The effect of hypoxia on the production of matrix metalloproteinase-7 protein by human macrophages

*** Elbarghati, Laila** ****Azzouz, Lubna**

Abstract:

Macrophages have been shown to accumulate in hypoxic areas of solid tumors. These cells appear to undergo marked phenotypic changes and upregulate expression of a number of pro-tumor genes when exposed to hypoxia *in vitro* and *in vivo* as VEGF.

Aim The aim of this study is to detect the release of MMP-7 protein in hypoxic macrophages (not only the intracellular levels) using zymography, western blotting and ELISA. Intracellular and secreted forms of pro-MMP-7 were shown to be upregulated by human MDM following 16 hours of exposure to hypoxia *in vitro*. The hypoxia up-regulated genes identified could be important for the survival and functioning of macrophages in hypoxic diseased tissues.

Keywords: hypoxia, matrix metalloproteinase-7, macrophage, tumor-associated macrophages

*Staff member Medical School, Tripoli University, Tripoli, Libya
**Staff member Medical School, Tripoli University, Tripoli, Libya

Introduction

Structurally, MMP-7 (matrilysin) is one of the smallest MMPs, consisting of two domains, a pro-domain and a catalytic domain **(Woessner, JF Jr. 1998 pp69-73)**. The protein localizes in normal tissues to secretory and ductal epithelium in the endometrium, in various exocrine glands and in macrophages (Rodgers et al, 1994 pp9087-9092). It is expressed in a variety of tumors ranging from breast, colon, prostate, stomach, lung, skin and soft tissue tumors **(Denys et al, 2004 pp 1443-1449)** and contributes to proliferation, angiogenesis and metastasis. The transcription of the gene is activated by ETS transcription factors **(Crawford, H. 2001, 1370-1383)**. MMP-7 mRNA has been shown to be up regulated in hypoxic macrophages as quantified by cDNA array and real time RT-PCR analysis, the promoter of MMP-7 is hypoxia- inducible in macrophages, and element from this promoter may prove useful in the future development of hypoxia inducible therapeutic constructs optimized for use in macrophages **(Burke, B., 2003 pp1233-1243).**

MMP-7 expression has been linked to resistance to doxorubicin chemotherapy. Owing to the ability of MMP-7 to cleave Fas ligand from the surface of the tumour cells, blocking the action of the drug **(Mitsiades, N 2001, pp577-581)**. TAMs express high level of MMP7 in hypoxic areas of human breast carcinoma **(Burke, B., 2003 pp1233-1243)** suggesting that hypoxic induction of this protease in macrophage may contribute to the resistance of hypoxic tumour cells to treatment with doxorubicin.

The aim of this study is to detect the MMP-7 protein in hypoxic macrophages by zymography, western blotting and ELISA as MMP-7 has an important role in tumor growth, spread and response to some forms of chemotherapy.

Material and methods

Monocytes isolation & hypoxia incubation:

Human monocytes were isolated from leukocyte enriched buffy coats obtained from healthy blood donors (National Blood Service, Sheffield, UK). Blood was diluted 1:1 with HBSS (without calcium or magnesium), layered on Ficol-Paque Plus (Amersham Biosciences, UK) and centrifuged for 40 min at 400g. The mononuclear cell-rich band was removed, washed twice with HBSS and re-suspended in Iscove's modified Dulbecco's media (IMDM) supplemented with 2% heat inactivated, human AB serum, 2-mM

L-glutamine (all from Sigma, Poole, UK). Totally $8x10^7$ mononuclear cells were seeded into 10-cm tissue culture plates (Iwaki Inc., Iwaki, Japan) and cultured for 2 h after which non-adherent cells were removed by washing and the culture medium replenished. Monocytes were either used in experiments at this stage or cultured for 7 days to allow differentiation into momocytes derived macrophages (MDM). The purity and full differentiation of the resultant MDM was checked using CD68 by immunocytochemistry and carboxypeptidase M by flow cytometry **(Rehli, M.1995, pp15644-15649)**. In all experiments the purity of differentiated MDM was greater than 90%.

MDM were subjected to 0.1% (hypoxia) or 20.9% (normoxia) O2 in 5% CO2 humidified multi-gas incubators (Heto, Camberley, UK) for 1, 6, 16 and 24 h. Incubator oxygen levels were confirmed during and immediately after all experiments using mobile oxygen analyzers (Analox Sensor Technology, Cleveland, UK). Culture medium depths of less than 2mm were used throughout this study to ensure rapid removal of oxygen from the culture media during hypoxic experiments.

