

Patterns of MMP-2 and MMP-9 expression in human cancer cell lines

M.W. ROOMI, J.C. MONTERREY, T. KALINOVSKY, M. RATH and A. NIEDZWIECKI

Dr. Rath Research Institute, 1260 Memorex Drive, Santa Clara, CA 95050, USA

Received October 20, 2008; Accepted February 3, 2009

DOI: 10.3892/or_00000358

Abstract. MMP-2 and MMP-9 secretion is elevated in several types of human cancers and their elevated expression has been associated with poor prognosis. Expression of MMPs is highly regulated by cytokines and signal transduction pathways, including those activated by phorbol 12-myristate 13-acetate (PMA). The aim of this study was to examine the effect of PMA on MMP-2 and MMP-9 secretion in 42 different human cancer cell lines, selected on the basis of their organ malignancies. They were cultured in the recommended media supplemented with 10% FBS and antibiotics in 24-well tissue culture plates. At near confluence, the cells were washed with PBS, 0.5 ml of medium was added, and the cultures were incubated. Parallel sets of cultures were also treated with PMA for induction of enzymes. After 24 h the media were collected and MMP-2 and MMP-9 levels were assayed by gelatinase zymography. Based on MMP-2 and MMP-9 secretion without and with PMA treatment, the various human cancer cell lines fell into one of two major groups. The first group characterized by low basal MMP-9 secretion fell into three different categories of susceptibility to PMA induction of MMP-9 expression: resistant, moderately susceptible and highly susceptible. High basal MMP-9 levels responsive to PMA induction characterized the second group. Most cancer cell lines examined exhibited basal levels of MMP-2, MMP-9 or both. MMP-2 secretion was not induced by PMA in any of the cancer cells examined.

Introduction

Metastasis is responsible for a majority of cancer-related deaths, due to tumor involvement of critical organs or to complications in therapies aimed at controlling tumor growth and spread. An understanding of the role of MMPs in tumor invasion and metastasis is important for devising therapies for preventing tumor cell spread. Metastasis is a complex

multi-step process including: detachment of cancer cells from the primary tumor and disruption of the basement membrane and subsequent invasion into the surrounding stroma; cancer cell entry into the vascular or lymphatic system and transport to distal sites such as the liver, lungs and brain; and extravasation, tumor cell proliferation and angiogenesis at distal sites (1-5).

A critical event in tumor cell invasion is degradation of the extracellular matrix (ECM), a complex network of extracellular macromolecules such as collagen, proteoglycans, fibronectin, laminin and many other glycoproteins that acts as a barrier to the spread of cancer cells to distal sites by restricting tumor growth and invasion (6-8). Although several different proteases are implicated in ECM degradation, a special group of metalloproteinases, matrix metalloproteinases (MMPs), a family of zinc and calcium-dependent proteolytic enzymes, digest various components of ECM, including collagen, laminin, fibronectin, vitronectin, elastin and proteoglycans. MMPs are crucial to invasion, migration, metastasis and tumorigenesis (8-10).

MMPs are initially expressed as inactive pro-enzymes that require proteolytic processing to release the active enzyme (11). Over twenty different MMPs act on a broad spectrum of substrates, including collagen type I, II, III, IV and stromelylin and are divided into subgroups based on their structure and substrate specificity (12). These subgroups include collagenases, stromelysins and stromelysin-like, matrilysins, gelatinases and membrane-type MMPs (MT-MMPs) (12). Among the many MMPs that have been identified, gelatinases, especially MMP-2 (gelatinase A) and MMP-9 (gelatinase B), are thought to play a key role in degradation of type IV collagen and gelatin, the two main components of ECM. MMP-2 and MMP-9 are secreted in their latent zymogenic form, 72 and 92 kDa, respectively. MMP-2 and MMP-9 are cleaved by other MMPs or proteases to yield the activated forms of 68, 58 and 54 kDa for MMP-2, and 84 kDa for MMP-9. Increased expression of MMP-2 and MMP-9 is reported in many human tumors, including ovarian, breast and prostate tumors, and melanoma (13-15). Type IV collagen is a major structural protein for ECM and basement membrane. Type IV collagenases MMP-2 (72-kDa gelatinase A) and MMP-9 (92-kDa gelatinase B) have been the focus of research since MMP-2 and MMP-9 expression is associated with cancer cell invasion and elevated in a variety of malignancies (4,5). A significant association has been reported between tumor aggression and increased levels of MMP-2 and MMP-9 in many experimental and clinical studies (8,9,16-19).

Correspondence to: Dr Aleksandra Niedzwiecki, Dr. Rath Research Institute, 1260 Memorex Drive, Santa Clara, CA 95050, USA

E-mail: author@drath.com

Key words: MMP-2, MMP-9, human cancer cell lines

Table I. Cancer cell lines classified by organ malignancy.

Male cancers	Gastrointestinal	Head and neck	Adult sarcoma		
Prostate	Liver	Laryngopharynx	Fibrosarcoma		
PC-3	Hep-G2	FaDu	HT-1080		
LNCAP	Sk-Hep-1	Tongue	Liposarcoma		
DU-145	Pancreas	SCC-25	SW872		
22RV1	MiaPaCa-1	Thyroid	Synovial sarcoma		
Testis	Colon	SW579	SW982		
NTER-2	HCT-116		Chondrosarcoma		
	HCA-7		SW1353		
Female cancers	Lung and Mesothelioma	Pediatric sarcoma	Leukemia	Glioma	Others
Breast	Lung	Osteosarcoma	HL-60	A-172	Bladder
MDA-MB-231	A-549	U2OS	Jurkat	T-98	T-24
MCF-7	Calu-3	MMNG	Raji	LN-18	Melanoma
Cervix	Mesothelioma	Ewing sarcoma	U-937		A-2058
HeLa	MSTO-211H	SK-ES-1			Kidney
DoTc2-4510		Rhabdomyosarcoma			786-0
Ovarian		RD (embryonic)			
SK-OV-3		Neuroblastoma			
Uterine		SK-N-MC			
SK-UT-1		Retinoblastoma			
		Y-79			

Increased MMP-2 and MMP-9 expression has also been documented to correlate with cancer invasion.

The aim of this study was to examine the pattern of MMP-2 and MMP-9 expression in human cancer cell lines in light of the cells' malignancy and organs of origin. Since expression of MMPs is highly regulated by cytokines and signal transduction pathways, including those activated by phorbol 12-myristate 13-acetate (PMA), the effect of PMA on MMP-2 and MMP-9 expression in several human cancer lines was assessed.

Materials and methods

Cancer cell lines and reagents. Forty-two different cancer cell lines were selected on the basis of organ malignancies, and included carcinomas, sarcomas and leukemias (Table I). The cancer cell lines and their recommended media were purchased from ATCC (Manassas, VA, USA). Penicillin, streptomycin, PMA and fetal bovine serum (FBS) were obtained from Sigma (St. Louis, MO). All other reagents used were of high purity and were obtained from Sigma, unless otherwise indicated.

Cell culture. Cancer cell lines were grown in recommended media, supplemented with 10% FBS, penicillin (100 units/ml) and streptomycin (100 µg/ml) in 24-well tissue culture plates (Coster, Cambridge, MA). The cells were plated at a density 1×10^5 cells/ml and grown to confluency in a humidified atmosphere at 5% CO₂ at 37°C. Serum-supplemented media were removed and the cell monolayer was washed

once with PBS and with the recommended serum-free medium. The cells were then incubated for 24 h in 0.5 ml serum-free media. Parallel sets of cultures were treated with PMA (100 ng/ml) for induction of MMP-9. Control and PMA treatments were done in triplicates. The plates were then returned to the incubator. The conditioned media were collected separately, pooled and centrifuged at 4°C for 10 min at 3000 rpm to remove cells and cell debris. The supernatant was collected and used for gelatinase zymography, a highly sensitive assay of gelatinolytic enzymatic activity able to detect both pro and active forms of MMP-2 and MMP-9.

