SECRETORY PROTEIN-RELATED HYPOXIA IN T47D BREAST CANCER CELL LINE

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Submission date: 23-Jun-2022 11:30AM (UTC+0700)
Submission ID: 1861649051
File name: 2022-06-01_english_ver_Proteomics.docx (729.99K)
Word count: 3555
Character count: 19541

SECRETORY PROTEIN-RELATED HYPOXIA IN T47D BREAST CANCER CELL LINE

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Abstract

Background: Increased proliferation can cause hypoxic areas in solid tumor cells. Hypoxia adaptation in cancer cells mediated by Hypoxia Inducible factor 1 (HIF-1) that induced the expression of various proteins. Analysis of secretory proteins in T47D breast cancer cells is still limited. The aim of this study is to compare the protein profile secreted by T47D breast cancer cell line under hypoxic and normoxic conditions and identify proteins as candidate hypoxia marker proteins in T47D breast cancer cell lines.

Methods: T47D breast cancer cell lines were cultured under standard conditions up to passage three. Cells were subcultured and divided into normoxic and hypoxic. The normoxic group was

incubated with 20% oxygen concentration, while the hypoxic group was incubated in a hypoxic chamber with 0.5% and 5% oxygen concentration for 6 hours, 24 hours, and 48 hours in serum free medium. Proteins in the culture medium were isolated and precipitated with trichloro acetic acid (TCA). The concentration of secreted protein was measured by Bicinchoninic Assay (BCA). Protein bands were visualized by SDS-PAGE method. The bands that had differences between the hypoxic and normoxic groups were further analyzed by Liquid chromatography-Mass spectrophotometer (LC-MS).

Result: Hypoxic group showed higher secretory protein than normoxic group. Protein bands were found in the 0.5% hypoxic group with a size of 50-75 kDa. LC-MS analysis of the eluent protein identifies keratin 1, 2, 9, and 10.

Conclusion: The T47D cell line under 0.5% hypoxic treatment showed higher secretory proteins that identified as keratin 1, 2, 9, and 10.

Keywords: Hypoxia, secretory protein, liquid chromatography-mass spectrophotometer, T47D, breast cancer cell line

1. Introduction

The state of lack of tissue oxygen supply is called hypoxia. In solid tumors, excess proliferation increases the distance of tissue oxygen diffusion from the vessels leading to insufficient angiogenesis in the growing tumor lesion.[1] Areas of hypoxia in solid tumors can cause various changes in tumor cell regulation as an adaptation to hypoxic conditions. Hypoxia in tumor cells is known to play an important role in the development, resistance to anti-tumor agents and metastasis.[2]

Cellular responses to hypoxic conditions are mediated by the transcription factor HIF1 which can influence the expression of various proteins. Proteins can be released by tumor cells into the extracellular matrix to support the tumor microenvironment. Protein release under hypoxic conditions can occur directly through the activation of HIF, as well as the activation of cellular signaling pathways. Hypoxia in tumor cells is known to increase the release of proteins and other effectors that can trigger changes in cell behavior. These proteins can cause tumor cells to develop with unrestricted growth characteristics and can invade surrounding tissues.[3] Increased HIF expression as a result of hypoxia is known to occur in 56-57% of breast cancers.[4]

Identification of candidate biomarker secreted protein released by cancer cells using mass spectrophotometry already used in human endometrial cancer, cervical cancer cells (Li Hongyan. 2005), lung cancer (Ling, Jin Huang. 2006), head and neck cancer (Ralhan, Ranju. 2011), colorectal cancer (Shin, Jihye. 2014), and many more. Meanwhile, secretome analysis of previous in vitro study in the hypoxic cancer secretome from MDA-MB 231 breast cancer cell analysis revealed that high expression

of lysyl oxidase in breast cancer may contribute to osteolytic lesion formation.[4] Meanwhile the secretory protein analysis of T47D breast cancer cell line never been identified before. Studies comparing hypoxic secretory protein profiles in breast cancer with modeling of the T47D cell line versus normoxia are not available yet. The aim of this study was to compare the secretory proteins produced by the T47D breast cancer cell line cultured under hypoxic and normoxic conditions.

