miRNA and protein biomarkers in hypoxia for obesity studies

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Introduction

Obesity is a serious health problem generally associated with diseases such as type 2 diabetes, hypertension, cardiovascular diseases and various types of cancer. A better understanding of the molecular mechanisms underlying obesity is essential. Investigating the development and function of adipose tissue has revealed biomarkers and possible targets for therapeutic strategies. Adipose tissue secretes adipokines and microRNAs which are involved in mechanisms regulating adipose tissue metabolism (1, 2). Obesity leads to hypoxic adipose tissue due to tissue mass expands, with adipocytes becoming distant from the vasculature. Such variation in oxygen pressure has drastic effects on inflammation and modulation of adipokines and miRNA secretion (3, 4). Primary cell culture is the best in vitro model to mimic the physiological state of tissues. Adipocytes with specific Body Mass Index (BMI), have been cultured at 21% or 1% of oxygen. We compared the biomarkers secretion from different adipocytes in hypoxic and normoxic conditions by the analysis of cell supernatants using a Human Obesity Antibody Array and an innovative 3D-structure microarray technology.

Material & Method

Cell culture conditions

Human subcutaneous preadipocytes, derived from adipose tissue from six donors, were obtained from Zen-Bio. Cells were plated in 6-well plates in preadipocyte medium. Cell differentiation was induced for 7 days at confluence by incubation in differentiation medium composed of adipose medium (AM) supplemented with 0.25 mM isobutyl methylxanthine and 10 µM of a PPARγ agonist. Cells were then cultured with AM. Mature adipocytes were fully differentiated at day 14 post-induction.
For exposure to hypoxia, the mature adipocytes were transferred into an INVIVO2 400 workstation (Ruskinn Technology Ltd) and were cultured with fresh AM at 37°C, 1% O_2 for 3 days to use fully hypoxia adapted cells. After this incubation time, the AM was replaced by basal medium without FBS. The cells were then cultured for 48h before collecting the basal medium which was used for protein and miRNA profiling. All media used in the workstation were preconditioned using HypoxyCOOL (Ruskinn Technology Ltd) to reduce oxygen concentration to 1%. Control cells were cultured in a standard incubator (21% O_2) as normoxic culture conditions. After the 48h incubation in basal medium, microscopic observation was done using a Zeiss Axiovert 40C inverted microscope and photos from each condition were taken.

Protein and miRNA profiling

In order to analyse the cytokine secretion, the collected basal media were assayed with obesity adipokine antibody array (Raybiotech, ref. AAH-ADI-G1) according to the manufacturer's protocol. From images, the fluorescence intensities of spots without local background were extracted and used for protein profiling analysis. These intensities were normalized in order to compare values from different arrays. The normalized data were then used to determine fold change of expression levels of each analyte between adipocytes cultured in hypoxic and normoxic conditions.
In order to analyse the miRNA secretion, the collected basal media were assayed with 3D-Gene® Human miRNA microarray (Toray, ref. TRT-XR516) according to the manufacturer's protocol. This oligo chip can detect about 2,000 types of human miRNAs selected from the miRBase database, release 19. From images, the extracted fluorescence intensities of spots were normalized to compare values from different arrays. The normalized data were used to calculate relative differences in expression levels for each miRNA, comparing data from adipocytes cultured in hypoxic and normoxic conditions. Data were expressed as Log2 of fold change ratio.

Results

I / Adipocytes features

The table 1 summarizes the subcutaneous and omental preadipocytes used in this study with features of donors. After 2 weeks of differentiation, the adipocytes are mature (Figures 1 & 2). Microscopic observation showed no significant difference of cell morphology and of lipid droplet numbers between hypoxic and normoxic conditions whereas OP-F-2 and OP-F-3 seem less fully differentiated.

Reference	Lot	Adipose tissue	Gender	Age	BMI (kg/m²)
SP-F-1	L020206	subcutaneous	female	25	18.8
SP-F-2	L060503	subcutaneous	female	29	25.1
SP-F-3	L070804	subcutaneous	male	42	33
OP-F-1	OMM092612B	omental	male	16	19.2
OP-F-2	OM111006	omental	female	28	26.6
OP-F-3	OMM032305	omental	female	43	51

Table 1: features of preadipocytes obtained from Zen-Bio.