Gelatinase zymography. Gelatinase zymography was performed in 10% NOVEX Pre-Cast SDS Polyacrylamide Gel (Invitrogen Corp.) in the presence of 0.1% gelatin under non-reducing conditions. Culture media (20 µl) were mixed with sample buffer and loaded for SDS-PAGE with tris glycine SDS buffer as suggested by the manufacturer (Novex). Samples were not boiled before electrophoresis. Following electrophoresis the gels were washed twice in 2.5% Triton X-100 for 30 min at room temperature to remove SDS. The gels were then incubated at 37°C overnight in substrate buffer containing 50 mM Tris-HCl and 10 mM CaCl₂ at pH 8.0 and stained with 0.5% Coomassie Blue R250 in 50% methanol and 10% glacial acetic acid for 30 min and destained. Upon renaturation of the enzyme, the gelatinases digest the gelatin in the gel and give clear bands against an intensely stained background. Protein standards were run concurrently and approximate molecular weights were determined by plotting the relative mobilities of known proteins.

Table II. Basal levels of MMP-9 (0-2%) secretion and resistance to PMA induction (0-20%).

Cell line	MMP-2 (%)	MMP-9 (%)	MMP-2 (w/PMA) (%)	MMP-9 (w/PMA) (%)
Ovarian cancer (SK-OV-3)	100	0	100	0
Retinoblastoma (Y-79)	100	0	100	0
Thyroid carcinoma (SW-579)	100	0	100	0
Osteosarcoma (SK-ES-1)	100	0	91	9
Glioblastoma (A-172)	100	0	87	13
Glioblastoma (T-98G)	100	0	87	13
Testis carcinoma (NT2-D1)	100	0	85	15
Glioblastoma (LN-18)	100	0	79	21
Synovial sarcoma (SW-982)	98	2	80	20

Gelatinase zymograms were scanned using CanoScan 9950F Canon scanner at 300 dpi. The intensity of the bands was evaluated using the pixel-based densitometer program Un-Scan-It, Version 5.1, 32-bit, by Silk Scientific Corp. (Orem, UT, USA), at a resolution of 1 Scanner Unit (1/100 of an inch for an image that was scanned at 100 dpi). The pixel densitometer calculates the optical density of each pixel (values 0-255) using the darkly stained background of the gel as a pixel value of 0. A logarithmic optical density scale was used since the optical density of films and gels is logarithmically proportional to the concentration. The pixel densitometer sums the optical density of each pixel to give a band's density. In all graphs, band densities were reported as percentages of the sums of all pixels in a given lane (treatment) of a gel.

Statistics. Pearson's correlation coefficient was determined between MMP-2 and MMP-9 expression using MedCalc Software (Mariakerke, Belgium)

Results

In the present study, only MMPs secreted into the medium were determined, and these levels were interpreted to reflect both expression and subsequent secretion. Based on MMP-2 and MMP-9 secretions, and in the absence and presence of PMA, the various human cancer cell lines were found classifiable on the basis of their basal level of MMP-9 expression and sensitivity to MMP-9 induction by PMA. Cell lines with low basal levels of MMP-9 expression fell into three different categories of PMA susceptibility for further MMP-9 expression: resistant, moderately susceptible or highly susceptible. A second category of cell lines was found containing cell lines with higher basal levels of MMP-9 expression and high susceptibility to PMA for further induction of MMP-9.

Cell lines expressing 0-2% basal MMP-9 levels and resistant to PMA induction of MMP-9. Ovarian cancer SK-OV-3, retinoblastoma Y-79, thyroid carcinoma SW-579, osteosarcoma SK-ES-1, glioblastoma A-172, T-98G, LN-18, testis carcinoma NT2-D1, synovial sarcoma SW-982 exhibited near 0% basal MMP-9 levels and resistance to PMA stimu-

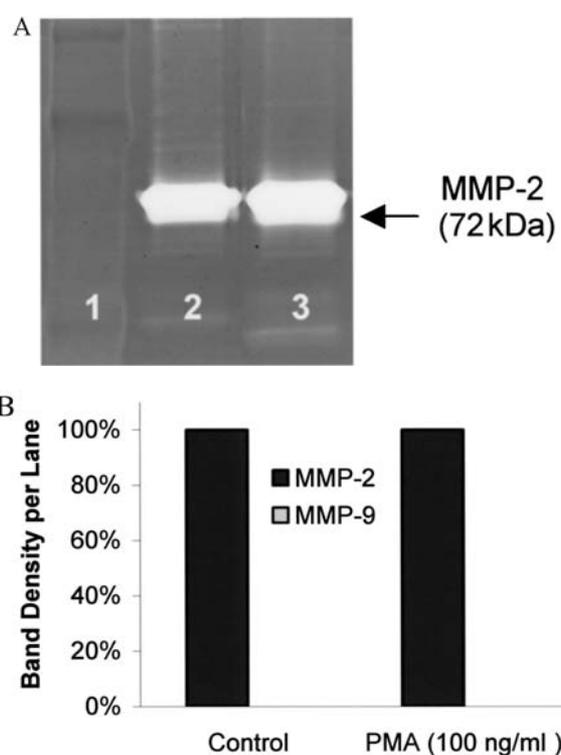


Figure 1. Thyroid carcinoma (SW-987) - representative cell line with zero basal MMP-9 secretion levels and resistant to PMA stimulation. (A) Gelatinase zymogram of thyroid carcinoma expressing only a single band corresponding to the molecular weight of MMP-2 (72 kDa) in both uninduced and induced media. (1-Markers, 2-Control, 3-100 ng/ml PMA treatment). (B) Quantitative densitometric analysis of MMP expression of thyroid carcinoma gelatinase zymogram. MMP expression is reported as a percentage of the sum of all band densities in a given lane.

lation of MMP-9. See Table II for the list of cancer cell lines in this category and the quantitative densitometric analyses of MMP-2 and MMP-9 expression without and with PMA treatment. Analysis of the control conditioned media (without PMA) by gelatinase zymography showed no bands for MMP-9 (92 kDa) and exhibited only one band corresponding to the molecular weight of MMP-2 (72 kDa). On PMA treatment, the pattern did not change and the cell lines still showed bands for MMP-2 and only a faint band for MMP-9 (0-20%) if at all. Fig. 1 present a gelatinase zymogram and

Table III. Basal levels of MMP-9 (0-7%) and moderate MMP-9 induction by PMA treatment (40-50%).

