Effects of DADS on expression of matrix metalloproteinases in prostate cancer cells

Chapter IV
7.1 Introduction

During the last few decades, the discovery of new anticancer agents has been based on the development of compounds that interfere with nonspecific intracellular processes (i.e., nucleotide turnover, DNA synthesis and replication, and microtubular functions). More recently, new understanding of tumor cell biology has permitted the identification of cellular processes that are specifically altered in cancer cells and that are responsible for autonomous growth and replication, invasion of surrounding tissues and formation of metastases. Several classes of proteinases, including serine proteinases, cysteine proteinases and matrix metalloproteinases (MMPs) have been implicated in tumor cell invasion process (Hojilla et al., 2003).

Potential targets for new drugs include the matrix metalloproteinases (MMPs), a group of proteinases that have physiologic roles in degrading and remodeling the extra cellular membrane. The MMPs are over expressed in a variety of malignant tumor types and their over expression is associated with tumor aggressiveness and metastatic potential (Chambers and Matrisian, 1997; Kahari and Saarialho-Kere, 1999; Kleiner and Stetler-Stevenson, 1999). The specific alteration of the MMPs observed in malignant tissues and their participation in some of the major oncogenic mechanisms have fueled interest in the design and evaluation of MMP inhibitors as anticancer agents (Denis and Verweij, 1997; Brown, 1999).
MMPs are highly regulated at the levels of both gene expression and protein activation. Transcriptional regulation of MMP genes is mediated by an AP-1 regulatory element in their proximal promoter regions (Fini et al., 1998). In general, the MMP genes are not expressed constitutively in vivo, and the basal production of MMPs in cell cultures is low (Shapiro, 1998; Kahari and Saarialho-Kere, 1999). MMP gene transcription is induced by a variety of extra cellular stimuli, such as cytokines (interleukin [IL] 4 and IL-10), growth factors (EGF, bFGF, TGF-α and TGF-β), and cell-cell or cell-matrix interactions (Kurogi et al., 1996; Kondapaka et al., 1997; Sundareshan et al., 1999). Binding of these stimulatory ligands to their receptors triggers a cascade of intracellular reactions that are mediated through at least three different classes of mitogen-activated protein (MAP) kinases: extra cellular signal regulated kinase, stress-activated protein kinase/Jun N-terminal kinases, and p38 (Reddy et al., 1999). Activation of these kinases culminates in the activation of the nuclear AP-1 transcription factor, which binds to the AP-1 cis element and activates the transcription of the corresponding MMP gene (Sato et al., 1997).

Role of matrix metalloproteinases in tumor growth, invasion, and metastasis

Tumor growth, invasion, and metastasis are a multistep and complex process that includes cell division and proliferation, proteolytic digestion of the extra cellular matrix, cell migration through basement membranes to reach the circulatory system, and remigration and growth of tumors at the metastatic sites (Folkman, 1995).
The proposed role of MMPs in this process is based on *in vitro* and *in vivo* preclinical studies as well as on studies of clinical specimens. MMPs degrade the basement membrane and extracellular matrix, thus facilitating the invasion of malignant cells through connective tissues and blood vessel walls and resulting in the establishment of metastasis (Chambers and Matrisian, 1997). In rat embryo fibroblasts, overexpression of MMP-9 degraded the matrix, resulting in enhanced metastatic potential (Bernhard *et al.*, 1994; Hua and Muschel, 1996). In addition, transfection of MMP genes into malignant human tumor cell lines, such as the MYV3L (rat bladder carcinoma), Madison 109 (mouse lung carcinoma), and DU-145 (human prostate carcinoma), increased the number and size of pulmonary metastasis in *in vivo* studies (Powell *et al.*, 1993; Kawamata *et al.*, 1995). Tumors in knockout mice lacking specific MMPs exhibit reduced tumorigenesis, angiogenesis, and tumor growth by increasing the bioavailability of growth factors that reside in the extracellular matrix and are released during extracellular matrix degradation (Manes *et al.*, 1997; Martin *et al.*, 1999).