2. Methods

2.1. T47D Cell Culture

T47D breast cancer cell line were obtained from Elabscience (EP-CL-0228) with a certificate of analysis for authentication. T47D cells were grown in 100 mm dishes using culture media (RPMI 1640 with 10% FBS, 0.2 U/ml insulin, 1% Penicillin-Streptomycin, Amphotericin; all materials obtained from Capricorn Scientific). T47D subculture was carried out when it reached 70-90% confluent with a planting density of $4x10^4$ cells/cm^2. After reaching the third passage, cells were harvested and 2.2 $x10^6$ cells were grown in 100 mm petri dishes (Iwaki). The culture cell medium was replaced with new media every 48 hours and incubated in an incubator at 37°C with 5% CO2.

2.2. Hypoxic treatment

Cells were harvested and 2.2 x 10^6 cells then planted and grown in 100 mm plates until confluence reached 60-70%. When confluency reached 70%, cells were washed with PBS three times then the medium was replaced with serum free medium. The normoxic group was incubated in 37ºC and 20% O_2. The hypoxic treatment group

was incubated in a hypoxic chamber (Stem Cell®) which flowed with a mixed gas with an oxygen concentration of 0.5% and 5% for 4 minutes at a flow of 20 L/min. The medium from T47D cell cultured was then collected and transferred to the tube according to the hypoxic treatment time 6 hours, 24 hours and 48 hours for further analysis. All treatment groups were conducted in triplicate.

2.3. Protein Isolation and Precipitation

The culture media were centrifuged at 800 rpm for 10 minutes and filtered with a 0.2 μm syringe filter to eliminate cells and debris. Media were added with 10% trichloroacetic acid (Sigma). The medium then precipitated on ice for 2 hours followed by centrifugation for 30 minutes at 14000 rpm. Washing was carried out with cold acetone 2 times. The washed precipitate protein was resuspended with 50 Mm ammonium bicarbonate. Protein concentration was measured by Bicinchoninic Assay/BCA kit (Thermo).

2.4. *BCA Assay*

Measurement of protein precipitate concentration was conducted by the BCA kit (Thermo). After reagent preparation by mixing reagent A and B, a total of 25 μl of standard protein and samples were put into the 96-well microplate (Iwaki). Working reagent (200 μl) was added to each well and incubated in an incubator at 37°C for 30 minutes. Samples were read with a spectrophotometer at a wavelength of 570 nm.

2.5. Protein separation with SDS-PAGE

We used TGX FastCast Acrylamide Kit 10% (BioRad) for SDS-PAGE methods. The step begins with making Running gel/Resolving Gel and Stacking Gel according to the product manual. A total of 20 µl of sample protein was transferred into the well and electrophoresis was performed at 100 v for 2 hours. The gel was then stained with 1% Coomassie Brilliant Blue R250 on a shaker until the bands were visible. The gel bands with different expressions were further diced using scalpel and transferred to a microtubule containing 50mM Ammonium Bicarbonate/50% acetonitrile then vortexed for 10 minutes.

2.6. LC-MS preparation

Protein samples were incubated for 60 minutes in 10 mM Dithiothreitol (DTT) for protein unfolding. After reduction, protein samples were alkylated by adding 54 mM Iodoacetamide/IAM and incubated in the dark at room temperature for 30 minutes. Supernatant was removed and gel cubes were vortexed in 400 µl 50 mM NH_4HCO_3 for 10 minutes for two times. Supernatant then discharged and alkylated protein samples were digested using trypsin in 50 Mm ammonium bicarbonate with incubation at 37°C overnight. Digested protein then dried using the freeze drying and redissolved in 0.1% formic acid.