Cell line	MMP-2 (%)	MMP-9 (%)	MMP-2 (w/PMA) (%)	MMP-9 (w/PMA) (%)
Neuroblastoma (SK-N-MC)	100	0	62	38
Osteosarcoma (MNNG-HOS)	100	0	62	38
Hepatocellular carcinoma (Hep-G2)	100	0	61	39
Cervical cancer (HeLa)	100	0	54	46
Bladder carcinoma (T-24)	100	0	44	56
Melanoma (A-2058)	97	3	44	56
Prostate cancer (PC-3)	93	7	52	48

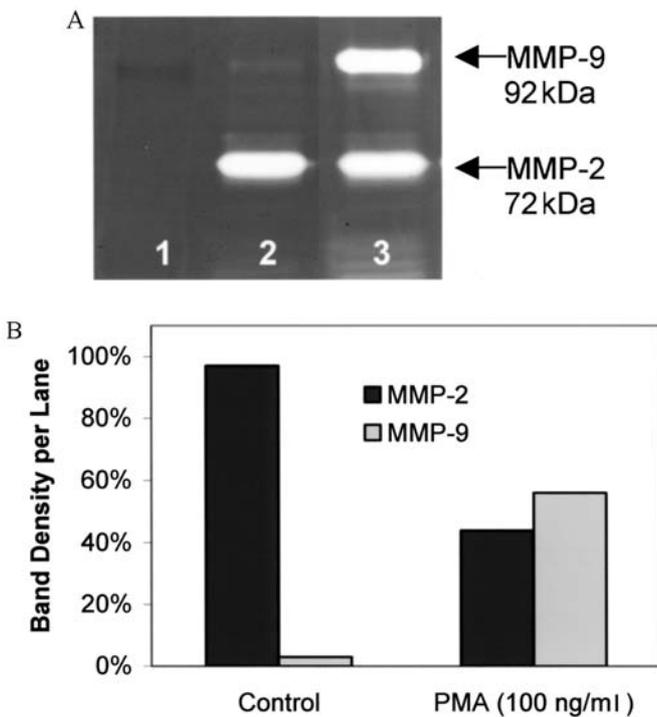


Figure 2. Melanoma (A-2058) - representative cell line expressing low basal MMP-9 levels and moderate response to PMA stimulation. (A) Gelatinase zymogram of melanoma cells show greater expression of MMP-2 over MMP-9 in uninduced media (control). PMA treatment led to a great increase in MMP-9 expression compared to control. (1-Markers, 2-Control, 3-100 ng/ml PMA treatment). (B) Quantitative densitometric analysis of MMP expression of melanoma gelatinase zymogram showing MMP expression as a percentage of the sum of all band densities in a given lane.

densitometric analysis of thyroid carcinoma SW-987, a representative cancer cell line of this group. This zymogram demonstrates undetectable basal MMP-9 levels and resistance to PMA induction of MMP-9, as well as MMP-2 secretion.

Cell lines expressing 0-7% basal MMP-9 and moderately susceptible to PMA stimulation. Melanoma A-2054, neuroblastoma SK-N-MC, osteosarcoma MNNG-HOS, hepatocellular carcinoma Hep-G2, cervical cancer HeLa, prostate cancer PC-3, bladder carcinoma T-24 demonstrated 0-7% basal level of MMP-9 and a moderate increase in MMP-9 expression (40-56%) with PMA stimulation (Table III). The

quantitative densitometric analyses of the conditioned media show only one band with a molecular weight corresponding to MMP-2 (72 kDa) and a very faint band for MMP-9 (92 kDa) if at all. Shown in Fig. 2 are the zymogram and densitometric analysis of melanoma A-2054, a representative cell line of this pattern. The control media (without PMA treatment) shows only one band for MMP-2 and a very weak band for MMP-9. On PMA treatment, the gelatinase zymogram exhibited two bands, corresponding to MMP-2 and MMP-9 with equal intensity.

Cell lines expressing 0-17% basal MMP-9 levels and highly susceptible to PMA stimulation. Table IV lists the cancer lines in this category with densitometric analyses of conditioned and PMA-treated media. Analysis of conditioned media detected low levels of MMP-9 in some cell lines of this category: chondrosarcoma SW-1353, mesothelioma MSTO-211H, osteosarcoma U2-OS, renal adenocarcinoma RCC-786-0, rhabdomyosarcoma RD, fibrosarcoma HT-1080), and none in others: lung carcinoma A-549, head and neck carcinoma FaDu, leukemia HL-60, breast cancer MCF-7 and MDA-MB-231, and uterine cancer SK-U-T1. All cell lines in this category, however, were very susceptible to PMA-induced MMP-9 expression. Fig. 3A and B show a zymogram and densitometric analysis of breast cancer cell line MCF-7, characterized with a band corresponding to MMP-9 (92 kDa) after stimulation by PMA and no bands in the conditioned media for either MMP-2 or MMP-9. Fig. 3C and D show a zymogram and densitometric analysis of rhabdomyosarcoma (RD) cell line with two bands, a major one at 72 kDa corresponding to MMP-2 and a band at 92 kDa corresponding to MMP-9. On PMA treatment MMP-9 is induced to be the major band.

Cell lines expressing relatively high basal levels of MMP-9 and susceptible to PMA. The various cancer cell lines (tongue carcinoma SCC-25, hepatocellular carcinoma SK-Hep-1, liposarcoma SW-972, cervical cancer DoTc2-4510, Burkitt's lymphoma Raji, leukemia Jurkat, pancreatic cancer MIA-PaCa-2 and prostate cancer DU-145) that fell in this group are shown in Table V with their zymogram densitometric analyses. These cells exhibited high basal MMP-9 levels, between 51-100%, which were enhanced to 79-100% with PMA treatment. In addition, some cell lines (tongue carcinoma SCC-25, hepatocellular carcinoma SK-Hep-1, liposarcoma

Table IV. Basal levels of MMP-9 (0-17%) and strong MMP-9 induction by PMA (60-100%).

Cell line	MMP-2 (%)	MMP-9 (%)	MMP-2 (w/PMA) (%)	MMP-9 (w/PMA) (%)
Lung carcinoma (A-549)	100	0	18	82
Head and neck carcinoma (FaDu)	100	0	5	95
Leukemia (HL-60)	100	0	5	95
Leukemia (U-937)	0	0	0	100
Breast cancer (MCF-7)	0	0	0	100
Breast cancer (MDA-MB-231)	0	0	0	100
Uterine cancer (SK-UT-1)	0	0	0	100
Chondrosarcoma (SW-1353)	97	3	30	70
Mesothelioma (MSTO-211H)	96	4	5	95
Osteosarcoma (U2-OS)	92	8	5	95
Renal adenocarcinoma (RCC-786-0)	83	17	19	81
Rhabdomyosarcoma (RD)	83	17	12	88
Fibrosarcoma (HT-1080)	77	23	43	57