Cell lysis and protein assay:

Following hypoxic or normoxic incubation MDM were washed in PBS and total cell extracts obtained by lysing cells in lysis buffer (50-mM tris HCL, pH 8.0, 150-mM NaCl, 1% triton X-100 and 1 protease inhibitor tablet (**Roche, Mannheim, Germany**)). Cell lysates were incubated on ice for 20 min, sheered by repeated passage through a 25-gauge needle and then centrifuged at 400g for 10 min at 4ºC to remove cell debris. Nuclear and cytoplasmic extracts were prepared using Cellytic Nuclear extraction kits (N- XTRACT, Sigma, Poole UK). All extracts were stored at (-20ºC) until immunoblots analysis. Protein concentration of cell extracts was estimated using QuantiPro BCA reagent (Sigma, Poole, UK).

Western blotting:

Samples for immunoblotting were prepared by heating to 100 1ºC for 5 min in reducing loading buffer. Totally 60 μg sample was run on 10% SDS-PAGE gels and after separation the proteins were transferred onto polyvinylidene difluoride membranes (Amersham Biosciences, Little Chalfont, UK). The membranes were incubated for 16 h at 4ºC in blocking buffer (5% skimmed milk powder in 10-mM Tris, 180-mM NaCl, 0.05% Tween-20, pH 8) then

probed with MMP-7 mouse monoclonal antibody at 1:100 concentrations in blocking buffer and incubated for 2 h at room temperature. After washing with TBST the membranes were then exposed to anti-mouse secondary antibody conjugated with horseradish peroxidase (Dako Ltd., Copenhagen, Denmark) at a 1:2500 dilution in blocking buffer for 1 h at room temperature. The secondary antibody was detected using enhanced chemiluminescence reagent (Amersham, Little Chalfont UK). Expression of β -actin was used to determine equal loading of protein in all immunoblots. The expression of this constitutively expressed protein is not altered by hypoxia and can be used to examine protein levels in nuclear as well as cytoplasmic extracts **(Krauss et al, 2004 pp 10752-10757)**. To determine equal loading of proteins in whole cell, cytoplasmic and nuclear extracts, immunoblots were stripped with stripping buffer (100-mM b-mercaptoethanol, 2% SDS, 62.5-mM Tris HCl, pH6.7) at 50 1°C for 30 min and re-probed with a mouse monoclonal antibody to β-actin (Sigma, Poole, UK) at1:30,000 in blocking buffer. Protein expression was quantified by densitometry using Quantity One software (BioRad, Hercules CA).

Zymography:

30 μl samples are prepared in 10 μl standard, non reducing loading buffer for 15% SDS-PAGE with hypoxic T47D cells as a positive control. A suitable substrate (gelatin or casein (1%) for MMP7 detection is embedded in the resolving gel during preparation of acrylamide gel. No reducing agent or boiling are necessary since theses with interfere with refolding of the enzyme. Following 2 h electrophoresis at 120 V, the SDS is removed from the zymogram and washed three times in wash buffer with Triton X-100 (2.5%) for 1 h, followed by incubation in an appropriate developing buffer, for 16, 24 h at 37 C. The zymogram is subsequently stained with Coomassie brilliant blue for 2 h after wash with DH_2O twice 10 min each, and areas of digestion appear as clear bands against a darkly stained background where the substrate has been degraded by the enzyme.

ELISA Enzyme linked immunossorbent assay MMP-7:

Human 7-day MDM were exposed to 0.1% O2 for 1, 6 and 16 h. The supernatant above the cells was then collected and centrifuged for 10 min at 400 g and the cell debris discarded. The samples were concentrated 5 fold using vivaspin columns before running on ELISA (R&D Systems), It measures both pro-MMP-7 and active MMP-7.

All reagents and standards were prepared according to the manufacturer's instructions. Briefly, 100 μl of the assay diluent RD1-52 was added to each well of a 96-well plate, then 5 μl of standard or sample was then added per well in triplicate and incubated for 2 hours on a shaker at room temperature. Wells were then washed three times with 400 μl wash buffer before addition of 200 μl anti-MMP-7 HRP-conjugated antibody. The plate was then incubated for 2 hours room temperature with gentle agitation before washing three times with wash buffer. 200 μl of substrate solution was then added and the plate incubated for 30 minutes at room temperature in the dark. Finally 50 μl of the stop solution was added and the optical density determined using a micro-plate reader set at 450 nm.