2.7. Protein analysis using LC-MS

The redissolved digested samples are placed in the automatic sample injector on the LC-MS device. The tool used is the NanoLC Ultimate 3000 Series System

Tandem Q Exactive Orbitrap HRMS Thermo Scientific™. The chromatographic system used is reverse phase. The stationary phase used was C18 (PepMap RSLC). The mobile phase used was (A) water with 0.1% formic acid and (B) acetonitrile, 0.1% formic acid. Separation was carried out using a mobile phase gradient: gradient B 2% for 3 minutes, 2%-35% B for 27 minutes, 35-90% B for 15 minutes, 90% B for 15 minutes, 2%B for 15 minutes. LC-MS data is in the form of a chromatogram that shows the ratio of mass to electric charge (m/z) for each compound. The data were analyzed by Proteome Discoverer 2.2 to identify the protein present in the sample. (Figure 1)

Result

Secretory protein concentration in T47D cell culture medium that had been precipitated with 10% (v/v) TCA was measured by BCA assay (Bicinchoninic Assay). The total protein concentration in the culture media of the hypoxic treatment group is higher than that of the normoxic group (table 1) and compared with the normoxic group, there was an increment of protein ratio from the hypoxic group at the range of 1.73-22.74 folds. The increase in protein occurred along with the hypoxic concentration and the duration of treatment. The lowest protein concentration was found in the normoxic group while the highest secretory protein concentration was found in the 48 hours hypoxic 0.5 % group. There was an increased concentration of secretory proteins in the 5% hypoxic group compared to the normoxic group, but the increase was not as much as the hypoxic 0.5% group.

The proteomic analysis method in this study combines SDS-PAGE electrophoresis and mass spectrophotometry. The SDS-PAGE method can identify proteins based on their molecular weight and isoelectric strength. The SDS-PAGE gel showed a band in the 0.5% hypoxic treatment group with a molecular weight between 50 kDa-75 kDa which did not appear in the 5% hypoxic or normoxic groups (figure 2).

Protein identification was carried out using the LC-MS method on the eluted SDS-PAGE band. The proteins in the SDS-PAGE band were identified as keratin 1, keratin 2, keratin 9, keratin 10 and cDNA FLJ54371 proteins/ serum albumin (table 2).

Discussion

The increased secretory protein concentration obtained from culture medium under hypoxic conditions may be related to the expression of HIF1α. Previous study showed that HIF1α expression is known highest with 0.5% oxygen and significantly decreases above 2% to 6% oxygen. HIF1α expression in various types of breast cancer cells is known to reach a peak between 4 hours to 8 hours of hypoxia and begins to decrease after 12 hours of treatment.

Protein release by the tumor cells is carried out mainly through extracellular vesicles. Hypoxia is known to increase biogenesis and release of secretory vesicles. Several mechanisms that can increase the release of these secretory vesicles are through HIF, Rab-GTPase, NF-Kb and tetraspanin signaling. These extracellular vesicles have an important role in intercellular communication through carried biological molecules, including proteins. In tumor cells, these extracellular vesicles are mainly released into

the microenvironment around the tumor (tumor microenvironment) and play a role in angiogenesis, invasion and metastasis.

Factors that can cause the band in the 5% hypoxic group and the normoxic group not appear is small sample concentration or the protein has been degraded. The type of gel dye is also known to affect the quality of the band on the gel. The gel staining in this study used coomassie blue R250 which is known to have a sensitivity about 3-10 ng. Several stainings are known to have a higher sensitivity than coomassie blue staining such as silver stain (0,25-5ng).

Keratin (formerly known as cytokeratin), is an intermediate filament present in various epithelial cells. Keratin in humans is encoded by 54 functional genes, and is expressed specifically depending on the cell type. Keratin plays an important role in maintaining cellular integrity and protecting cells from injury. There are 2 types of keratin, namely type I (K9-K19), keratin type II (K1-K8).[12] Keratin is known to be very dynamic to cellular stress and environmental changes.[12]

Keratin was recognized as a fragmented secretory product due to the degradation of dead cells. Panabieres et al (2009) on several types of colorectal cancer cells (HT29, HCT116, Caco-2) and breast cancer cells (MCF-7, SKBR3, MDA MB-231) showed that cancer cells released cytokeratin 19 (CK-19) in a full length.[13] The cytokeratin released by breast cancer cells is thought to have a role in metastasis and tumor growth through an unexplained mechanism. [13]