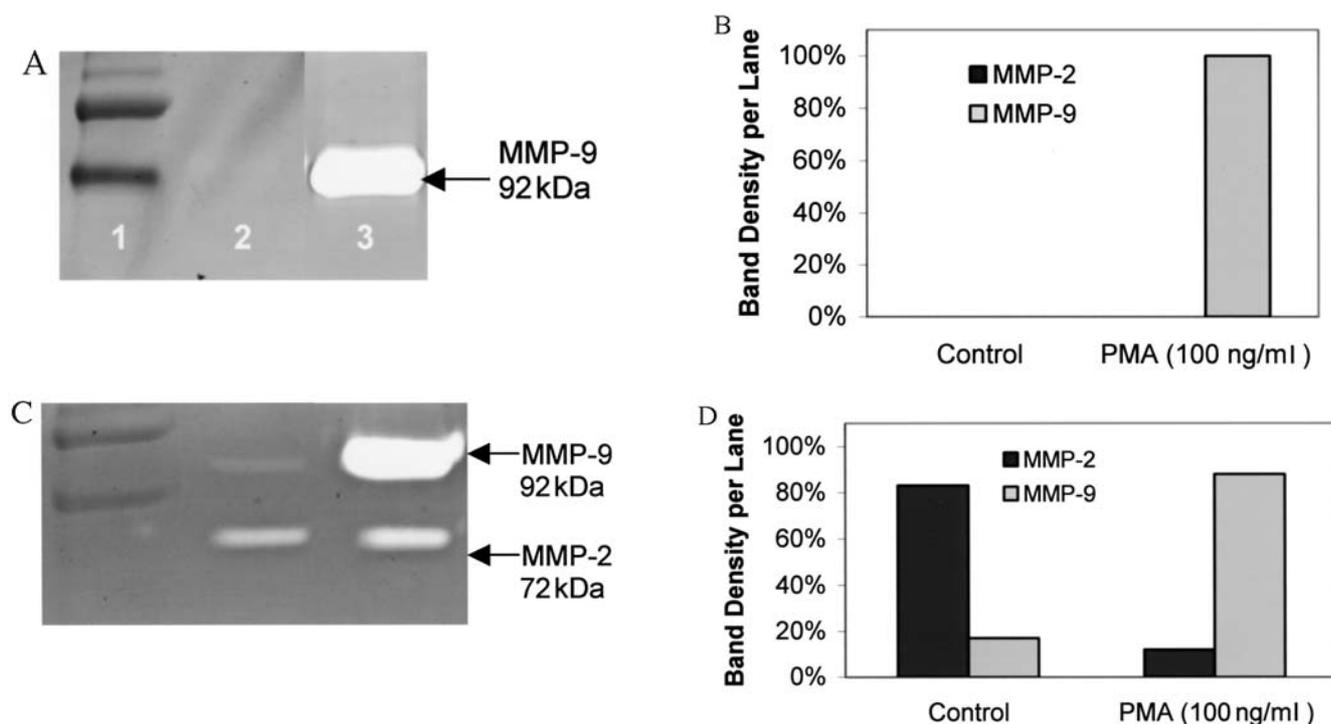


Figure 3. Breast cancer (MCF-7) and rhabdomyosarcoma (RD) - representative cell lines with low basal MMP-9 levels and high responsiveness to PMA. (A) Gelatinase zymogram of breast cancer cells show absence of MMP expression in uninduced media (control) and only a single band corresponding to the molecular weight of MMP-9 (94 kDa) in induced media. (1-Markers, 2-Control, 3-100 ng/ml PMA treatment). (B) Quantitative densitometric analysis of MMP expression of breast cancer gelatinase zymogram in a given lane. (C) Gelatinase zymogram of rhabdomyosarcoma cells shows two bands, a major one at 72 kDa (MMP-2) and a band at 92 kDa (MMP-9). PMA treatment significantly increased MMP-9 expression (1-Markers, 2-Control, 3-100 ng/ml PMA treatment). (D) Quantitative densitometric analysis of MMP expression of rhabdomyosarcoma gelatinase zymogram in a given lane.

SW-872) exhibited MMP-2 secretion in conditioned and PMA media. Fig. 4A and B show the zymogram and densitometry analysis of MMP-2 and MMP-9 in the unstimulated and stimulated media by tongue carcinoma (SCC-25) cell line. PMA-induced MMP-9 secretion greatly exceeded MMP-2 secretion. Some cancer cell lines showed only one band for MMP-9 (cervical cancer DoTc2-4510,

pancreas MIA-PaCa-2 and Burkitt's lymphoma Raji). Fig. 4C and D show the zymogram and densitometric analysis for pancreatic cancer MIA-PaCa-2, showing the expression of only one band corresponding to the molecular weight MMP-9 (92 kDa) in the control media, with further induction by PMA. However, a band at 72 kDa corresponding to MMP-2 was not observed.

Table V. High basal MMP-9 levels and strong MMP-9 induction by PMA.

Cell line	MMP-2 (%)	MMP-9 (%)	MMP-2 (w/PMA) (%)	MMP-9 (w/PMA) (%)
Tongue carcinoma (SCC-25)	40	60	21	79
Hepatocellular carcinoma (SK-Hep-1)	19	81	21	79
Liposarcoma (SW-872)	49	51	14	86
Cervical cancer (DoTc2-4510)	0	100	0	100
Human Burkitt lymphoma (Raji)	0	100	0	100
Leukemia (Jurket)	0	100	0	100
Pancreatic cancer (MIA-PaCa-2)	0	100	0	100
Prostate cancer (Du-145)	0	100	0	100