Statistical analysis

A parametric t-test was used to evaluate significance between experimental groups and

P values exceeding 0.05 were not considered significant.

Results

MMP-7 detection by western blotting

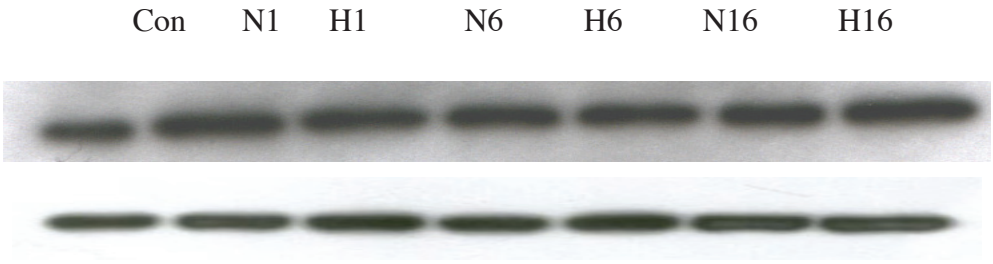
MMP-7 was detected in the MDM and the media collected from macrophages after hypoxic incubation of 1, 6, and 16 hours. The MMP-7 protein was found to be up-regulated within both the total cell extract more than two folds (Figure 1) and the media more than two folds after 16 of hypoxia (0.1% O_2) (Figure 2). There were no changes after 1 and 6 hours of hypoxia within both the cell extract and the media. The positive control was stimulated endothelial cells (HUDMECS) with 10 mM PMA. The MMP-7 expression was compared to the expression of the constitutively expressed protein β-actin to ensure equal loading.

MMP-7 detection by zymography

MMP-7 protein up regulation at 16 hours (0.1% O_2) was confirmed by zymography. The expression of the protein was more than two folds. The control was hypoxic T47D cells (Figure 3).

MMP-7 detection by ELISA

A commercial ELISA was also used to detect the amount of MMP-7 released by 7-day MDM following exposure to normoxia or hypoxia for 1, 6 and 16 hours *in vitro*.



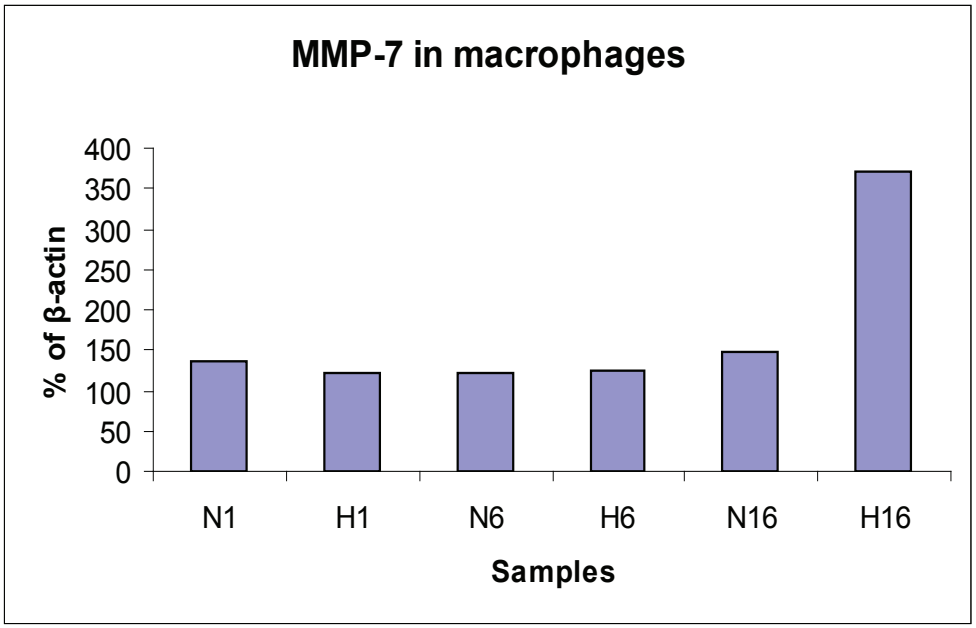
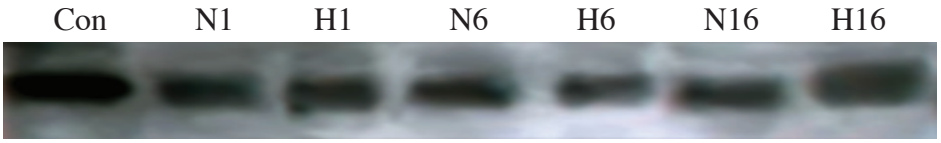


Figure 1: MMP-7 in macrophages (total cell extract) by Western blotting. MMP-7 was found to be up-regulated after 16 hours of hypoxia (0.1%O_2). There was no up-regulation after 1 and 6 hours of hypoxia. The β-actin protein was used as a loading control.