MMP expression, although low or undetectable in most normal tissues, is substantially increased in the majority of malignant tumors. Numerous studies in a variety of tumor types, including lung, colon, breast, and pancreatic carcinomas, demonstrate overexpression of MMPs in malignant tissues in comparison to adjacent normal tissues (Bernhard *et al.*, 1994; Kugler *et al.*, 1998; Hashimoto *et al.*, 1998). In addition, the plasma and urine levels of MMPs are elevated in patients with cancer compared with healthy subjects.
The MMPs in tumor tissues are produced not only by malignant tumors but also by stromal fibroblasts and inflammatory cells (Dano et al., 1999). These cells may produce cytokines and proteins that induce the production of MMPs by surrounding cells, creating intracellular networks of MMP secretion and activation (Johansson et al., 1997; Uria et al., 1997). In addition, parallel analyses of tissue samples spanning the process from normal tissue to tumor formation have demonstrated that overexpression of MMPs is a feature of progression to the malignant phenotype (Airola et al., 1999; Ozdemir et al., 1999). Furthermore, analyses of cellular components derived from primary tumor tissues or their corresponding lymph node metastases demonstrated increased expression of MMPs in the metastatic tissue, indicating that the MMP expression is a component of the metastatic process (Iwata et al., 1996). In addition to the well-documented overproduction and activation of MMPs in malignant tissue, there is now ample clinical evidence that overproduction of these molecules confer a poor prognosis in patients with a variety of malignancies (Murray et al., 1998).

Whether specific members of the MMP family are associated with oncogenesis is a matter of debated and varies among the tumor types and stage of lesions studied. Some of these variabilities can be attributed to the different experimental conditions and techniques used in different studies. In general, the gelatinases (MMP-2 and MMP-9) have been most consistently detected in malignant tissues and associated with tumor aggressiveness, metastatic potential, and a poor prognosis. More recently, matrilysin (MMP-7) has been the focus of attention because its preferential expression in early
stage tumors and premalignant lesions may make it a suitable target for chemopreventive strategies (Wilson et al., 1997; Fingleton et al., 1999).

**Inhibition of matrix metalloproteinases as anticancer therapy**

The pharmacologic inhibition of MMP activity could markedly inhibit the invasiveness of primary and metastatic tumors and therefore, be of therapeutic benefit to patients with cancer. MMP expression and activation involve multiple steps including transcription of MMP genes, secretion of the zymogen into the extra cellular matrix, and activation of the zymogen, several of which are amenable to pharmacologic intervention. Inhibition of signal transduction transmitted through the MAP kinases markedly inhibits the expression of MMPs and the invasive potential of cancer cell lines (Simon et al., 1998). Cancer cell lines treated with specific inhibitors of the MAP kinases, such as PD 98059 and SB 203560, or other nonspecific tyrosine kinase inhibitors, such as PD 166285, have reduced expression of MMP in vitro (Simon et al., 1998).

At present, inhibition of the function of MMPs in the extra cellular matrix is being most actively pursued for anticancer therapy. The naturally occurring inhibitors of MMP activity were the first compounds to be considered for clinical development. Theoretically, the ability of MMP inhibitors to potently and specifically inhibit the activities of several MMPs could result in a beneficial therapeutic effect (Ahonen et al., 1998). The development of synthetic inhibitors of MMPs has been actively pursued and widely tested in clinical trials (Brown, 1998).
The presence of MMPs in prostate cancer has been elucidated in a number of studies. It has been shown that serum level and/or tissue expression of several MMP members such as MMP-1, MMP-2, MMP-3, MMP-7 and MMP-9 are correlated to the progression or metastasis capacity of prostate cancer (Stearns and Stearns, 1996; Jung et al., 1997; Still et al., 2000). Despite the uncertainty regarding the expression of MMPs in prostate cancer, it has been shown that synthetic inhibitors of MMPs can reduce the growth of prostate cancer in animal models (Lokeshwar, 1999; Zucker et al., 2000). This strongly indicates that MMPs might still play an important role in prostate cancer progression. Further investigations regarding specific MMP that play a role in prostate cancer progression will help us to design a more specific therapy for the disease.

The MMPs represent an attractive target for cancer treatment, and a number of MMP inhibitors are undergoing clinical trials to establish whether any of these compounds are therapeutically useful. Independent of the conclusions from the first generation of studies, the field of MMP inhibitors remains attractive for creative and innovative research. The development of novel, less toxic and more effective MMP inhibitors as well as the combination of conventional agents with these novel anticancer agents will constitute the main focus of future research efforts. MMP-2 and MMP-9 have been frequently associated with the invasive metastatic potential of tumor cells including prostate cancer (Stetler-Stevenson, 1999; Zhang et al., 2004). Several epidemiological and laboratory studies have shown that many vegetables, fruits and grains as well as phytochemicals offer significant
protection against various cancers including prostate cancer (Taraphdar et al., 2001). DADS, an organosulfur compound of garlic has shown to induce apoptosis in many tumor cell lines including prostate cancer (Sundaram and Milner, 1996; Xiao et al., 2004). DADS inhibited the proliferation and induced DNA damage of prostate cancer cell line PC 3 (Arunkumar et al., 2005). The PC 3 cell line is highly invasive and metastatic and androgen independent was derived from the ascites fluid of a patient with advanced prostate cancer that had metastasized to the lungs, pancreas, liver, kidney and bones (Kaighn et al., 1979). This cell line produces high levels of MMP-2 and MMP-9 (Aalinkeel et al., 2004; Vayalil et al., 2004). The present study was aimed to investigate the role of DADS on the levels of MMP-2 and MMP-9 in prostate cancer cells (PC 3) and this study suggesting its role as antimetastatic in cancer progression.
7.2 Materials and methods

7.2.1 Western blot analysis of matrix metalloproteinases 2 and 9 (MMP 2 and 9)

MMP 2 and 9 proteins expression in PC 3 cells was assessed by Western blot method as described by Fido et al. (1995).