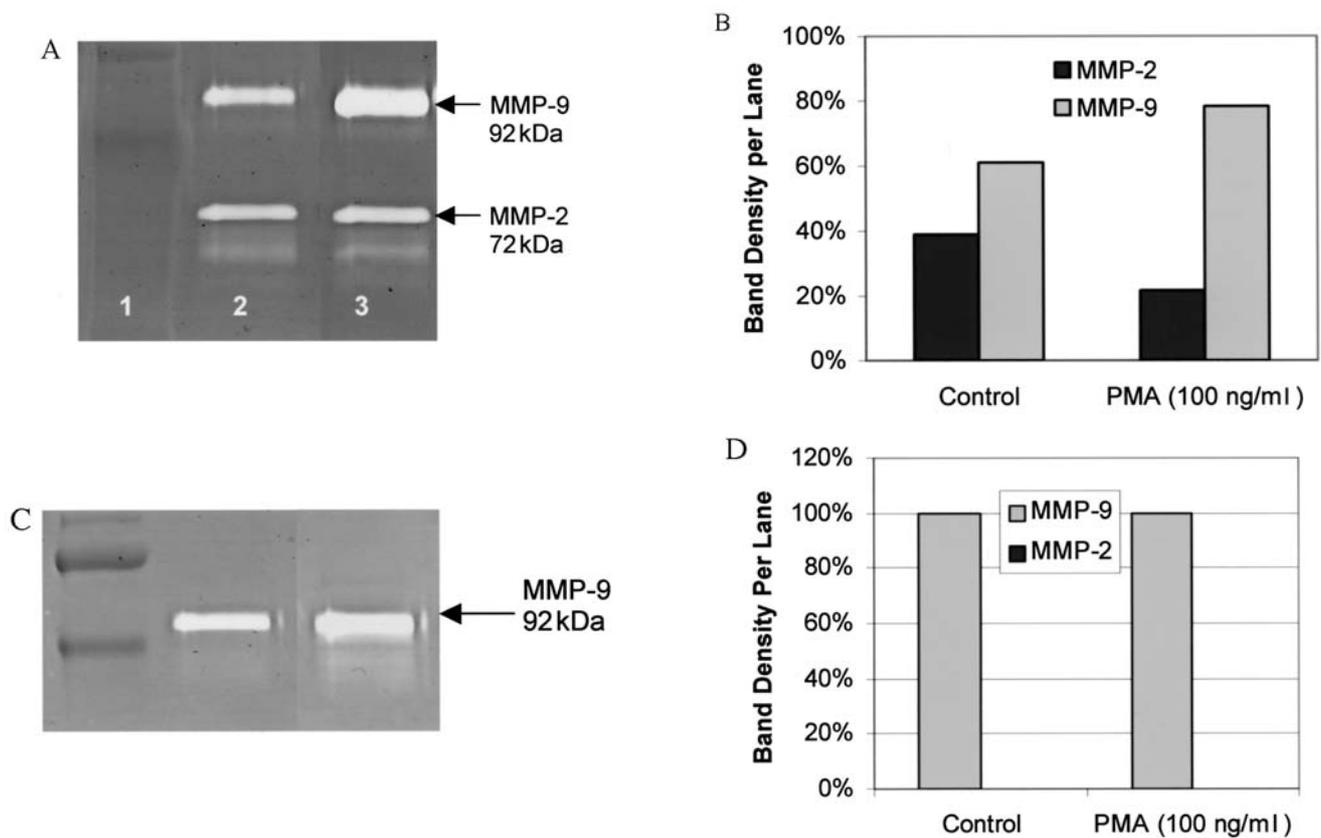


Figure 4. Tongue carcinoma (SCC-25) and pancreatic cancer (MIA-PaCa-2) - representative cell lines expressing high MMP-9 basal levels and high susceptibility to MMP-9 induction by PMA. (A) Gelatinase zymogram of tongue carcinoma expressing two bands corresponding to the molecular weight of MMP-9 (94 kDa) and MMP-2 (72 kDa) in uninduced and induced media. The MMP-9 band in induced media (PMA treated) is more prominent, correlating to further MMP-9 induction by PMA. (1-Markers, 2-Control, 3-100 ng/ml PMA treatment). (B) Quantitative densitometric analysis of MMP expression of tongue carcinoma gelatinase zymogram. MMP expression is reported as a percentage of the sum of all band densities in a given lane. (C) Gelatinase zymogram of pancreatic cancer cells expressing only a single band corresponding to the molecular weight of MMP-9 (92 kDa) in uninduced and induced media. The MMP-9 band in induced media (PMA treated) is more prominent, correlating to further MMP-9 induction by PMA. (1-Markers, 2-Control, 3-100 ng/ml PMA treatment). (D) Quantitative densitometric analysis of MMP expression of pancreatic cancer gelatinase zymogram. MMP expression is reported as a percentage of the sum of all band densities in a given lane.

Cell lines exhibiting no expression of MMP-2 or MMP-9. Interestingly, the prostate cancer cell lines PC-3, LNCAP, and 22RV-1, colon cancer cell lines HCT-116 and HCA-7, the lung cancer cell line Calu, and the leukemia cell line U-937 showed no MMP-2 or MMP-9 activity in either control or stimulated media.

Correlation between MMP-2 and MMP-9 levels. Another important finding that has emerged from this study is an apparent inverse relationship between basal MMP-2 levels and the susceptibility of cells to PMA-induced MMP-9 expression. As the basal MMP-2 levels increased, the susceptibility of a cell line to induction of MMP-9 by PMA

Table VI. Correlation of MMP-2 levels with MMP-9 induction by PMA.

Cell line	MMP-2 (%)	MMP-9 (PMA) (%)
Group A		
Breast cancer (MCF-7)	0	100
Breast cancer (MDA-MB-231)	0	100
Uterine cancer (SK-UT-1)	0	100
Leukemia (U-937)	0	100
Cervical cancer (DoTc2-4510)	0	100
Human Burkitt lymphoma (Raji)	0	100
Leukemia (Jurket)	0	100
Pancreatic cancer (Mia-PaCa-2)	0	100
Prostate cancer (Du-145)	0	100
Hepatocellular carcinoma (SK-Hep-1)	19	79
Tongue carcinoma (SCC-25)	40	79
Group B		
Synovial sarcoma (SW-982)	98	20
Ovarian cancer (SK-OV-3)	100	0
Retinoblastoma (Y-79)	100	0
Thyroid carcinoma (SW-579)	100	0
Osteosarcoma (SK-ES-1)	100	9
Glioblastoma (A-172)	100	13
Glioblastoma (T-98G)	100	13
Testis carcinoma (NT2-D1)	100	15
Glioblastoma (LN-18)	100	21
Neuroblastoma (SK-N-MC)	100	38
Osteosarcoma (MNNG-HOS)	100	38
Hepatocellular carcinoma (Hep-G2)	100	39
Group C		
Liposarcoma (SW-872)	49	86
Fibrosarcoma (HT-1080)	77	57
Renal adenocarcinoma (RCC-786-0)	83	81
Rhabdomyosarcoma (RD)	83	88
Osteosarcoma (U2-OS)	92	95
Prostate cancer (PC-3)	93	48
Mesothelioma (MSTO-211H)	96	95
Melanoma (A-2058)	97	56
Chondrosarcoma (SW-1353)	97	70
Cervical cancer (HeLa)	100	46
Bladder carcinoma (T-24)	100	56
Lung carcinoma (A-549)	100	82
Head and neck carcinoma (FaDu)	100	95
Leukemia (HL-60)	100	95

decreased (Table VI). This inverse relationship is independent of basal levels of MMP-9. These cell lines can be further divided into three groups (A, B and C). In Group A, the basal MMP-2 levels are zero and upon exposure to PMA, MMP-9, levels are induced to 100%. A typical example, Burkitt's lymphoma (Raji), is shown in Fig. 5A and B. Burkitt's lymphoma shows the absence of MMP-2 and only the

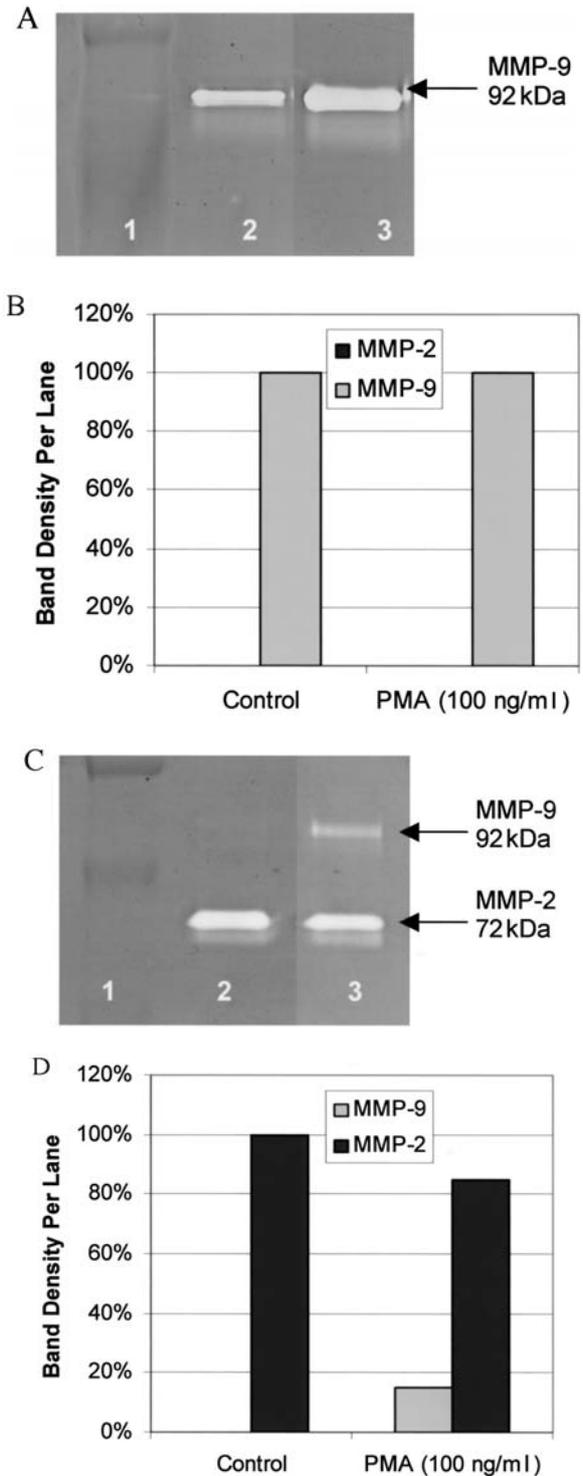


Figure 5. Burkitt's lymphoma (Raji), testes carcinoma (NT2-D1) - representative cell lines with zero or basal MMP-2 expression and high susceptibility to MMP-9 induction by PMA. (A) Gelatinase zymogram of Burkitt's lymphoma cells expressing only a single band corresponding to the molecular weight of MMP-9 (94 kDa) in uninduced and induced media. The MMP-9 band in induced media (PMA treated) is more prominent, correlating to further MMP-9 induction by PMA. (1-Markers, 2-Control, 3-100 ng/ml PMA treatment). (B) Quantitative densitometric analysis of MMP expression of Burkitt's lymphoma gelatinase zymogram. MMP expression is reported as a percentage of the sum of all band densities in a given lane. (C) Gelatinase zymogram of testes carcinoma (NT2-D1) cell line expressing only a single band corresponding to the molecular weight of MMP-2 (72 kDa) in uninduced, and two bands, one corresponding to MMP-2 and MMP-9 (94 kDa), in PMA treated media. (1-Markers, 2-Control cells, 3-100 ng/ml PMA treatment). (D) Quantitative densitometric analysis of MMP expression of testes carcinoma gelatinase zymogram. MMP expression is reported as a percentage of the sum of all band densities in a given lane.