Proteomic analysis of keratin as a secretory product of cancer cells is still limited. Palazzolo (2012) who conducted a proteomic analysis of breast cancer cell

exosomes found the expression of keratin 9.[18] Green (2015) who performed a proteomic characterization of breast cancer exosomes associated with metastatic ability also found the expression of keratin 9 and several other cytoskeletal proteins such as tropomyosin, and transgelin. These proteins are known to play a role in signaling and regulation of cellular structures.[19]

Keratin on hypoxia cancer cell

Effect of hypoxia on keratin release research is still very limited and has not been fully elucidated. Hypoxia through the HIF transcription factor is known to increase the transcription of various genes. Hypoxia can cause increases in the expression of several keratin coding genes including KRT14, KRT16, KRT18, KRT19 and KRT20.[20] There is no data yet regarding KRT1, KRT2, KRT9, KRT10.

The mechanism of keratin release under hypoxic conditions in tumor cells has not been fully elucidated. Ni et.al (2010) who examined the effect of hypoxia on alveolar cells revealed that hypoxia is known induce cellular stress by increasing reactive oxygen (ROS) which can cause reorganization of keratin.[22] Through the activation of mitogen-activated protein kinase (MAPK) p38, keratin 8 will be phosphorylated resulting in keratin disassembly and degradation. Disassembly and degradation of keratin will reduce cell integrity and increase the ability to migrate. The mechanism of keratin release under hypoxic conditions of tumor cells may have the same mechanism and further research needs to be done.[22]

Evidence about the expression of keratin as a secretory product of cancer cells under hypoxic conditions is limited. On the other hand, keratin is often referred to as a

contaminant in proteomic examination. Contamination of keratin in proteomic examination can come from skin, hair or air during sample processing. Contamination is difficult to avoid even though it is carried out in a laboratory with a high standard protocol.[23] Possible sources of keratin as a contaminant in this study can occur and have not been ascertained.

Comparison of cells in the hypoxic treatment group with the normoxia group was carried out to assess the protein released by breast cancer cells is the protein released due to hypoxia treatment. Several materials in this experiment which are not mass spectrophotometry/MS-grade become the weakness of this study and can increase the risk of contamination. Additional examination can be done by specific examinations such as immunoepithelial spots combined with keratin expression in culture medium. Immunoepithelial spots can be performed to observe the expression of keratin in cells. Expression of keratin in cells could be associated with the release of proteins identified through LC-MS. In this study, immunoepithelial spot examination could not be performed because T47D cells were used for other research. The role of hypoxia on keratin expression, especially keratin 1, 2, 9 and 10 needs to be studied further.

Conclusion

The T47D cell line under 0.5% hypoxic treatment showed higher secretory proteins that identified as keratin 1, 2, 9, and 10. The concentration of secretory protein in the

hypoxic group was higher than the normoxic group. Expression of keratin 1, 2, 9, and 10 proteins were obtained on T47D culture medium with 0.5% hypoxia treatment.

Acknowledgment

We are grateful to Histology Department staff Dewi Kartikawati Paramita, PhD for handling T47D until cell stock achieved and the technical assistant of all laboratory staff Laboratorium Riset Terpadu, Dr. Sardjito General Hospital and in Histology and Cell Biology, UGM, Yogyakarta especially Mrs. Sumartiningsih.

Author Contribution

DP contributed in planning and supervised the study. EH supervised the experiment method. AN working in the laboratory experiment and drafting the manuscript. DP, EH reviewed and revised the manuscript. All authors discussed the results and commented on the manuscript.

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Figure/tables

Figure 1. Flowchart of mass spectrophotometric liquid chromatography examination. The sample is injected and passed through an ion source for ionization. The peptides are separated based on the charge of the ions in the analyzer. The mass analyzer will measure the Mass-to-charge ratio (m/z). The detector captures the ions and measures the intensity of each ion. Proteins were identified through bioinformatics analysis.

