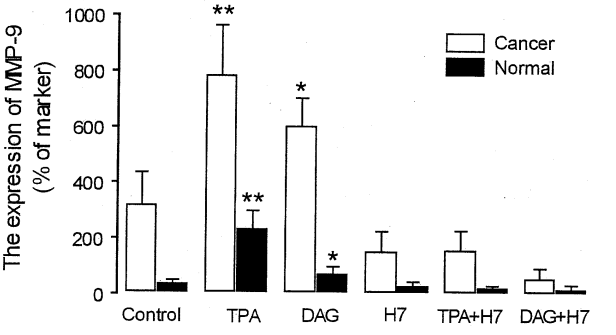

Fig. 4. The quantitative data of the effect of PKC activators and/or inhibitor on the MMP-9 expression in organotypic cultures of human breast cancer tissue and normal breast tissue. Data are expressed as mean±SEM. *, $P < 0.05$ vs. ethanol-treated control; **, $P < 0.01$ vs. ethanol-treated control. The results are from patients 2, 4, 8, 9, 10 and 12. Refer to legend of figure 1 for details.

by H7. However, no significant changes were found either in the H7-treated alone (Fig. 3 and 4). Similarly, elevation of proMMP-9 expression was observed in the DAG-treated culture, which was significantly blocked by H7. However, the expressions of proMMP-2 and active MMP-2 were not significantly affected by the treatment of TPA, DAG, H7 or their combinations.

Effects of Transcription and Translation Inhibitors

Either actinomycin D, or cyclo-heximide alone, or combined TPA with actinomycin D, or TPA with cycloheximide decreased the expression of proMMP-9 in human breast cancer tissue culture (Fig. 5 and 6). There is no change in the expression of proMMP-9 observed in the culture when treated with mitomycin C alone. However, the expression was still stimulated by the treatment of TPA or combined TPA with mitomycin C. However, all these transcription and translation inhibitors did not show significant effects on the expressions of proMMP-2 and MMP-2 (Fig. 5).

Relationships between the Expression of proMMP-9 in Organotypic Cultrues and Clinical Factors

The release of proMMP-9 was elevated by the treatment of TPA in both cultures of human breast cancer and the adjacent normal breast tissue. No significant differences were found in the level of proMMP-9 by the stage of carcinoma, lymphatic metastasis, or the presence of estrogen or progestrone receptors (Tables 1 and 2).

Discussion

In this study, the tissues were cultured for 24 h to evaluate the effects of activators and inhibitors of PKC and inhibitors of transcription and translation on the expression of MMPs. This culturing time may be sufficient to demonstrate these effects, since no reaction was observed at 4 h after incubation and no significant difference was observed between results obtained by 24-h and 48-h incubations (data not shown).

Results of organotypic culture indicated that the expression of proMMP-9 in the human breast cancer

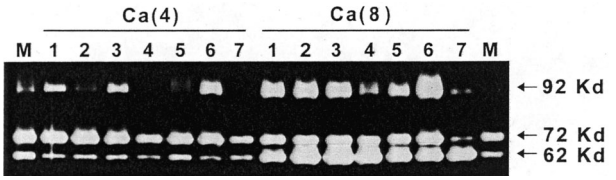

Fig. 5. Effect of transcription and translation inhibitors on the gelatinase expression in organotypic cultures of human breast cancer tissue (Ca). Each lane represents as follow: Lane 1, control; lane 2, actinomycin D; lane 3, mitomycin C; lane 4, cycloheximide; lane 5, TPA plus actinomycin D; lane 6, TPA plus mitomycin C; lane 7, TPA plus cycloheximide. The number in the parentheses represents the patient number. Refer to legend of figure 1 for details.