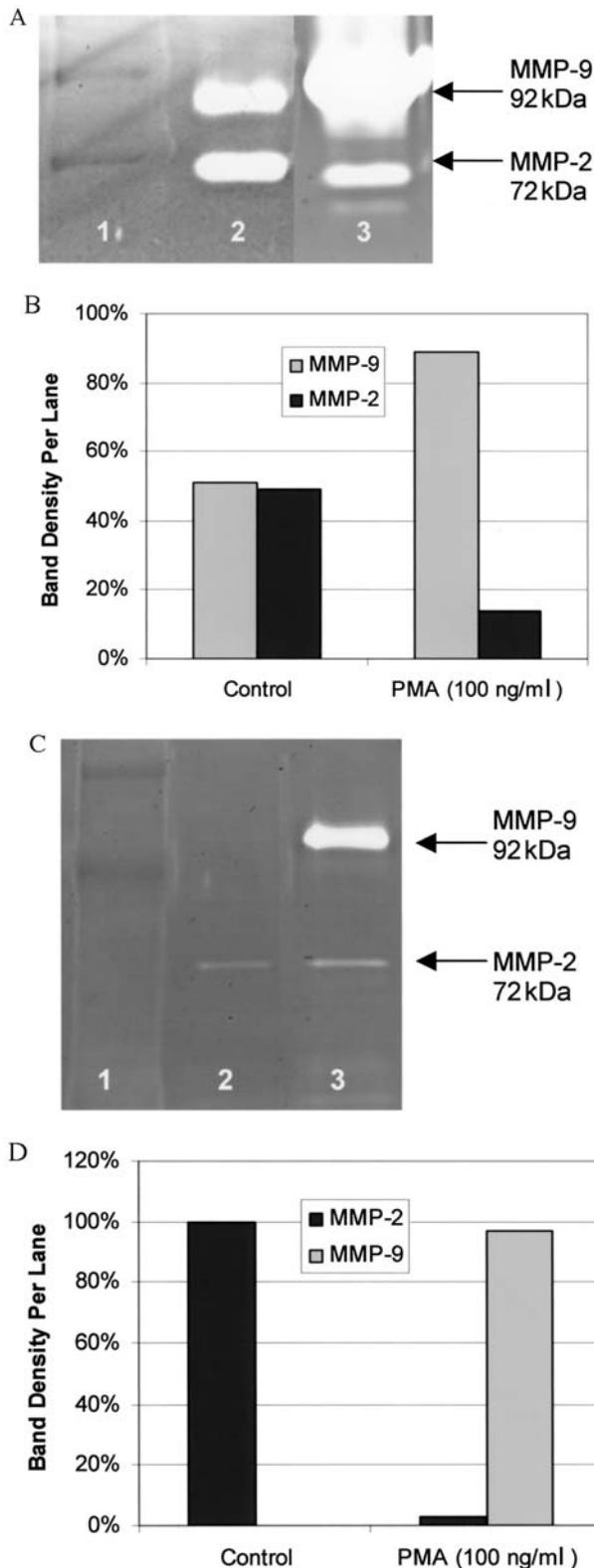


Figure 6. Liposarcoma (SW-872), Head and neck (FaDu) - representative cell lines with moderate MMP-2 and MMP-9 expression. (A) Gelatinase zymogram of liposarcoma cells shows equally prominent expression of MMP-2 and MMP-9 in uninduced media. PMA treatment led to a significant increase in MMP-9 expression compared to MMP-9 expression in untreated samples. (1-Markers, 2-Control, 3-100 ng/ml PMA treatment). (B) Quantitative densitometric analysis of MMP expression of liposarcoma gelatinase zymogram showing MMP expression as a percentage of the sum of all band densities in a given lane. (C) Gelatinase zymogram of FaDu cells. (1-Markers, 2-Control, 3-100ng/ml PMA treatment). (D) Quantitative densitometric analysis of MMP expression of head and neck (FaDu) gelatinase zymogram showing MMP expression as a percentage of the sum of all band densities in a given lane.

Table VII. Correlation of MMP-2 and MMP-9 levels without and with PMA.

Variables analyzed	Correlation coefficient (R)	Significance (P)
MMP-2 vs. MMP-9	-0.82	<0.001
MMP-2 w/PMA vs. MMP-9 w/PMA	-1.00	<0.001
MMP-2 vs. MMP-9 w/PMA	-0.65	<0.001
MMP-2 w/PMA vs. MMP-9	-0.55	<0.001
MMP-2 vs. MMP-2 w/PMA	+0.65	<0.001
MMP-9 vs. MMP-9 w/PMA	+0.55	<0.001

presence of a band of molecular weight corresponding to MMP-9 (92 kDa) in both PMA-treated and untreated media. In Group B, as basal MMP-2 levels increase (from 0 to 100%), PMA-induced MMP-9 levels are significantly decreased (0-40%). A representative of this group, testis sarcoma (NT2-D1), shown in Fig. 5C and D, expresses MMP-9 both in PMA-treated and untreated media. In Group C, as basal MMP-2 levels vary from 50 to 100%, so do MMP-9 expression levels as induced by PMA (50-95%). Typical examples, such as liposarcoma (Fig. 6A and B) and head and neck carcinoma cell line (Fig. 6C and D), demonstrate variable MMP-2 and MMP-9 expression without and with PMA treatment.

MMP-2 and MMP-9 expression data for the various cell lines from these three groups were pooled and evaluated for correlation (Table VII). A strong negative correlation ($r=-1.0$, $p<0.001$) between MMP-2 and MMP-9 after PMA induction suggests that as basal MMP-2 levels increase, susceptibility to PMA induction of MMP-9 decreases (Fig. 8). Analyzing expression values between MMP-2 and MMP-9, without PMA treatment, further validated this. A linear negative correlation between MMP-9 and MMP-2 expression without the presence of PMA was obtained with correlation coefficient $r=-0.82$, $p<0.001$ (Fig. 7). Figs. 7 and 8 show negative correlations suggesting that as basal MMP-2 expression increases, susceptibility to MMP-9 induction decreases in both untreated and PMA-treated conditions. Other significant correlations that resulted suggest that as basal MMP-2 expressions increase (both without and with PMA treatment), susceptibility to MMP-9 induction by PMA decreases. Furthermore, MMP-2 expression as a result of PMA treatment positively correlates to basal MMP-2 levels, as does PMA-induced MMP-9 expression with basal MMP-9 levels.

Discussion

Numerous clinical and experimental studies have demonstrated an increase in particular MMPs, especially MMP-2 and MMP-9 with cancer progression. Although the pattern of MMP expression can vary from tumor to tumor, specific MMPs increase in particular organ-related cancers. Recent clinical studies have focused on developing potential anti-neoplastic agents for specific cancers.