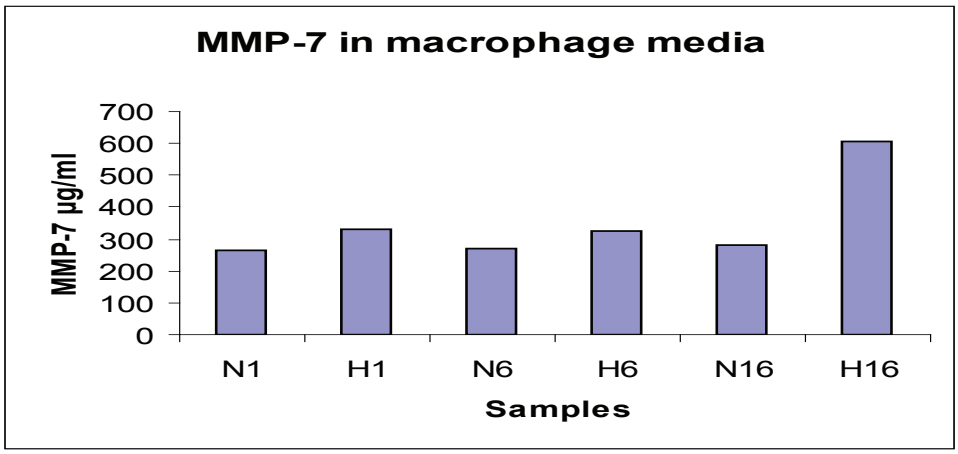


Figure 2: MMP-7 in MDM media by Western blotting. MMP-7 was found to be up-regulated after 16 hours of hypoxia (0.1%O_2). There was no up-regulation after 1 and 6 hours of hypoxia.

This is fully quantitative and more sensitive than Western blotting (it detects as little as 0.15 ng/ml MMP-7, compared to 5 ng/lane on Western blots. In agreement with the Western blot data, no difference in MMP-7 production was observed between normoxia and hypoxia at 1 and 6 hours. MMP-7 production in hypoxic macrophages was double than that seen in normoxic counterparts at 16 hours (*P* value <0.05) (Figure 4).

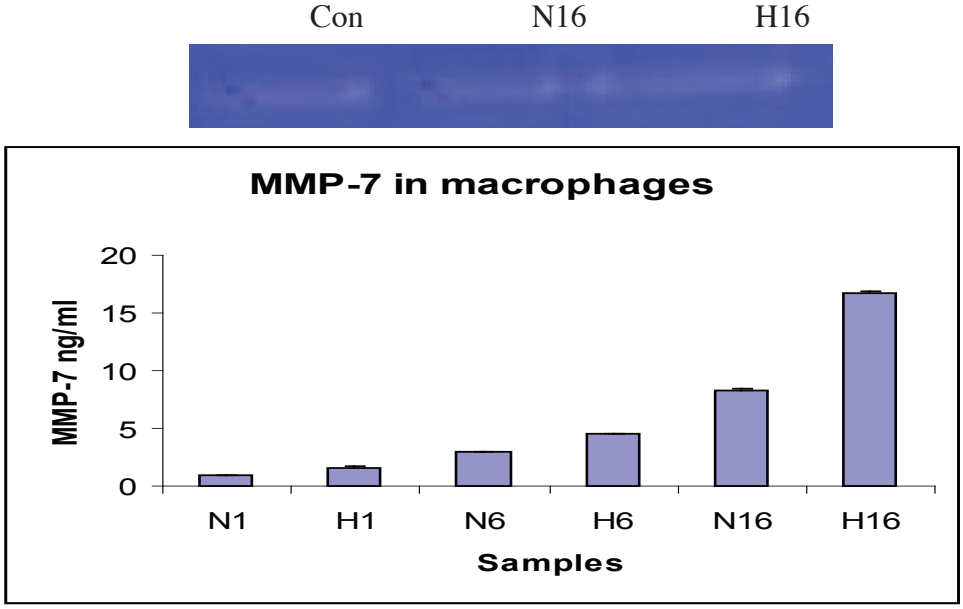


Figure 3: MMP-7 in MDM media by using zymography. The protein was found to be up-regulated at 16 hours of hypoxia within the media collected from the macrophages.