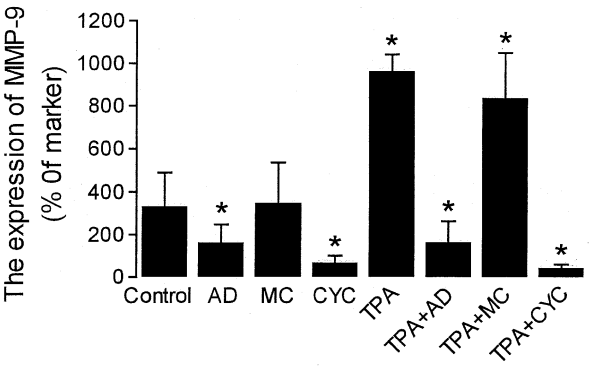

Fig. 6. The quantitative data of the effect of transcription and translation inhibitors on the MMP-9 expression in organotypic cultures of human breast cancer tissue. Data are expressed as mean±SEM. *, $P < 0.05$ vs. ethanol-treated control. AD, actinomycin D; MC, mitomycin C; CYC, cycloheximide. The results are from patients 4, 5 and 8. Refer to legend of figure 1 for details.

tissue became higher than that in the adjacent normal breast tissue or firbroadenoma after TPA treatment for 24 h. This finding in the cultured media was consistent with previous observations on cell lines (12, 14, 28). Moreover, the relatively high levels of proMMP-9 in the cultured medium and cytosol and particulate fractions from the cancer culture suggested that the tissue of breast cancer may have a high sensitivity to producing this enzyme. Therefore, TPA treatment not only elevates the level of this gelatinase in the tissue but also releases the gelatinase to the culture medium.

In addition to TPA, DAG participates in the activation of PKC and is one of the secondary messengers produced in the body and transmission of messages. After addition of DAG into the culture, the expression of proMMP-9 elevated significantly, and was inhibited by PKC inhibitor H7, although the expressions of proMMP-2 and active MMP-2 were not affected by the treatment of DAG. This provided strong evidence to prove the direct association between the proMMP-9 expression and the PKC activation. However, the basal level of proMMP-9 release was not affected by the treatment of H7. Since multiple signaling pathways are involved in the expression of MMP-9 in human breast cancer cells (31), our findings suggested that the spontaneous release of proMMP-9 may be compensated by some Factors other than PKC pathway during the presence of PKC inhibitors.

After treatment with the transcription inhibitors, we observed that these compounds blocked the expression of proMMP-9 but did not affect those of proMMP-2 and active MMP-2. Moreover, the augmentation of proMMP-9 level by TPA was attenuated after the treatments of these compounds. Mitomycin C is an inhibitor of the DNA replication and an anticancer drug (26). This drug did not affect the expression of proMMP-9 even under the influence of TPA. These findings suggest that the regulation of MMP-9 in tissue may be at the transcription level. It has been reported that the promoter region of the proMMP-9 contains TPA response elements (TRE) and may act as a binding domain of transcription factor AP-1 (15). Transcription is carried out when TRE is initiated in the presence of TPA. Since TPA is an activator of PKC, the activation of PKC also activates Raf, c-jun, and c-fos (2). After activation, c-jun and c-fos bind to the AP-1 site and elevates the expression of the proMMP-9.

Con A may activate MMP-2 indirectly through the activation of membrane-type matrix metalloproteinase (MT-MMP) (24, 32). However, we observed that the expressions of proMMP-2 and active MMP-2 decreased slightly in Con A-treated organotypic cultures. Since the results of previous studies were based on the observations of cell cultures, it is possible that Con A may not exert the same effect in tissue. However, this suggestion requires further investigation.

In summary, the results of this study indicated that the MMP-9 expression in breast cancer may be highly sensitive response to PKC activators and it may be regulated at the transcription level. However, the mechanism is unknown. It has been reported that the expression of PKC activity in breast cancer is higher than that in adjacent normal breast tissue (19). This may account for the altered responses in breast cancer. Moreover, although the higher expression of MMP-9 in breast cancer is connected with the node-negative patients (25), the increase of the expression and secretion of MMP-9 by treatment with TPA is correlated with the increase in the ability of human breast cancer cell invasiveness and motility (22). Thus, the organotypic culture technique may provide a feasible model for the investigation of tumor progression in human breast cancer, and may serve as a potential tool for screening anti-cancer drugs.

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