Effects of Hypoxia on Invasion, Metabolism, Proliferation, Proteinase Activities and Gene Expression of a Human Prostate Cancer Cell Line

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Introduction: Human cancers are often characterized by acute or chronic hypoxia and low oxygen tensions have been associated with increased metastasis [1, 2]. Using the Metabolic Boyden Chamber (an MR-compatible invasion assay [3]), we investigated the effects of hypoxia on differences in prostate cancer cell invasion and metabolism in the presence or absence of human umbilical vein endothelial cells (HUVECs). To better understand the mechanisms underlying the effects of hypoxia as observed by MR, we performed cell cycle analysis, measured the endogenous activities of the proteinases uPA, MMP-2, and MMP-9, and analyzed the influence of hypoxia on the gene expression of PC-3 cells.

Methods: PC-3 cells (ATCC) were originally derived from a bone metastasis of a prostate cancer patient. For MR experiments, adherently grown cancer cells were layered on either side of an ECM gel chamber in a customized 10-mm NMR tube and perfused with cell culture medium. In some experiments, HUVECs (Clonetics) were added on the surface of the ECM gel ca. 16 h before the experiment forming a lumen-like structure. The temperature was kept at 37°C. The oxygen tensions were kept above 20 % for oxygenated conditions, and below 1.5 % for hypoxia. Cancer cell invasion was quantified from changes in the profiles of intracellular water along the sample. Cell metabolism was studied by 1D ^{1}H MRS and 1D ^{31}P MRS. Endogenous uPA, MMP-2, and MMP-9 activities were measured after exposure to 48 h of hypoxia using the uPA activity assay (Chemicon) and the MMP-2 and MMP-9 Biotrak Assay (Amersham). Cell cycle analysis was performed by flow cytometry using the BrDU Flow Kit (Becton Dickinson). Changes in gene expression levels of PC-3 cells exposed to 48 h hypoxia were assessed by oligonucleotide microarray analysis.

***Figure 1**: Invasion Index of PC-3 cells with or without HUVECs on ECM gel under oxygenated ($O_2 \geq 20$ %) and ($O_2 \leq 1.5$ %) hypoxic conditions. (Mean ± SE, n=4)*

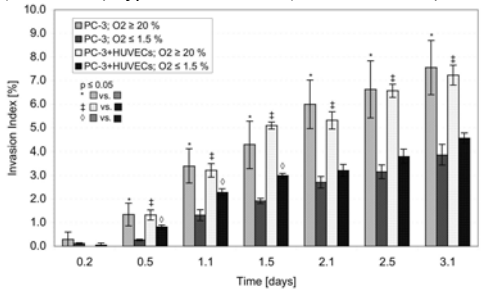

***Figure 2**: Cell growth of PC-3 cells (Mean ± SE, n =4) obtained by MR from the intracellular water signal.*

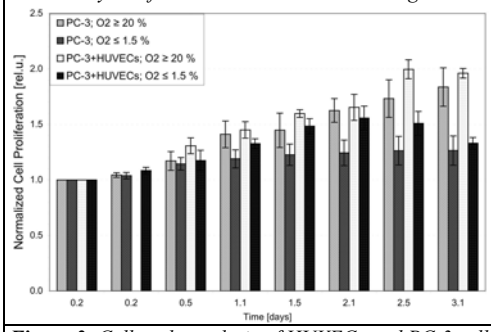

***Figure 3:** Cell cycle analysis of HUVECs and PC-3 cells grown in tissue culture. After 48 h hypoxia, the S phase fraction of PC-3 cells was significantly decreased; for HUVECs, the G0/G1 phase fraction was decreased and G2/M phase fraction increased. * $p \leq 0.1$, ** $p \leq 0.05$, Mean ± SE, n = 3*

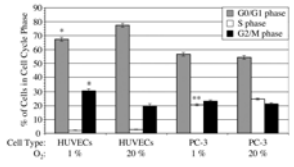

***Figure 4:** Presented are the number of genes related to a known function in invasion, metabolism, angiogenesis, and cell cycle regulation that are down-regulated and up-regulated as a result of 48 h of hypoxia.*

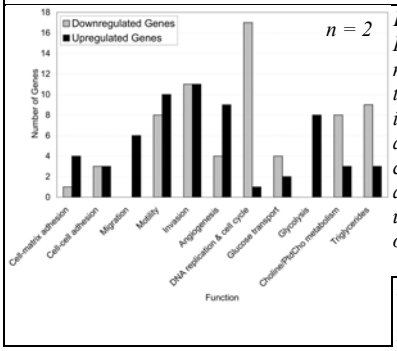

***Table 1:** Changes in Enzyme Activities for PC3 cells and HUVECs under different conditions set in the context of the Invasion Index. For all experimental conditions, HUVECs demonstrated higher endogenous MMP-2, MMP-9, and uPA activity per cell than PC-3 cells.*