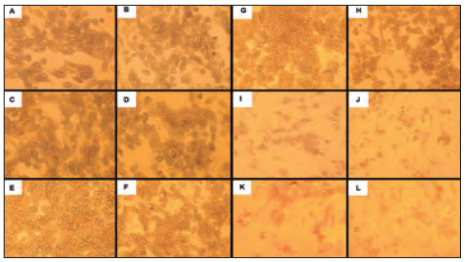

Figure 1: Microscopic observation of mature subcutaneous (A, B, C, D, E, F) and omental (G, H, I, J, K, L) adipocytes. Cells were cultured in normoxic (A, C, E, G, I, K) and hypoxic conditions (B, D, F, H, J, L). Cells came from donors with different BMI: SP-F-1 and OP-F-1, BMI<25 (A, B, G, H); SP-F-2 and OP-F-2, 25<BMI<29 (C, D, I, J); SP-F-3 and OP-F-3, BMI>30 (E, F, K, L).

II / Profiling of secreted adipokines

	SP-F-1	SP-F-2	SP-F-3	OP-F-1	OP-F-2	OP-F-3
Adiponectin	0,51	0,38	0,18	0,18	0,50	0,78
Adipsin	0,82	1,00	0,78	0,88	1,90	1,71
ANGPTL4	3,36	3,21	1,31	2,39	1,64	3,48
IL-6	3,05	1,07	1,32	1,38	1,21	1,14
IL-8	1,98	2,01	1,66	1,61	0,91	1,24
Leptin	177,29	11,98	7,35	18,79	15,28	40,59
MCP-1	3,37	2,52	1,06	1,38	1,09	0,73
MIF	2,95	1,30	0,94	0,55	0,40	1,05
OPG	8,16	5,67	5,59	1,92	0,81	3,90
PAI-I	5,37	3,48	4,20	1,76	0,66	2,80
TIMP-1	1,70	1,55	1,21	0,89	0,95	2,73
TIMP-2	2,09	1,16	1,88	1,26	2,62	2,80
TNF-alpha	0,99	0,94	1,37	0,96	0,94	1,45
VEGF	13,85	9,84	13,79	5,13	2,18	5,88

Table 2: Fold change of secreted adipokines. The fold change was calculated as the ratio of the protein level in hypoxic condition to the protein level in normoxic condition.

Table 2 shows the well-known adipokines secreted by adipocytes (5) and significant proteins detected with the obesity adipokine antibody array. Fold changes over and below 1 show an increase or decrease of adipokine secretion under hypoxia, respectively. Fold changes higher than 2 and lower than 0,5 are considered as significant. Adipocytes secrete a number of adipokines as adiponectin, IL-6, IL-8, leptin, TNF-alpha, MCP-1, MIF, VEGF and PAI-1 (4, 5). In this array, ANGPTL4, IL-6, IL-8, MIF and TNF-alpha were weakly detected and their calculated fold changes must be considered carefully. Adiponectin, adipsin, leptin, MCP-1, OPG, PAI-1, TIMP-1, TIMP-2 and VEGF were clearly detected (data not shown). Hypoxia up-regulated the secretion of leptin, OPG, PAI-1, VEGF, down-regulated the secretion of adiponectin and had no clear effect on TNF-alpha and adipsin secretion. These data confirm some previous studies (4, 5). TIMP-1 were also secreted by adipocytes (6) as well as TIMP-2 of which the secretion level increased in response to oxidative stress (7). These fold change levels are strongly dependent on donor without clear connection to BMI.

III / Profiling of secreted miRNA

a/ Advantage of 3D-gene® technology from Toray

Toray has developed a new structure for oligo or DNA microarray (Figure 2). The 3D Gene® microarray is made with black resin substrate which substantially decreases background noise and enables the detection of low expression genes or miRNA. The adoption of uneven columnar structure for the detection area of substrate enables the stabilization of spot morphology and the acquisition of a uniform detected image. The probes are spotted on top of the micro-columns which are treated with special chemicals so that the probes are firmly immobilized on the substrate in a dense and uniform fashion. A microbead agitation system maximizes the hybridization efficiency and signal intensity of detected genes or miRNA dramatically increases.

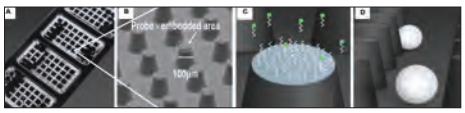

Figure 2 : Structure of 3D Gene® microarray. The microarray is made with black resin substrate (A). The detection area is constituted by columnar structure (B). The top surface of the micro-columns are treated with special chemicals so that the probes are firmly immobilized (C). A bead agitation system is used to increase the signal intensity (D).

b/ Profiling of secreted miRNA

The 3D Gene® microarray technology allowed to detect between 200 and 400 miRNA in each supernatant (Data not shown). The figure 3 shows the detected miRNAs with Log2 ratio higher or equal to 1 and lower or equal to -1.
It is difficult to discern a general trend from these data according to oxygen level, tissue origin of the cells or BMI of donor. Nevertheless, some miRNAs showed interesting differences of secretion in function of oxygen level and among cell batches like miR-1298, miR-3679-5p, miR-4648, miR-4672, miR-4725-3p, miR-6722-5p or miR-6723-5p. These miRNAs are potential new biomarkers for hypoxia. Only miR-940 has recently been reported as down-regulated in MCF7 cells exposed to hypoxia (8). In this study, miR-4466 was also down-regulated. Our results showed that miR-4466 is one of the most detected microRNA in culture medium without influence of oxygen level (Data not shown). The major hypoxia-inducible miR, miR-210, also known as hypoxamirs, was one of the first discovered as a direct transcriptional target of HIF (9). miR-210 was detected in the supernatant but with no significant negative Log2 ratio (between -1 and 0).