The PC 3 cells were plated in 100 mm petriplates at a concentration of $1 \times 10^6$ cells/plate and grown in RPMI 1640 with 10% FBS. 24 h after plating the cells (70-80% confluent), cells were treated with DADS (25 µM and 40 µM) for 24 h.

At the end of treatment, cells were washed once with ice-cold PBS and 600 µl of ice-cold RIPA buffer containing protease inhibitor was added. 2x concentrated conditioned media of control and DADS treated PC 3 cells were also collected. Samples were collected into a 1.5 ml tube, and centrifuged for 10 min at 14,000 rpm at 4°C. The supernatants were saved in new cold tubes, and protein concentration of supernatants was determined by the method of Lowry et al. (1951).

50 µg of total proteins from cell lysate was subjected to SDS-PAGE. The samples were mixed with 2x sample buffer and boiled for 5 min. The sample mixture was run on 12% SDS-PAGE gels in 1x running gel buffer at 100 V for 2.5 h and electrotransferred to a PVDF (Millipore, Germany) at 30 V for 7 h. The membrane was blocked with blocking buffer containing 5% BSA overnight. The blocked membranes were incubated with primary
antibodies for matrix metallo proteinase-2 and 9 (MMP-2 and 9, Rabbit polyclonal) for 3 h. After incubation with primary antibodies, the membranes were washed three times with blocking buffer for 10 min each and incubated with horse radish peroxidase-conjugated secondary antibody (HRP-Goat anti-Rabbit IgG, 1:2000 dilutions) (GENEI, Bangalore, India). Following two intermittent washes with 1X TTBS, membranes were developed using ECL (Perkin Elmer, USA) and films exposed for appropriate times to detect signal.

7.2.2 Statistical analysis

The data were analyzed using the SPSS 7.5 Windows Students version software. For all the measurements, One-way ANOVA followed by Student’s Newman Keuls (SNK) test was used to assess the statistical significance of difference between control and DADS-treated groups. A statistically significant difference was considered at the level of $P<0.05$. 
Fig. 1B  Quantification of protein expression of MMP 2 and 9 in PC 3 cells

Each value is mean ± SEM. All experiments were carried out three times * represents statistical significance between control Vs DADS treatment groups at P<0.05 level using Student-Newman-Keuls test.
7.3 Results

Effects of DADS on protein expression of MMP 2 and 9

Western blotting was performed to determine MMP 2 and MMP 9 proteins expression in DADS treated PC 3 cells. The expression of active MMP 2 (72 kDa) and MMP 9 (83 kDa) proteins were decreased but not significantly in DADS treated PC 3 cells in both 25 and 40 μM doses (figure 1A and 1B).
7.4 Discussion

The search for superior drugs for the treatment of cancer has focused recently on the process of angiogenesis and metastases. Folkman (1971) proposed over 30 years ago that angiogenesis was critical for tumor development and that limiting the development of new blood vessels to a tumor could be a valuable target for anticancer therapy because without a new blood supply, tumors are limited to 1–2 mm in size. Angiogenesis is an attractive target for cancer chemotherapy because it provides an opportunity for more specific, less toxic drugs. The development of drug resistance in the genetically unstable tumor tissue is a cause of treatment failure. Targeting the genetically stable endothelial cells of the developing tumor holds great promise for more specific and less toxic treatment that will not become susceptible to multidrug resistance (Kerbel, 1991). Several new antiangiogenic agents are currently in the clinic, and data show that some of the more useful cancer chemotherapeutic drugs have antiangiogenic properties (Miller et al., 2001). As predicted from the in vitro studies, DADS has antiproliferative activity in several cell lines and animal models.

In the present study, the investigation on the effects of DADS on the expression of MMPs from prostate cancer cells and the finding indicated that DADS could decrease MMP 9 and MMP 2 in prostate cancer cells. DADS does not have antiangiogenic/antimetastatic effect on PC 3 cells. It needs further studies, to explore other factors involved and affected by DADS in the process of angiogenesis in prostate cancer cells.