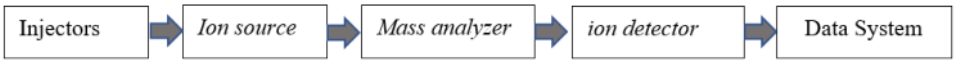
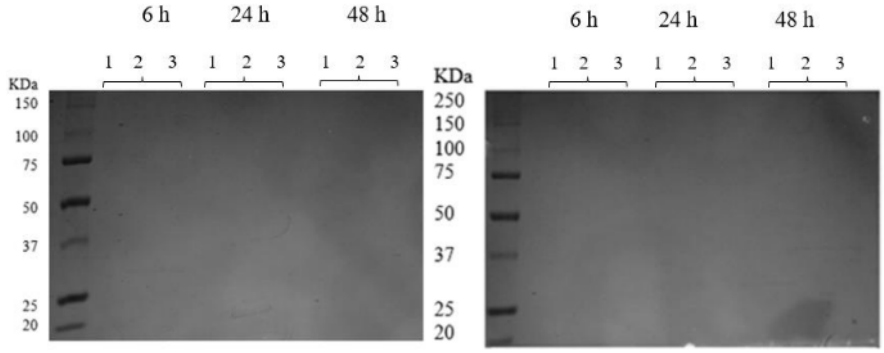
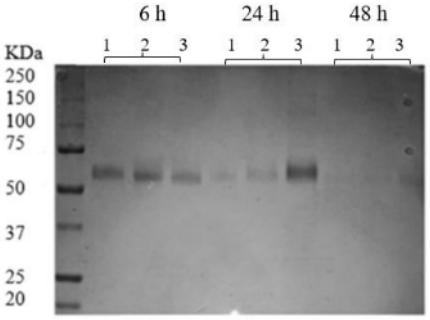


Figure 2. Protein bands visualization on SDS-PAGE gel. a. Normoxic group, b. 0.5% hypoxic condition, c. 5% hypoxic condition. There was a band between 50-75 kDa in the 0.5% hypoxic group, but not in the 5% hypoxic and normoxic groups.



a. Normoxic

b. 5% hypoxic



c. 0.5% hypoxic

Table 1. Protein concentration from T47D cell culture medium with 0.5%, 5% hypoxic, and normoxic treatment

Sample	Protein Concentration (µg/mL)			Protein Concentration Average (µg/mL)	Protein Ratio (Hypoxic/normoxic)
	1	2	3		
Hipoxia 0.5% 6 hours	130.63	92.51	83.43	102.19	1.95
Hipoxia 0.5% 24 hours	119.49	111.83	128.11	119.81	2.29
Hipoxia 0.5% 48 hours	178.97	126.33	124.67	143.33	2.74
Hipoxia 5% 6 hours	132.35	128.91	79.31	113.52	2.17
Hipoxia 5% 24 hours	130.63	72.71	69.48	90.94	1.73
Hipoxia 5% 48 hours	87.53	170.03	82.60	113.39	2.16
Normoxia	61.33	35.00	60.55	52.3	1

Table 2. Protein identified from SDS-PAGE band of T47D cell culture medium under 0.5% hypoxic treatment for 24 hours and 48 hours

No.	Accession Number	Protein Identity	Protein Coverage (%)	Peptide Number	Molecule Weight (kDa)
Hypoxic group 0.5% 24 hours					
1.	H6VRG1	Keratin 1	11	6	66.1
2.	P35527	Keratin 9	4	2	62
3.	B4DPP6	Serum Albumin	2	1	70.3
Hypoxic group 5% 48 hours					
1.	P35527	Keratin 9	9	3	62
2.	H6VRG1	Keratin 1	7		66.1
3.	P35908	Keratin 2	4	2	65.4
4.	P13645	Keratin 10	3	1	58.8
5.	B4DPP6	Serum Albumin	2	1	70.3

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