Experimental Condition	*Invasion Index I(t) (MR experiments)*	*Endogenous Enzyme Activity per Cell* *MMP-2*	*MMP-9*	*uPA*
$PC\text{-}3_{Bb}$ on ECM	20 % O_2 > 1 % O_2	20 % O_2 < 1 % O_2	20 % O_2 < 1 % O_2	20 % O_2 < 1 % O_2
$PC\text{-}3_{T}$	N/A	20 % O_2 < 1 % O_2	20 % O_2 < 1 % O_2	20 % O_2 < 1 % O_2
$PC\text{-}3_{Bb}$ on HuvECM	20 % O_2 > 1 % O_2	20 % O_2 > 1 % O_2	20 % O_2 > 1 % O_2	20 % O_2 > 1 % O_2
PC-3, 20 % O_2	N/A	Plastic > ECM	Plastic > ECM	Plastic > ECM
PC-3, 1 %O_2	N/A	Inconclusive	Plastic ≤ ECM	Plastic < ECM
$PC\text{-}3_{Bb}$, 20 % O_2	ECM = HuvECM	Inconclusive	ECM < HuvECM	ECM < HuvECM
$PC\text{-}3_{Bb}$, 1 %O_2	ECM < HuvECM	ECM ≥ HuvECM	ECM > HuvECM	ECM > HuvECM
HuvECM	N/A	20 % O_2 > 1 % O_2	20 % O_2 = 1 % O_2	20 % $O_2 \leq$ 1 % O_2
$HUVECs_T$	N/A	20 % O_2 > 1 % O_2	20 % O_2 > 1 % O_2	20 % O_2 > 1 % O_2
HUVECs, 20 % O_2	N/A	Plastic < ECM	Inconclusive	Plastic > ECM
HUVECs, 1 %O_2	N/A	Plastic < ECM	Plastic < ECM	Plastic < ECM
No. of experiments	*n = 4*	*n = 2*	*n = 2*	*n = 1*

$PC\text{-}3_{Bb}$, PC-3 cells grown adherently on Biosilon® beads; $PC\text{-}3_{T}$, or $HUVECs_{T}$, PC-3 cells or HUVECs grown adherently on tissue culture plastic; HuvECM, HUVECs on ECM gel; N/A, not applicable.

***Table 2:** Intracellular LacTG (mean ± SE), calculated from global 1D ^{1}H NMR spectra obtained from STEAM-based pulse sequence (a) or lactate-edited spin-echo-based pulse sequence (b); n = 4*

Experimental Condition	*time [day]*	*LacTG[(a)]* [rel.u.]	*Lac[(b)]* [rel.u.]	*TG[(b)]* [rel.u.]
PC-3, n=4 $O_2 \geq 20$ %	1	1.00 ± 0.47	1.81±0.32	6.9±2.3
	2	0.90 ± 0.54	1.81±0.32	6.0±3.4
	3	1.00 ± 0.72	1.79±0.31	4.8±2.7
PC-3, n=4 $O_2 \leq 1.5$ %	1	2.48 ± 0.61*	1.95±0.23	11.4±1.0
	2	2.47 ± 0.88*	2.01±0.35	10.9±1.7
	3	2.3 ± 1.1(*)	1.78±0.35	9.4±1.8
PC-3 and HUVECs on ECM gel, n=4 $O_2 \geq 20$ %	1	1.66 ± 0.24	1.98±0.39	7.82±0.66
	2	1.41 ± 0.29	1.83±0.13	6.59±0.54
	3	1.29 ± 0.32	1.94±0.10	6.2±1.0
PC-3 and HUVECs on ECM gel, n=4 $O_2 \leq 1.5$ %	1	3.04 ± 0.44‡	2.55±0.22	14.4±1.4‡
	2	3.05 ± 0.56‡	2.70±0.32‡	13.7±1.8‡
	3	2.96 ± 0.70‡	2.71±0.33‡	13.7±1.1‡

* $p < 0.05$, PC-3, $O_2 \geq 20$ % *versus* $O_2 \leq 1.0 \pm 0.5$ %; (*) $p < 0.1$, PC-3, $O_2 \geq 20$ % *versus* $O_2 \leq 1.0 \pm 0.5$ %; ‡ $p < 0.05$, PC-3 and HUVECs on ECM gel, $O_2 \geq 20$ % *versus* $O_2 \leq 1.0 \pm 0.5$

Results: Hypoxia decreased invasion of PC-3 cells irrespective of the presence of HUVECs (Fig.1). However, in the presence of HUVECs, invasion of hypoxic PC-3 cells was significantly increased in the first 2 days and remained slightly higher in the last day compared to hypoxic PC-3 cells alone (Fig. 1). Endogenous activities of uPA, MMP-2, and MMP-9 rose for PC-3 cells on ECM after 48 h of hypoxia (Table 1). This effect was reversed by the presence of HUVECs on the ECM gel (Table 1). HUVECs expressed much higher endogenous proteinase activities per cell than PC-3 cells. Cell growth of PC-3 cell decreased under hypoxia, which was partially ameliorated by the presence of HUVECs on ECM (Fig.2). Decreased cell proliferation of PC-3 as a result of hypoxia was confirmed by flow cytometry (Fig.3) and by the down regulation of genes such as CDC6, CDC7 and MCM2 to MCM7 (Fig.4). 48 h hypoxia upregulated genes involved in glycolysis, migration and motility, but down-regulated genes involved in Choline/PtdCho and lipid metabolism (Fig.4). Despite the results observed on a genetic level, intracellular triglyceride (TG) levels increased significantly during hypoxia, thus being mainly responsible for the increase in the lactate/triglyceride signal (LacTG) (Table 2). **Conclusion:** Endothelial cells can confer an advantage of cancer cell invasion under hypoxia, which might be partly due to their higher expression of endogenous active proteinases. However, the observed changes in invasion could not be explained solely by the activity of selected proteinases, demonstrating the importance of combining gene and protein expression analysis with functional assays.

References: [1] P Vaupel *et al.* Cancer Res, *49:* 6449-65, 1989. [2] EK Rofstad. Int J Radiat Biol, *76:* 589-605, 2000. [3] U Pilatus *et al.* Neoplasia, *2:* 273-9, 2000. **Acknowledgements:** We acknowledge support by NIH Grant 2RO1CA73850, technical assistance by Dr. Chacko & Mr. Cromwell, and Dr. Shungu and Ms. Mao for the software XsOsNMR.