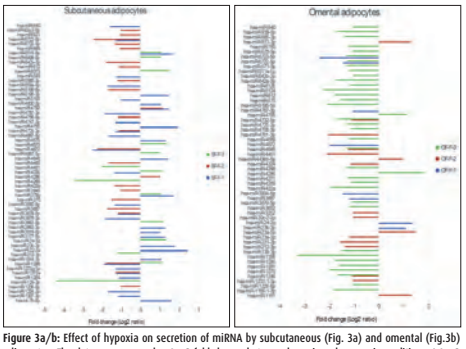

Figure 3a/b: Effect of hypoxia on secretion of miRNA by subcutaneous (Fig. 3a) and omental (Fig.3b) adipocytes. The data are expressed as Log2 fold change between hypoxic and normoxic conditions. A Log2 ratio of 1 means hypoxia 2 fold up-regulates the secretion of the miRNA. A Log2 ratio of -1 means hypoxia 2 fold down-regulates the secretion of the miRNA.

Discussion

Adaptation of adipocytes to hypoxia is already well referenced and is an important issue for obesity. Obesity is characterized by hypoxic white adipose tissue and associated inflammation. Hypoxia-induced dysregulation of adipokines plays a key role in obesity (1, 4, 10). Recently, even more evidences support the role of miRNAs as inflammatory mediator (2, 3, 11, 12). Hypoxia clearly regulates the miRNAs expression (8). Indeed, miRNAs are involved in cellular signal transduction via exosomes whose content can serve as biomarkers (13, 14).
To analyse the effect of low level of oxygen on the secretion of biomarkers, we screened the adipokines and miRNAs present in culture supernatants of mature adipocytes using microarrays. Secretion of adipokines is already well described and our study showed similar results. The secreted miRNA profiling highlighted some miRNAs as potential biomarkers for hypoxia. However, this biomarker screening was done on only one sample per condition and further analyses have to be done to statistically confirm these first results. Several additional assessments must also be done to reduce the donor-dependent variability even if some results are common to every donor like hypoxia-induced adiponectin down-regulation or leptin up-regulation. The adipogenesis itself is dependent on donor. Indeed, the differentiation of OP-F-2 and OP-F-3 seems not to be fully achieved as illustrated by low levels of lipid droplets formation. We have not seen clear effect of cell features like BMI, tissue origin or gender and age of donor on secretion of biomarkers. To smooth the donor-dependent variations, pools of preadipocytes with average BMI can be used. We also have access to a wide variety of donors and other cell types.
Toray's technology enables miRNA profiling from a wide range of samples as plasma, serum, cells or FFPE samples. Genomic analysis can also be done with 3D Gene® microarray, it enables the analysis of the whole genome on one array.

Conclusion

The combination of hypoxia cell culture models and biomarker profiling tools, for both proteins and miRNAs, opens new avenues for understanding molecular pathways and for drug discoveries. Such studies are suitable for a broad range of diseases such as type 2 diabetes, hypertension, cardiovascular diseases and cancer.

References

1. Blüher M. (2014). Molecular Metabolism.
2. Ge Q, *et al.* (2014). Journal of Immunology Research.
3. Alexander R, *et al.* (2011). Expert Opin Ther Targets.
4. Trayhurn P. (2013). Physiol Rev.
5. Wang B, *et al.* (2007). Pflugers Arch - Eur J Physiol.
6. Weise S, *et al.* (2008). J Endocrinol.
7. Choi S, *et al.* (2010). Anal Biochem.
8. Camps C, *et al.* (2014). Molecular Cancer.
9. Kulshreshtha R, *et al.* (2007). Mol. Cell. Biol.
10. Deng Y. and Scherer P.E. (2010). Ann. N.Y. Acad. Sci.
11. Nallamshetty S, *et al.* (2013). Free Radic Biol Med.
12. Xie H, *et al.* (2009). Expert Opin Ther Targets.
13. Vickers KC and Remaley AT. (2012). Curr Opin Lipidol.
14. Müller G, *et al.* (2011). Cell Signal.

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TORAY Innovation by Chemistry 3D-Gene

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