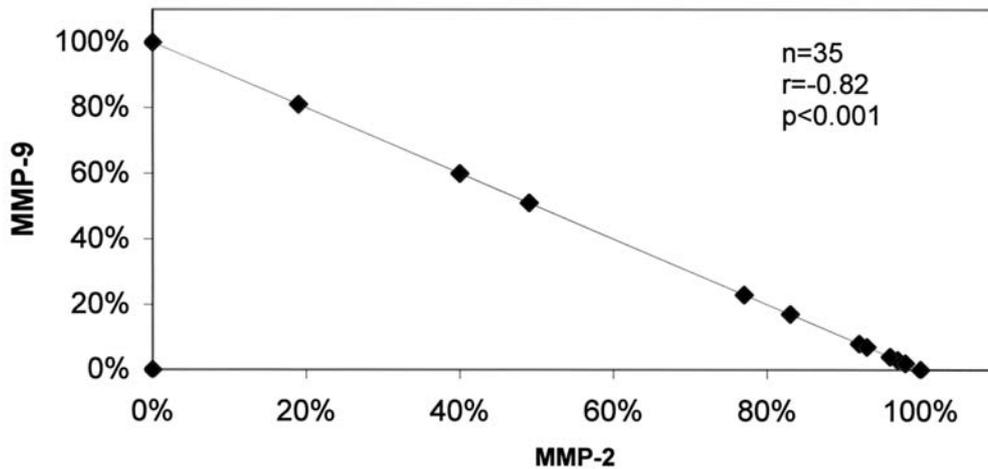


Figure 7. Increasing MMP-2 expression correlates with decreasing MMP-9 expression. A linear negative correlation between MMP-9 and MMP-2 expression without the presence of PMA was obtained with correlation coefficient $r=-0.82$, $p<0.001$.

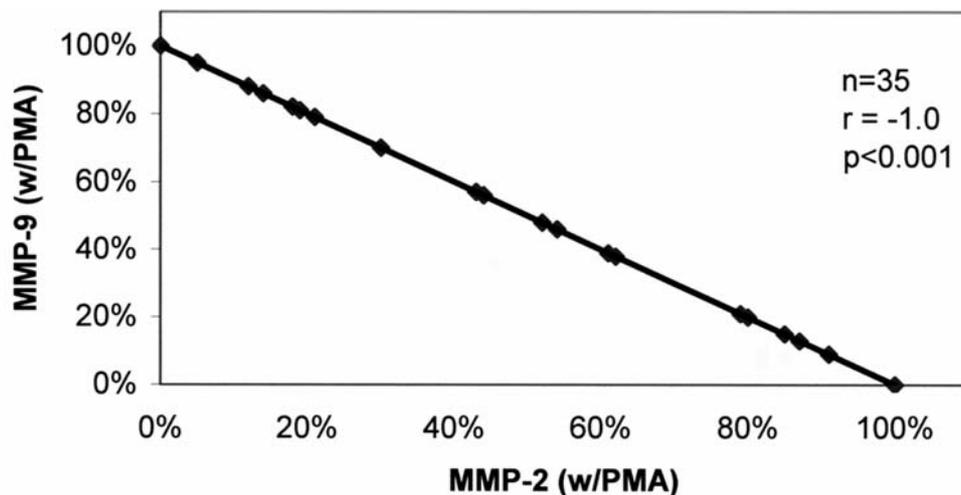


Figure 8. Increasing MMP-2 expression correlates with decreasing MMP-9 expression in the presence of PMA. A linear negative correlation between MMP-9 and MMP-2 expression in the presence of PMA was obtained with correlation coefficient $r=-1.0$, $p<0.001$.

Most MMPs appear to be secreted in inactive pro-forms which are then activated extracellularly by either the plasminogen cascade system or by other members of the MMP family, such as MT-MMPs (12). Of the MMP family, MMP-2 and MMP-9 have been given great attention because their expression can be used diagnostically and prognostically. This is in part due to the fact that MMP-2 and MMP-9 are type IV collagenases, and thus can degrade the major structural protein of ECM and basement membrane. Several studies have documented enhanced expression of these MMPs with tumor progression and metastasis (8,10,16-19).

In this study, PMA was used as a model agent to stimulate the expression of MMPs by the cytokine pathway. The results clearly indicate that MMP-2 is not induced by PMA and that MMP-9 was induced by PMA but to different degrees. Thus a study of these distinct groups of cancer cell lines will elucidate the molecular mechanisms of the resistance/susceptibility of MMP-9 induction by PMA, and explain to some degree the invasive/metastatic potential of the cancer

cells. To our knowledge this is the first study focused on MMP-9 expression of a large number of cancer cell lines in response to PMA. This information should be valuable for understanding the molecular mechanisms by which MMPs influence tumor invasion/metastasis.

Different cancers express distinct levels of MMP-2 and MMP-9. In ovarian cancer, for example, MMP-9 expression is elevated when compared to breast and bladder cancer (20). Increased levels of MMP-2 expression have been reported in colon, pancreas, prostate and skin cancer (13). In our study, MMP-2 was constitutively expressed by most cell lines, whereas MMP-9 levels were usually low but could be induced by PMA. Interestingly, the prostate cancer cell lines PC-3, LNCAP, and 22RV-1, colon cancer cell lines HCT-116 and HCA-7, lung cancer cell line Calu and leukemia cell line U-937 showed no MMP-2 or MMP-9 activity in either control or stimulated media. However, when cancer cells that express no MMPs are co-cultured with stromal cells, representative of the clinical picture, induction of

MMPs is observed. *In vitro* co-culture experiments of normal fibroblasts with tumor cell lines such as prostate, breast, colon, lung and melanoma, have demonstrated high expressions of MMP-2 and MMP-9 otherwise not seen; neither fibroblasts nor these cancer cell lines express MMP-2 and MMP-9 at substantial levels when monocultured (21-23). These results suggest that co-culturing cancer cells with normal fibroblasts triggers alteration of the fibroblasts to support further cancer cell growth by secretion of MMPs, leading to ECM degradation, attachment to ECM components, and cellular motility.

Research on various cancers has localized gelatinases by *in situ* hybridization both in cancer cells themselves and in mesenchymal cells adjacent to the invading tumor front (15,24,25). *In situ* hybridization of breast cancer tissue, detected stromelysin-3 mRNA exclusively in stromal cells not cancer cells (26) and colon adenocarcinoma u-PA immunoreactivity and mRNA were found in the tumor stroma and not in the cancer cells (25). A glycoprotein, extracellular matrix metalloproteinase inducer (EMMPRIN), at the surface of cancer cells was found to stimulate these peritumor fibroblasts (27). Thus, EMMPRIN plays a role in invasion and metastasis of cancer cells by stimulating nearby fibroblasts to secrete increased amounts of interstitial collagenase, stromelysin-1, and gelatinase A (28). Indeed, Liotta and Kohn (29) found that cells surrounding tumors often supported, if not promoted, tumor growth.

Also of interest is the inverse correlation between the basal levels of MMP-2 and the susceptibility of the cell line to MMP-9 induction by PMA. The significance of this inverse correlation is not clear. However, it indicates an unexplored mechanism by which MMP-2 regulates MMP-9. Given the importance of MMPs in cancer invasion/metastasis, it becomes very important to explore the regulation of one MMP by the other.