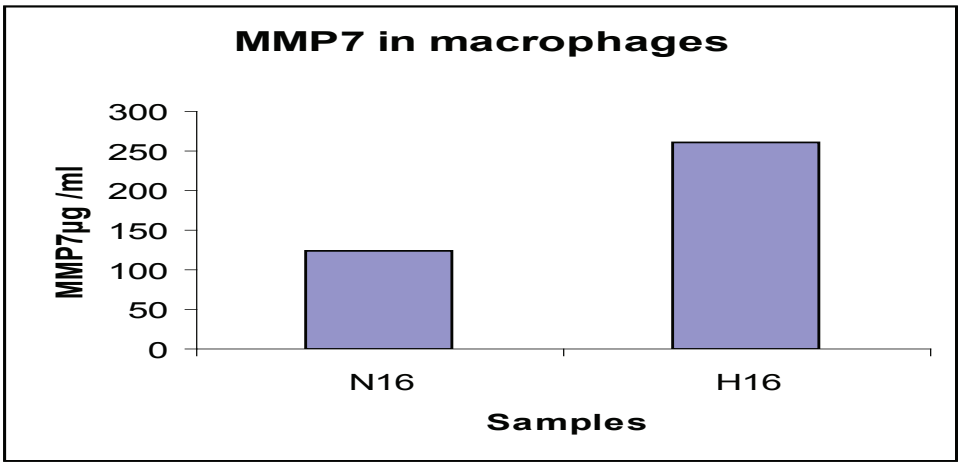


Figure 4: MMP-7 in MDM media by using MMP-7 ELISA. MMP-7 was up-regulated at 16 hours of hypoxia (0.1% 0_2) within the media of the macrophages with no changes in MMP-7 expression after 1 and 6 hours of hypoxia**.**

Discussion

In this study both the intracellular and secreted forms of pro-MMP-7 were shown to be upregulated by human MDM following 16 hours of exposure to hypoxia in vitro. However, this was a slow upregulation and was not seen at earlier time points (i.e. 1 and 6 hours of hypoxia). Pro-MMP-7 protein was also found to be constitutively expressed by MDM during normoxic cultures (i.e. at all time points). This may reflect their response to their culture conditions (i.e. be an experimental artifact) or reflect the ability of macrophages to continually participate in extracellular matrix turnover by their constitutive release of MMP-7 **(Busiek, 1992 pp9087-9092).** In addition, macrophages can survive prolonged hypoxia with no effect on the cell viability, so the increase in the amount of pro-MMP-7 released by hypoxic cells was not a result of cell death and release of cellular content, but a response to the hypoxic stress.

These data support the findings of Burke et al., (2003) who showed that MMP-7 mRNA is upregulated by human MDM after 16 hours of exposure to hypoxia in vitro. The present study shows that mRNA upregulation is accompanied by an increase in both intracellular pro-MMP-7 protein and release. Recently, using cDNA microarray analysis, (White, J. R., 2004 pp1-18) showed that macrophages subjected to hypoxia in vitro can upregulate mRNA expression for MMP-1 and MMP-12. However, their study failed to identify MMP-7 as being upregulated at the mRNA level. This may have been due to differences in the culture conditions used to generate MDM in the two reports.

The hypoxic upregulation of intracellular and secreted pro-MMP-7 at 16 hours is most likely due to activated gene transcription and protein translation rather than secretion from ready-made intracellular stores. In support of this, Sabha et al., (2006) reported a slow MMP-7 response involving the transcription of the pro-MMP-7 gene following 6 hours of exposure to hypoxia in primary rat bladder smooth muscle cells.

The recruitment of macrophages in hypoxic areas of tumors is well documented **(Ohno, S et al. 2004 pp3335-3342)**, although the mechanisms involved are only beginning to be elucidated **(Murdoch, C 2004 pp2224-2234)**. It is thought that macrophages are attracted to sites of hypoxia either by chemoattractant or necrotic debris. To migrate through tissues, macrophages need to degrade the constituents of ECM, like type-IV collagen, fibronectin and laminin. One mechanism by which macrophages are thought to do this is by release of the MMPs. Unlike most MMPs, MMP-7 lacks substrate specificity and can therefore efficiently digest many components of the ECM. MMP-7 can also activate other molecules such as TNF- α , uPA and MMPs 1, 2 and 9 **(von Bredow D. 1998 et al. pp965-972)** which may accelerate ECM degradation. Thus, production of these proteins by TAM is likely to affect the interaction of both endothelial and tumor cells with the ECM, contributing to cell proliferation, angiogenesis and metastasis in tumors. Furthermore, the upregulation of MMP-7 by macrophages in response to hypoxia may allow them to move more rapidly towards hypoxic sites. Once they reach such sites, macrophage movement is thought to be restricted due to inhibition of the chemotactic signal transduction mechanism and down-regulation of chemoattractant and chemoattractant receptor on hypoxic macrophages (**Grimshaw, M. J et al. 2001 pp480-489**).