In conclusion, the results clearly indicate that MMP-2 is not induced by PMA and that MMP-9 was induced by PMA but to different degrees dependent upon the specific cell line. These findings are important in determining whether MMP-2, MMP-9 or both should be targeted, and for diagnostic or pathological understanding. The specific expressions of MMPs in various malignancies have generated great interest, and our findings will be helpful in further studies attempting to develop anti-neoplastic agents.

Acknowledgements

We would like to thank Dr D.S.R. Sarma for his valuable suggestions. The research study was funded by Dr Rath Health Foundation (Plantation, FL, USA) a non-profit organization.

References

- Fidler IJ: Molecular biology of cancer: invasion and metastasis. In: Cancer: Principles and Practice of Oncology. 5th edition. De Vita VT, Hellman S and Rosenberg SA (eds). Lippincott-Raven, Philadelphia, PA, pp135-152, 1997.
- Egeblad M and Werb Z: New functions for the matrix metalloproteinases in cancer progression. *Nat Rev Cancer* 2: 161-174, 2002.
- Folkman J: Role of angiogenesis in tumor growth and metastasis. *Semin Oncol* 29 (Suppl. 16): S15-S18, 2002.
- Chambers AF and Matrisian LM: Changing views on the role of matrix metalloproteinases in metastasis. *J Natl Cancer Inst* 89: 1260-1270, 1997.
- Kleiner DL and Stetler-Stevenson WG: Matrix metalloproteinases and metastasis. *Cancer Chemother Pharmacol (Suppl)* 43: S42-S51, 1999.
- Yurchenko PD and Schitny JC: Molecular architecture of basement membranes. *FASEB J* 4: 1577-1590, 1990.
- Barsky SH, Siegel GP, Jannotta F and Liotta LA: Loss of basement membrane components by invasive tumors but not by their benign counterparts. *Lab Invest* 49: 140-147, 1983.
- Liotta LA, Tryggvason K, Garbisa A, Hart I, Foltz CM and Shafie S: Metastatic potential correlates with enzymatic degradation of basement membrane collagen. *Nature* 284: 67-68, 1980.
- Stetler-Stevenson WG: The role of matrix metalloproteinases in tumor invasion, metastasis and angiogenesis. *Surg Oncol Clin N Am* 10: 383-392, 2001.
- Stetler-Stevenson WG: Type IV collagenases in tumor invasion and metastasis. *Cancer Metastasis Rev* 9: 289-303, 1990.
- Woessner JF Jr: The matrix metalloproteinase family. In: Matrix Metalloproteinases. Parks WC and Mechan RP (eds). Academic Press, San Diego, CA, pp1-14, 1998.
- Nelson AR, Fingleton B, Rothenberg ML and Matrisian LM: Matrix metalloproteinases: biologic activity and clinical implications. *J Clin Oncol* 18: 1135-1149, 2000.
- Bérubé M, Deschambeault A, Boucher M, Germain L, Petitclerc E and Guérin SL: MMP-2 expression in uveal melanoma: differential activation status dictated by the cellular environment. *Mol Vis* 11: 1101-1111, 2005.
- Sato T, Sakai T, Noguchi Y, Takta M, Hirakawa S and Ito A: Tumor-stromal cell contact promotes invasion of human uterine cervical carcinoma cells by augmenting the expression and activation of stromal matrix metalloproteinases. *Gynecol Oncol* 92: 47-56, 2004.
- Di Nezza LA, Misajon A, Zhang J, Jobling T, Quinn MA, Ostör AG, Nie G, Lopata A and Salamonsen LA: Presence of active gelatinases in endometrial carcinoma and correlation of matrix metalloproteinase expression with increasing tumor grade and invasion. *Cancer* 94: 1466-1475, 2002.
- Cottam DW, Rennie IG, Woods K, Parsons MA, Bunning RA and Rees RC: Gelatinolytic metalloproteinase secretion patterns in ocular melanoma. *Invest Ophthalmol Vis Sci* 32: 1923-1927, 1992.
- Fishman DA, Bafetti L, Banionis S, Kearns AS, Chilukuri K and Stack MS: Production of extracellular matrix degrading proteinases by primary cultures of human epithelial ovarian carcinoma cells. *Cancer* 80: 1457-1463, 1997.
- Garzetti G, Ciavattini A, Lucarini G, Goteri G, De Nicolis M, Garbisa S, Masiero L, Romanini C and Graziella B: Tissue and serum metalloproteinase (MMP-2) expression in advanced ovarian serous cystadenocarcinomas: clinical and prognostic implications. *Anticancer Res* 15: 2799-2804, 1995.
- Gohji K, Fujimoto N, Hara I, Fujii A, Gotoh A, Okada H, Arakawa S, Kitazawa S, Miyake H, Kamidono S and Nakajima M: Serum matrix metalloproteinase-2 and its density in men with prostate cancer as a new predictor of disease extension. *Int J Cancer* 79: 96-101, 1998.
- Schmalfeldt B, Prechtel D, Härting K, Späthe K, Rutke S, Konil E, Fridman R, Berger U, Schmitt M, Kuhn W and Lengyel E: Increased expression of matrix metalloproteinases (MMP)-2, MMP-9, and the urokinase-type plasminogen activator is associated with progression from benign to advanced ovarian cancer. *Clin Cancer Res* 7: 2396-2404, 2001.
- Maneva-Radicheva L, Ebert U, Dimoudis N and Altankov G: Fibroblast remodeling of adsorbed collagen type IV is altered in contact with cancer cells. *Histol Histopathol* 23: 833-842, 2008.
- Ko K, Yazumi S, Yoshikawa K, Konda Y, Nakajima M, Chiba T and Takahashi R: Activation of fibroblast-derived matrix metalloproteinase-2 by colon cancer cells in no-contact co-cultures. *Int J Cancer* 87: 165-171, 2000.
- Sato H, Kida Y, Mai M, Endo Y, Sasaki T, Tanaka J and Seiki H: Expression of genes encoding type IV collagen-degrading metalloproteinases and tissue inhibitors of metalloproteinases in various human tumor cells. *Oncogene* 7: 77-83, 1992.

24. Pyke C, Ralfkiaer E, Huhtala P, Hurskainen T, Danø K and Tryggvason K: Localization of messenger RNA for Mr 72,000 and 92,000 type IV collagenases in human skin cancers by in situ hybridization. *Cancer Res* 52: 1336-1341, 1992.
25. Pyke C, Kristensen P, Ralfkiaer E, Grøndahl-Hansen J, Eriksen J, Blasi F and Danø K: Urokinase-type plasminogen activator is expressed in stromal cells and its receptor in cancer cells at invasive foci in human colon adenocarcinomas. *Am J Pathol* 138: 1059-1067, 1991.
26. Basset P, Bellocq JP, Wolf C, Stoll I, Hutin P, Limacher JM, Podhajcer OL, Chenard MP, Rio MC and Chambon P: A novel metalloproteinase gene specifically expressed in stromal cells of breast carcinomas. *Nature* 348: 699-704, 1990.
27. Biswas C, Zhang Y, De Castro R, Guo H, Nakamura T, Kataoka H and Nabeshima K: The human tumor cell-derived collagenase stimulatory factor (renamed EMMPRIN) is a member of the immunoglobulin superfamily. *Cancer Res* 55: 434-439, 1995.
28. Kataoka H, De Castro R, Zucker S and Biswas C: Tumor cell-derived collagenase-stimulating factor increases expression of interstitial collagenase, stromelysin and 72-kDa gelatinase. *Cancer Res* 53: 3154-3158, 1993.
29. Liotta LA and Kohn EC: The microenvironment of the tumor-host interface. *Nature* 411: 375-379, 2001.