Interestingly, MMP-7 expression has been linked to resistance of tumor cells to doxorubicin chemotherapy. Doxorubicin kills tumor cells by inducing Fas ligand activation on the surface of tumor cells, which results in cell death. MMP-7 can cleave Fas ligand from the surface of the tumor cells, so blocking the action of the drug **(Mitsiades, N., and Yu, W. 2001 pp577-581)**. The fact that macrophages release high levels of MMP-7 when subjected to hypoxia suggests that hypoxic TAM may contribute to the resistance of tumor cells to such forms of chemotherapy.

The data presented above show that only the latent pro-MMP-7 form could be detected in Western blotting. This was expected as the cleavage of active MMP-7 from released pro-MMP-7 is extracellular, requiring the presence of proteolytic enzymes in the culture fluid. This analysis relies on

the SDS-PAGE separation of pro-MMP-7 from other proteins which may bind it, so the possibility of an inhibitory protein (e.g. TIMP-1) binding to the soluble pro-MMP-7 and inhibiting its activity is unlikely. The presence of the pro-MMP-7 zymogen form is frequently observed in culture cells **(Imai, K., Yokohama, Y., 1995 pp6691-6697)**. Although primary macrophages are known to contain potential pro-MMP-7 activators such as endoproteinases and plasmin **(Lijnen, H. R. 2000 pp175-181)**, it could be that tissue culture conditions do not favor pro-MMP-7 activation as potential soluble activators are often too dilute to activate the zymogen form or the use of the heat-inactivated serum throughout the experiments. However, it is highly likely that active MMP-7 would form rapidly when macrophages release pro-MMP-7 in hypoxic areas of tissues where such enzymes are present. For example, addition of exogenous plasminogen to 5 day-old cultured MDM generated plasmin via MDM-derived uPA. Plasmin was then able to process MDM secreted pro-MMP-7 to its active form and this coincided with a 10-fold increase in elastin degradation compared to plasminogen-free MDM culture supernatants **(Filippov, S., Caras, I.2003 pp925-935)**. Interestingly, despite the fact that MDM only secrete the pro-form of MMPs, these were still able to degrade elastin over a 5-day culture period, suggesting that even pro-MMP-7 may be proteolytically active, albeit with much reduced activity than the cleaved active form.

In a rodent model that mimics the osteoblastic and osteolytic changes associated with human metastatic prostate cancer, MMP-7 produced by osteoclasts was capable of processing the receptor activator of a nuclear factor-kappa B ligand (RANKL) to a soluble form that promoted osteoclast activation **(Lynch, C. C., 2005 pp485-496).** Therefore, the upregulation of MMP-7 release by hypoxic macrophages could have a role in prostate cancer and associated bone metastasis. The role of MMP-7 in such diseases indicates that inhibition of MMP-7 activity at these sites may be a future therapeutic target. In addition to its involvement in tumor progression, induction of MMP-7 protein by hypoxia may play a role in other disease states. For example, high levels of MMP-7 are expressed by macrophages in human atherosclerotic lesions (Halpert, I., 1996 pp9748-9753) and these sites are known to be hypoxic (Halpert, I., 1996 pp9748-9753). Thus elevated levels of MMP-7 at these sites may aid plaque rupture and therefore may play an important role in the progression of certain cardiovascular diseases **(. Shah, P. K. 1998, pp 199-206).**

The data presented here support the overall hypothesis that macrophages within hypoxic tumor sites upregulate not only the transcription factors (such

as HIF- 1/2, ATF-4 and Egr-1) (Elbarghati L., 2008 pp899-908) but also such pro-tumor proteins as MMP-7. The latter can stimulate tumor invasion, growth and metastasis. The hypoxic up regulated gene identified (MMP-7) could be important for the survival and functioning of macrophages in hypoxic diseased tissues and their promoters could prove useful in macrophage delivery gene therapy.

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